AMP-Activated Protein Kinase: A Target for Drugs both Ancient and Modern

D. Grahame Hardie,[1,*] Fiona A. Ross,[1] and Simon A. Hawley[1]
[1]Division of Cell Signalling & Immunology, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK
*Correspondence: d.g.hardie@dundee.ac.uk
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The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status. It is activated, by a mechanism requiring the tumor suppressor LKB1, by metabolic stresses that increase cellular ADP:ATP and/or AMP:ATP ratios. Once activated, it switches on catabolic pathways that generate ATP, while switching off biosynthetic pathways and cell-cycle progress. These effects suggest that AMPK activators might be useful for treatment and/or prevention of type 2 diabetes and cancer. Indeed, AMPK is activated by the drugs metformin and salicylate, the latter being the major breakdown product of aspirin. Metformin is widely used to treat diabetes, while there is epidemiological evidence that both metformin and aspirin provide protection against cancer. We review the mechanisms of AMPK activation by these and other drugs, and by natural products derived from traditional herbal medicines.

The AMP-activated protein kinase (AMPK) is a highly conserved sensor of cellular energy status, and genes encoding the three subunits of the kinase are found in essentially all eukaryotic genomes (Hardie, 2011). AMPK is switched on by metabolic stresses and xenobiotic compounds that cause a cellular energy imbalance, which is detected as increases in the ratios of ADP:ATP and AMP:ATP. Because the energy status of the cell is a crucial factor in all aspects of cell function, it is not surprising that AMPK has many downstream targets whose phosphorylation mediates dramatic changes in cell metabolism, cell growth, and other functions. In general, AMPK switches on catabolic processes that provide alternative pathways to generate ATP, while switching off anabolic pathways and other processes consuming ATP, thus acting to restore cellular energy homeostasis. The kinase evolved in single-celled eukaryotes and is still involved in multicellular organisms in regulating energy balance in a cell-autonomous manner. However, it is now clear that new functions were acquired during the development of metazoans so that AMPK is also regulated by hormones and adipokines that regulate energy balance at the whole body level, a topic reviewed in more detail elsewhere (Hardie et al., 2012). Metabolic disorders, such as obesity and type 2 diabetes, which are becoming increasingly prevalent in modern society, are essentially problems caused by a positive energy balance, and it was predicted—in a review written in the 1990s (Winder and Hardie, 1999)—that activators of AMPK might be useful for treating these disorders. This suggestion has been taken up by the pharmaceutical industry, which has been developing novel activators, but what was not predicted was that several existing drugs, as well as many natural plant products with reputed health benefits, would also turn out to be AMPK activators. Findings that AMPK caused inhibition of progress through the cell cycle (Imamura et al., 2001), and that the mechanism of AMPK activation required the presence of the tumor suppressor LKB1 (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004), also introduced the idea (discussed further below) that AMPK activators might be useful in the prevention and/or treatment of cancer.

The major aim of this review is to discuss the various drugs and xenobiotics that regulate AMPK, to clarify the various mechanisms by which they achieve this, and to discuss the evidence that these agents might be useful in the treatment of obesity, type 2 diabetes, and cancer.

AMPK: Subunit Structure and Regulation

AMPK appears to exist in all eukaryotes as heterotrimeric complexes composed of a catalytic α subunit and regulatory β and γ subunits (Figure 1). In mammals all three subunits have multiple isoforms (α1, α2; β1, β2; γ1, γ2, and γ3) encoded by distinct genes, and all twelve possible heterotrimeric combinations are able to form when these are coexpressed in cells. The functions of the different subunit isoforms remain unclear, although there is tissue-specific expression of some isoforms, and (as discussed below) there is evidence that different isoforms may target complexes to specific subcellular locations. Moreover, some AMPK-activating drugs show selectivity for certain isoform combinations.

The Catalytic α Subunits

The catalytic subunits are encoded by two alternate genes in mammals (*PRKAA1* and *PRKAA2* in humans). The α1 isoform appears to be universally expressed, whereas α2 is more abundant in tissues such as skeletal and cardiac muscle, and appears to be absent in cells of the blood and endothelial cell lineages. Both isoforms contain conventional serine/threonine kinase domains at the N terminus, and their kinase activity is increased >100-fold by phosphorylation of a conserved threonine residue within the "activation loop," a sequence segment also critically involved in regulation of many other kinases (Johnson et al., 1996). This threonine residue is usually referred to as Thr-172 because of its position in the original rat sequence (Hawley et al., 1996), although the exact residue numbering may differ in other species. The upstream kinases that phosphorylate this site were first identified by whole kinome screens in yeast (*Saccharomyces cerevisiae*), where three protein kinases with partially redundant functions were identified (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003).
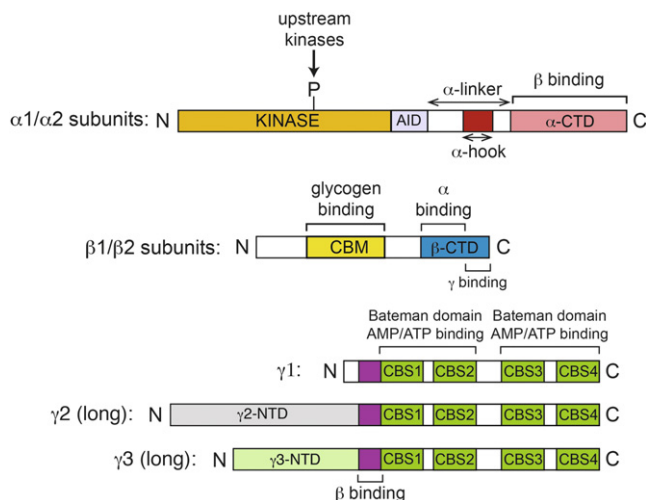
Figure 1. Domain Structure of the α, β, and γ Subunits of AMPK and Their Isoforms

α-subunits: AID, autoinhibitory domain; α-CTD, α-terminal domain; β-subunits: CBM, carbohydrate-binding module; β-CTD, C-terminal domain; γ subunits: CBS1, CBS2, CBS3, CBS4, cystathione β-synthase repeats; γ2-NTD, N-terminal extension of "long" version of γ2 subunit; γ3-NTD, N-terminal extension of "long" version of γ3 subunit.

Although there were no clear orthologs of these in the human kinome, searching using their kinase domain sequences revealed that the closest matches were to LKB1 and the two isoforms of the calmodulin-dependent kinase kinases, CaMKKα and CaMKKβ. Indeed, all three of these can act as upstream kinases in mammalian cells, although CaMKKα is less active that the other two (Hawley et al., 2003, 2005; Woods et al., 2003, 2005; Shaw et al., 2004; Hurley et al., 2005). Thr-172 can also be phosphorylated by transforming growth factor-β activated protein kinase-1 (TAK1) (Momcilovic et al., 2006; Herrero-Martín et al., 2009), although the exact physiological significance of that mechanism remains uncertain.

The discovery of LKB1 as an upstream kinase was particularly interesting because LKB1 had previously been identified as the product of a tumor suppressor gene (Alessi et al., 2006), and AMPK was its first downstream target to be identified. It was subsequently discovered that, in addition to the α1 and α2 subunits of AMPK, LKB1 also phosphorylates and activates 12 other enzymes with kinase domains closely related to those of AMPK, now known as the AMPK-related kinase or *ARK* family (Lizcano et al., 2004). LKB1 is only active in the form of a complex with two other subunits, STRAD and MO25 (Hawley et al., 2003). This complex appears to be constitutively active (Sakamoto et al., 2004), with Thr-172 phosphorylation normally being regulated instead by binding of ligands to the substrate, AMPK (see below). By contrast, CaMKKβ appears to have a very low basal activity against AMPK in intact cells and only phosphorylates Thr-172 in response to treatments that increase intracellular Ca^{2+} (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005).

The identities of the protein phosphatase(s) dephosphorylating Thr-172 are not fully resolved, although there is genetic evidence in *S. cerevisiae* that there are two phosphatases dephosphorylating this site. One is a complex between Glc7

(the single PP1 catalytic subunit) and its targeting subunit Reg1 (Sanz et al., 2000), whereas the other is Sit4, the ortholog of the mammalian phosphatase PP6 (Ruiz et al., 2011, 2012). In MIN6 cells (a mouse pancreatic β cell line) evidence from siRNA knockdowns suggests that a complex between PP1 and the targeting subunit PPP1R6 dephosphorylates Thr-172 and, despite no clear sequence relationship, DNA-encoding PPP1R6 complements a *reg1* mutant yeast strain (Garcia-Haro et al., 2010). It has also been reported that RNAi-based depletion of PPM1E (a member of the PPM family of protein phosphatases, which are not related to the PPP family containing PP1 and PP6) increases Thr-172 phosphorylation in HEK293 cells (Voss et al., 2011).

The kinase domain (KD) of AMPK is immediately followed by a small region termed the autoinhibitory domain (AID) (Figure 1). It was given this name because truncated α subunits containing the KD plus the AID have very low activity even when phosphorylated on Thr-172, whereas constructs containing the KD alone exhibit full activity (Göransson et al., 2007; Pang et al., 2007; Chen et al., 2009). In a crystal structure of a fission yeast KD-AID construct, the AID forms a small domain with three α helices, which interacts with the small and large lobes of the KD on the opposite face to the active site, appearing to constrain the kinase domain in a less active, "open" conformation. Mutations in the predicted KD:AID interface in a heterotrimeric complex from mammals also increased the basal activity in the absence of AMP, suggesting that the AID is involved in the mechanism of allosteric activation by AMP (Chen et al., 2009). However, in the structure of an active mammalian heterotrimeric complex, the AID appeared to be unstructured (Xiao et al., 2011), so its role in the overall regulation of the AMPK heterotrimer remains unclear.

The C-terminal region of the α subunit (α-CTD) forms a globular domain that interacts with the C-terminal domain of the β subunit and is thus essential for formation of the αβγ complex (Crute et al., 1998; Hudson et al., 2003; Xiao et al., 2007, 2011). The KD/AID and the α-CTD are joined by a linker peptide of extended conformation; in a recent structure (Xiao et al., 2011) this α-linker wraps around the γ subunit, rather like two arms stretching out from the KD/AID and the α-CTD that hold the γ subunit in a tight embrace. The "clasped hands" of these arms are formed by a structure called the "α hook," which contacts one of the regulatory nucleotides bound to the γ subunit (see below).

Several groups have reported that complexes containing the α2 isoform are enriched in the nucleus in different cell types (Salt et al., 1998; Turnley et al., 1999; Ai et al., 2002), although α1 complexes do not appear to be completely excluded from the nucleus. A short putative nuclear localization sequence (KKIR, residues 224–227) has been highlighted toward the C terminus of the α2 kinase domain (Suzuki et al., 2007); in α1 the equivalent sequence is KKIC. Interestingly, there are well-defined and functional nuclear export sequences at the C termini of both α1 and α2 (Kazgan et al., 2010). It seems likely that both the α1 and α2 complexes shuttle in and out of the nucleus, although this has not been studied in detail.

The β Subunits

The β1 and β2 subunits are encoded by two alternate genes in mammals (*PRKAB1* and *PRKAB2* in humans). Both are widely

expressed, although β1 predominates in some tissues, such as rodent liver (Thornton et al., 1998). As already indicated, their C-terminal domains (β-CTD) interact with the α-CTD, and the β subunits then terminate with a short sequence that associates with the γ subunit via an inter-subunit β sheet (Amodeo et al., 2007; Townley and Shapiro, 2007; Xiao et al., 2007, 2011). The β-CTD thus forms the "core" of the heterotrimeric complex, holding the α and γ subunits together. Both β subunits are subject to myristoylation at their N termini (Oakhill et al., 2010); this is required for the ability of AMP and ADP binding to enhance phosphorylation of Thr-172 (Oakhill et al., 2010, 2011). It has also been proposed that myristoylation affects the translocation of AMPK complexes containing α2 between the cytoplasm and the nucleus (Suzuki et al., 2007).

Along with the β-CTD, the other β subunit region that is well conserved between species is the central carbohydrate-binding module (CBM, Figure 1), originally termed the glycogen-binding domain (Hudson et al., 2003; Polekhina et al., 2003). This module is related to CBMs found in other proteins, where they are often fused to catalytic domains involved in the metabolism of glycogen and starch and thus colocalize the catalytic domains with their polysaccharide substrates (Machovic and Janecek, 2006). Although the CBM was missing from two partial structures of the mammalian heterotrimer (Xiao et al., 2007, 2011), it was present in a structure from budding yeast (Amodeo et al., 2007). The CBM from rat β1 has also been crystallized on its own, and conserved residues involved in carbohydrate binding have been identified (Polekhina et al., 2005). The CBM of β2 has a higher affinity for glycogen than that of β1 (Koay et al., 2010).

Although it is clear that the β subunit CBMs cause AMPK complexes to bind to the surface of glycogen particles (Hudson et al., 2003; Polekhina et al., 2003; Bendayan et al., 2009), the physiological role of this is not completely resolved. However, glycogen synthase (GS), the key enzyme of glycogen synthesis that is also bound to the glycogen particle, is a substrate for AMPK. Both the muscle and liver isoforms of glycogen synthase are phosphorylated by AMPK at N-terminal sites that cause their inactivation (Carling and Hardie, 1989; Jørgensen et al., 2004; Bultot et al., 2012), suggesting that one function of the CBM is to colocalize AMPK with this downstream target. Glycogen and synthetic oligosaccharides based on the structure of glycogen also inhibit AMPK, although inhibition by glycogen varies with different preparations of the polysaccharide (McBride et al., 2009). One hypothesis is that AMPK acts as a glycogen sensor that regulates glycogen synthesis according to certain aspects of glycogen structure, although the latter are not yet well defined.

The γ Subunits

The γ1, γ2, and γ3 subunit isoforms are encoded by three alternate genes in mammals (PRKAG1-3 in humans). The γ1 isoform appears to be expressed ubiquitously, whereas γ2 and γ3 are most abundant in muscle. The γ2 and γ3 isoforms contain N-terminal extensions that are unrelated to each other and are not present in γ1 (Figure 1); in both cases these extensions can also occur as "long" and "short" forms because of the use of alternate transcriptional start sites (Lang et al., 2000; Yu et al., 2004). Recently, a third γ2 variant (γ2-3B) has been identified that lacks the amino acids encoded by exons 1–3 in the "long" form but has 32 unique residues encoded by an alternate exon, 3B (Pinter et al., 2012). Both at the mRNA and the protein level, γ2 is expressed in adults in both cardiac and skeletal muscle, whereas γ3 appears to be restricted to skeletal muscle (Cheung et al., 2000; Milan et al., 2000; Yu et al., 2004). However, AMPK activity has been immunoprecipitated from other tissues, such as brain, using an anti-γ3 antibody (Cheung et al., 2000). Both γ3 and γ2-3B are also expressed in fetal mouse heart, and γ2-3B appears to account for almost half of total γ2 protein in adult mouse heart (Pinter et al., 2012).

All three γ subunit isoforms contain at their C termini four tandem repeats of a sequence known as a cystathione β-synthase (CBS) repeat; these are numbered CBS1-4 from the N terminus to the C terminus (Figure 1). CBS1 is immediately preceded by a short sequence involved in the interaction with the β subunit (Viana et al., 2007; Xiao et al., 2007). CBS repeats occur in around 20 other proteins in the human genome, invariably as tandem pairs. Single tandem pairs form structures known as Bateman domains, which are regulatory domains that bind adenosine-containing ligands, either AMP, ADP, ATP, or, in the case of cystathione β-synthase itself, S-adenosyl methionine (Scott et al., 2004). The γ subunits of AMPK are unusual in having four rather than two repeats, and these form two Bateman domains that assemble in a head-to-head manner (Amodeo et al., 2007; Townley and Shapiro, 2007; Xiao et al., 2007). Because of the 4-fold symmetry, there are four clefts that could potentially bind adenine nucleotides; these are numbered according to the number of the CBS repeat bearing an aspartate residue that interacts with the nucleotide ribose ring. Site 2 is unoccupied (CBS2 lacks an aspartate), whereas site 4 contains AMP that is very tightly bound and nonexchangeable and which may play a structural role. Sites 1 and 3 are the sites at which AMP, ADP, and ATP bind reversibly in competition with each other. They bind AMP, ADP, and ATP with quite similar affinity, although site 1 appears to have a higher affinity for all three nucleotides (Scott et al., 2004; Xiao et al., 2011).

Canonical Regulation of AMPK by Adenine Nucleotides and Calcium Ions

When cells are under metabolic stress (e.g., because of shortage of glucose or oxygen) or are treated with a pharmacological agent that inhibits ATP synthesis (e.g., almost any mitochondrial inhibitor) or are under stress because of accelerated ATP consumption (e.g., because of activation of motor proteins during muscle contraction), there will be an increase in the cellular ADP:ATP ratio. In unstressed cells the concentration of AMP is usually very low because the high ATP:ADP ratio drives the adenylate kinase reaction (ATP + AMP ↔ 2ADP) toward ADP synthesis. However, whenever the ADP:ATP ratio rises during energetic stress, the highly reversible adenylate kinase reaction will be partially displaced toward synthesis of AMP, causing a large rise in AMP. Increases in ADP and AMP (relative to ATP) are thus signals that cells are under energetic stress, and are the key signals that switch on AMPK in its canonical, energy-sensing role. In unstressed cells, the two exchangeable sites on the γ subunit (sites 1 and 3) are probably occupied by ATP. During a mild stress both ADP:ATP and AMP:ATP ratios will rise, but because AMP concentrations will still be low compared with ATP and ADP, it is most likely ADP that initially displaces ATP. The majority of cellular ATP is present as
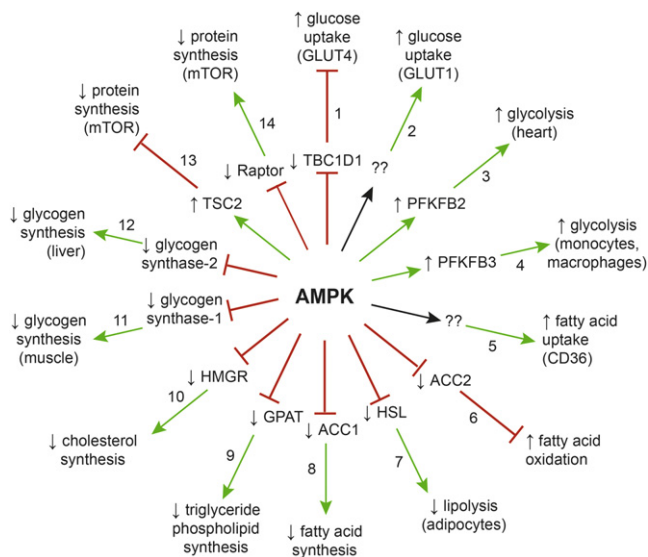
Figure 2. Acute Metabolic Effects of AMPK Activation
Upward/downward arrows indicate that the target protein (inner wheel) or pathway (outer wheel) are activated/inactivated. See text for numbering and key to acronyms. Green arrows on the "spokes" of the wheel indicate activation, red lines with a bar at the end indicate inhibition, and black arrows and "??" indicate that the direct target protein that explains the effect, is not known.
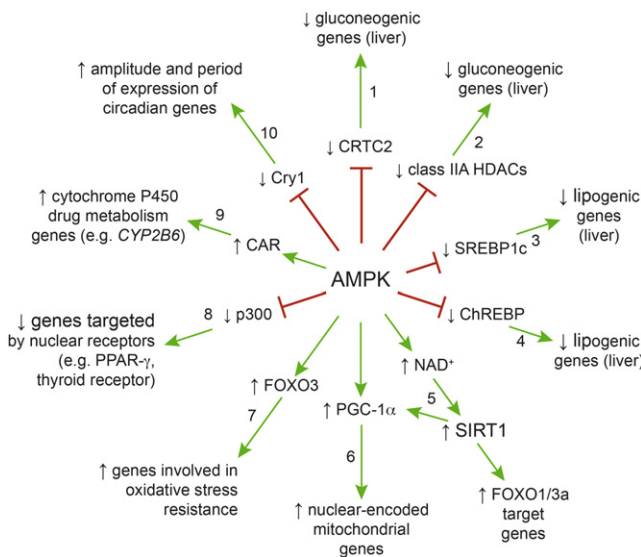

Figure 3. Effects of AMPK Activation on Transcription
Upward/downward arrows indicate that the target transcription factor or co-activator (inner wheel) or target genes (outer wheel) are activated/inactivated. Note that SIRT1 (on spoke 5) deacetylates both FOXO family transcription factors and the transcriptional coactivator PGC-1α.

a complex in which the β and γ phosphates bind a Mg^{2+} ion ($Mg.ATP^{2-}$), which has a lower affinity for the γ subunit than free ATP^{4-} (Xiao et al., 2011). Thus, when binding to the AMPK-γ subunits ADP does not have to compete with total ATP but only with the less abundant ATP^{4-}. Given its low concentration during a mild stress, AMP may only displace ADP or ATP as stress becomes more severe and its concentration rises further.

Binding of ADP or AMP causes a conformational change in the AMPK complex that both promotes phosphorylation of Thr-172 (Hawley et al., 1995; Oakhill et al., 2011) and inhibits dephosphorylation (Davies et al., 1995; Suter et al., 2006; Xiao et al., 2011). Complete phosphorylation can cause >100-fold activation, although the degree of phosphorylation may never come close to this in vivo. It is important to note that ADP and AMP appear to affect phosphorylation and dephosphorylation entirely by binding to AMPK, rather than by regulating the upstream kinase or phosphatase. The effect of phosphorylation is further amplified by allosteric activation, which is triggered only by binding of AMP, not ADP. It has been proposed (Xiao et al., 2011) that the allosteric effect is due to binding of AMP to the high affinity site (site 1), whereas the effect on phosphorylation state is mediated by binding of ADP or AMP to the lower affinity site (site 3). However, mutations of the aspartate residues that bind the nucleotide ribose rings in sites 1 and 3, and even the nonexchangeable site (site 4), have been found to reduce both responses (Oakhill et al., 2011). Thus, there appear to be complex, co-operative interactions between the nucleotides bound at these three sites, which are not yet fully understood. It has also been proposed that protection against Thr-172 dephosphorylation is mediated by the "α hook" region of the α subunit; a structure of a complex with AMP bound at site 3 suggests that the α hook would be able to interact with AMP

or ADP at this site but that the extra phosphate on ATP might prevent this interaction (Xiao et al., 2011).

The other "canonical" activation mechanism involves the phosphorylation of Thr-172 by CaMKKβ in response to a rise in intracellular Ca^{2+}. This occurs, for example, in response to depolarization of neurons (Hawley et al., 2005), activation of receptors coupled to phosphatidylinositol-specific phospholipases, and hence Ca^{2+} release, in various cell types (Stahmann et al., 2006; Thornton et al., 2008; Yang et al., 2011), and activation of the antigen receptor in T lymphocytes (Tamás et al., 2006). This mechanism can occur in the absence of any change in cellular nucleotides, although the two canonical mechanisms (AMP/ADP and Ca^{2+}) can also synergize with each other (Fogarty et al., 2010).

Downstream Targets and Use of AMPK Activators in Diabetes and Cancer

AMPK phosphorylates serine residues surrounded by a well-defined recognition motif (Scott et al., 2002; Gwinn et al., 2008). Space constraints do not permit a comprehensive discussion of the entire literature on downstream targets, and the reader is referred to other recent reviews (Hardie, 2007, 2011; Steinberg and Kemp, 2009; Mihaylova and Shaw, 2011; Hardie et al., 2012). However, Figures 2, 3, and 4 summarize some well-characterized targets that are also briefly discussed below, with one or two representative references provided in each case.

Figure 2 involves targets involved in the acute regulation of metabolism. Progressing clockwise around the spokes of the "wheel," starting at 12 o'clock:

(1) AMPK activates GLUT4-mediated glucose uptake in muscle via phosphorylation of TBC1D1 (Frøsig et al., 2010), a Rab-GAP protein that, under basal conditions, inhibits fusion of intracellular GLUT4-containing vesicles
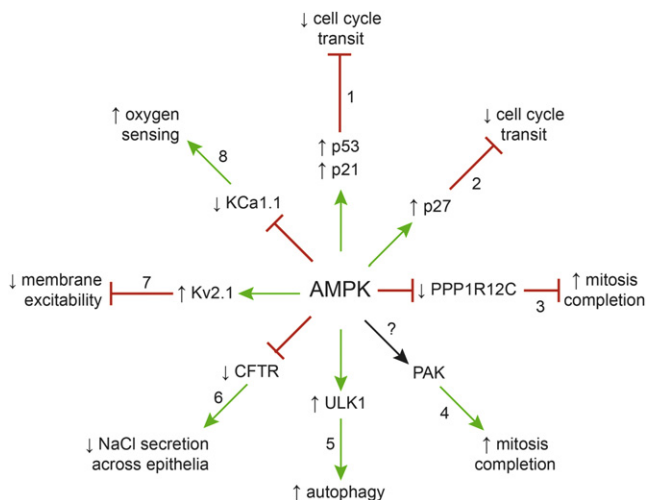
Figure 4. Effects of AMPK Activation Other than on Metabolism and/or Transcription
Upward/downward arrows indicate that the functions of the target protein (inner wheel) or target process (outer wheel) are activated/inactivated.

with the plasma membrane. Phosphorylation by AMPK triggers 14-3-3 binding, causing TBC1D1 to dissociate from the intracellular vesicles;

(2) AMPK also activates glucose uptake in other cells by activation of GLUT1 already present on the membrane (Barnes et al., 2002). However, GLUT1 does not appear to be a direct target for AMPK, and the molecular mechanism remains unknown;

(3) AMPK activates glycolysis in cardiac myocytes by phosphorylation and activation of the PFKFB2 isoform of 6-phosphofructo-2-kinase, which catalyzes the synthesis of fructose-2,6-bisphosphate, a key activator of the glycolytic enzyme 6-phosphofructo-1-kinase (Marsin et al., 2000);

(4) AMPK also activates glycolysis in monocytes and macrophages by phosphorylation and activation of the PFKFB3 isoform of 6-phosphofructo-2-kinase. This may allow macrophages to generate ATP efficiently in regions of infection or injury that are hypoxic (Marsin et al., 2002).

(5) AMPK activates fatty acid uptake via translocation of the transporter CD36 to the plasma membrane, although the molecular mechanism remains unknown (Habets et al., 2009);

(6) AMPK activates fatty acid oxidation by phosphorylating and inactivating the mitochondria-associated isoform of acetyl-CoA carboxylase (ACC2), thus lowering malonyl-CoA, an inhibitor of fatty acid uptake into mitochondria via the carnitine:palmityl-CoA transferase system (Merrill et al., 1997);

(7) Lipolysis (hydrolysis of triglyceride stores) in adipose tissue is catalyzed by desnutrin/ATGL, which removes the first fatty acid, and hormone-sensitive lipase (HSL), which removes the second (Zimmermann et al., 2004). AMPK inhibits lipolysis in rodent and human adipocytes by phosphorylation of HSL, antagonizing its activation by cyclic AMP-dependent protein kinase and its translo-

cation to the surface of the lipid droplet (Garton et al., 1989; Daval et al., 2005; Bourron et al., 2010). Because lipolysis is a catabolic pathway, it might seem illogical that AMPK should inhibit it. However, this may be a mechanism to limit the production during lipolysis of free fatty acids; if these are allowed to accumulate they recycle into triglycerides, creating a futile cycle that consumes ATP. Consistent with this, AMPK is activated in 3T3-L1 adipocytes by cyclic AMP-elevating agents, and this is associated with increases in cellular AMP:ATP ratios, but both effects are blunted by inhibition of lipolysis or of the acyl-CoA synthetase required for recycling of fatty acids back to triglycerides (Gauthier et al., 2008). Somewhat paradoxically, AMPK has been reported to phosphorylate and *activate* desnutrin/ATGL in mammalian cells (Ahmadian et al., 2011), although in nematode worms the enzyme appears to be inhibited by AMPK phosphorylation (Narbonne and Roy, 2009), which is more consistent with the known effects of AMPK on HSL function in mammals;

(8) AMPK inhibits fatty acid synthesis by directly phosphorylating and inactivating the cytosolic isoform of acetyl-CoA carboxylase (ACC1) (Davies et al., 1992); phosphospecific antibodies that recognize the key phosphorylation sites on both ACC1 and ACC2 are widely used as markers for AMPK activation.

(9) AMPK inhibits triglyceride and phospholipid synthesis by causing inactivation of the first enzyme involved in their synthesis, glycerol phosphate acyl transferase (GPAT); it remains unclear whether GPAT is a direct substrate for AMPK (Muoio et al., 1999);

(10) AMPK inhibits isoprenoid/cholesterol synthesis by direct phosphorylation and inactivation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) (Clarke and Hardie, 1990);

(11) AMPK inhibits muscle glycogen synthesis by phosphorylation and inactivation of glycogen synthase-1 (Jørgensen et al., 2004);

(12) AMPK inhibits liver glycogen synthesis by phosphorylation and inactivation of glycogen synthase-2 (Bultot et al., 2012);

(13) AMPK inhibits protein synthesis by phosphorylating tuberous sclerosis complex protein-2 (TSC2), causing Rheb (a small G protein that activates mammalian target-of-rapamycin complex-1, mTORC1) to be converted to its inactive GDP-bound form (Inoki et al., 2003); and

(14) AMPK also inhibits protein synthesis by directly phosphorylating Raptor, a subunit of the mTORC1 complex (Gwinn et al., 2008).

In addition to these acute effects on metabolism, AMPK also has numerous effects on transcription, which are summarized in Figure 3. Progressing clockwise around the spokes of the wheel, starting at 12 o'clock:

(1) AMPK phosphorylates cyclic AMP response element binding protein (CREB)-regulated transcription coactivator-2 (CRTC2, formerly called TORC2), causing it to bind

14-3-3 proteins, thus retaining it in the cytoplasm and inhibiting activation of genes encoding gluconeogenic enzymes in the liver (Koo et al., 2005);

(2) AMPK phosphorylates class IIa lysine deacetylases (HDAC-4, −5 and −7), causing them to bind 14-3-3 proteins that retain them in the cytoplasm and preventing formation of heterodimers with class I lysine deacetylases (e.g., HDAC3). The latter activate transcription of specific genes, including gluconeogenic genes, and this therefore represents a second mechanism (see 1 above) by which AMPK downregulates gluconeogenesis (Mihaylova et al., 2011);

(3) AMPK phosphorylates sterol response element binding protein-1c (SREBP-1c), preventing its proteolytic processing and nuclear translocation and thus inhibiting transcription of lipogenic genes, including those encoding ACC-1 and fatty acid synthase (Li et al., 2011);

(4) AMPK phosphorylates carbohydrate response element binding protein (ChREBP), inhibiting its DNA binding and consequent activation of transcription of lipogenic genes, including the liver isoform of pyruvate kinase (Kawaguchi et al., 2002);

(5) AMPK activates the class III deacetylase SIRT1, which deacetylates FOXO-1 and −3, activating genes modulated by those transcription factors (see 7 below). SIRT1 also deacetylates and activates PGC-1α (see 6 below). The mechanism by which AMPK activates SIRT1 remains uncertain, although it has been proposed that effects of AMPK on metabolism increase the cellular concentration of NAD^+, a cosubstrate of SIRT (Cantó et al., 2009);

(6) AMPK activation causes phosphorylation of peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α), a "master regulator" of expression of mitochondrial genes that triggers mitochondrial biogenesis. The phosphorylation sites identified do not closely conform to the AMPK recognition motif so this may not be a direct effect, but this phosphorylation is believed to activate transcription of PGC-1α by a positive feedback loop acting on its own promoter (Jäger et al., 2007). In addition, PGC-1α is deacetylated and activated by SIRT1 in response to AMPK activation (see 5 above);

(7) AMPK directly phosphorylates several sites on the transcription factor FOXO3 (see also 5 above), activating transcription of many genes, including genes involved in resistance to oxidative stress (Greer et al., 2007);

(8) AMPK phosphorylates the transcriptional coactivator p300, reducing its interaction with, and thus inhibiting transcription induced by, nuclear hormone receptors, such as PPAR-γ, and the thyroid hormone receptor (Yang et al., 2001);

(9) AMPK activation induces transcription from genes modulated by the constitutive androstane receptor (CAR), particularly those (e.g., CYP2B6) encoding cytochrome P450 enzymes that metabolize xenobiotic compounds, such as phenobarbital (Rencurel et al., 2005); and

(10) AMPK phosphorylates the cryptochrome protein Cry1, triggering its degradation and thus relieving its inhibitory effects on the BMAL1:CLOCK heterodimer,

a transcription factor involved in the establishment of circadian rhythms of gene expression (Lamia et al., 2009).

In addition to the effects on expression of messenger RNAs described above, AMPK phosphorylates the RNA polymerase I transcription factor TIF-1A, thus inhibiting the production of ribosomal RNA (Hoppe et al., 2009). In proliferating cells, rRNA accounts for around 80%–90% of all RNA synthesis, so this mechanism would conserve ATP, while also inhibiting cell growth under conditions of energy limitation.

Figure 4 summarizes regulation by AMPK of some targets involved in cellular processes other than metabolism or transcription. Progressing clockwise around the spokes of the wheel, starting at 12 o'clock:

(1) AMPK activation triggers phosphorylation of p53 at Ser-15 (human sequence numbering). This site does not fit the AMPK recognition motif, so this may not be a direct phosphorylation, but it causes p53 stabilization and thus activates transcription of p21[WAF1], a cyclin-dependent kinase inhibitor that triggers G1:S phase cell-cycle arrest (Imamura et al., 2001; Jones et al., 2005);

(2) AMPK activation causes phosphorylation of another cyclin-dependent kinase inhibitor, p27[KIP1], at a C-terminal threonine residue; this site does not fit the AMPK consensus motif, so this may not be a direct phosphorylation. However, it causes p27[KIP1] stabilization, inducing G1:S phase cell cycle arrest and autophagy (Liang et al., 2007);

(3) AMPK phosphorylates PPP1R12C, a regulatory subunit of protein phosphatase-1 (PP1) that targets PP1 to dephosphorylate myosin regulatory light chain (MRLC). Phosphorylation triggers binding of 14-3-3 proteins to PPP1R12C, and this may prevent the phosphatase complex from dephosphorylating MRLC. Phosphorylation of PPP1R12C appears to be necessary for completion of mitosis, as discussed further under (4) below (Banko et al., 2011);

(4) AMPK also phosphorylates PAK2, a protein kinase that phosphorylates MRLC at the N-terminal site that is dephosphorylated by the PPP1R12C:PP1 complex; phosphorylation of MRLC activates the ATPase activity of nonmuscle myosin, triggering the movement of this motor protein along actin fibers. Although it is not yet clear whether phosphorylation by AMPK regulates PAK2 activity, MRLC phosphorylation is required for completion of mitosis (Banko et al., 2011). This helps to explain defects in chromosomal segregation observed in Drosophila larvae that lack AMPK (Lee et al., 2007) and is consistent with findings that AMPK is found to be phosphorylated at the mitotic apparatus in M phase cells (Vazquez-Martin et al., 2011). Although mitosis is an energy-requiring event and it might appear paradoxical that AMPK should activate it, it may be crucial for cells undergoing mitosis to complete the process if energy stress occurs while it is happening so that they can arrest in a more orderly manner in the following G1 phase instead;

(5) AMPK phosphorylates the protein kinase ULK1, triggering autophagy. Autophagy is critical to recycle amino acids and other components in cells starved of nutrients, while autophagy of mitochondria (mitophagy) recycles components from damaged mitochondria that are no longer efficiently producing ATP (Egan et al., 2011);

(6) AMPK phosphorylates and inactivates the cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel gated by ATP that is involved in *trans*-epithelial salt transport (Hallows et al., 2003). Salt transport is an energy-requiring process, so this would represent an energy-conserving mechanism;

(7) AMPK phosphorylates the voltage-gated, delayed rectifier K^+ channel, Kv2.1, in its C-terminal tail; phosphorylation sensitizes the channel so that it opens at lower extents of membrane depolarization. This reduces membrane excitability and the consequent firing of action potentials in central neurons. Because firing of action potentials in the brain is a major consumer of energy, this mechanism may protect neurons during metabolic stress (Ikematsu et al., 2011); and

(8) AMPK phosphorylates another voltage-gated K^+ channel, $K_{Ca}1.1$ (also known as BK_{Ca}); in this case the phosphorylation inactivates the channel. This is believed to be part of the oxygen-sensing mechanism in type I cells of the carotid body: hypoxia activates AMPK, causing inactivation of $K_{Ca}1.1$ that then facilitates opening of voltage-gated Ca^{2+} channels. Increases in cell Ca^{2+} cause neurotransmitter release from the type I cells, triggering firing of the carotid sinus nerve that signals to the brain, where corrective changes in breathing patterns are initiated (Ross et al., 2011).

In summary, AMPK activation has numerous and complex effects on cell function, although these can mostly be viewed as mechanisms to restore cellular energy homeostasis when it becomes disrupted. By direct phosphorylation of metabolic enzymes and by effects on transcription, AMPK switches on catabolic pathways, such as the uptake of glucose and fatty acids, and their metabolism by mitochondrial oxidation and (in the case of glucose) by glycolysis. In addition, AMPK switches off biosynthetic pathways, such as the synthesis of glucose, glycogen, and lipids in the liver. By promoting muscle glucose uptake and metabolism and by inhibiting hepatic glucose production, AMPK activation can explain the antihyperglycemic actions of the drug metformin, which is used to treat type 2 diabetes and is discussed further below. In addition, type 2 diabetes is thought to be primarily caused by insulin resistance, which is strongly associated with excess triglyceride storage in liver and muscle. By switching off the synthesis of fatty acids and triglycerides and enhancing fat oxidation instead, AMPK activation might also explain the insulin-sensitizing actions of metformin.

The synthesis of fatty acids, triglycerides, cholesterol, RNA (especially rRNA), and proteins are all upregulated in rapidly proliferating cells, such as tumor cells. AMPK activation switches off all of these pathways and would therefore be expected to exert a cytostatic, antitumor effect, reinforced by its ability to cause cell-cycle arrest. These effects of AMPK might explain, at least in part, the tumor suppressor effects of the upstream kinase LKB1 (Alessi et al., 2006), as well as findings that metformin usage reduces the risk of cancer in diabetics (Evans et al., 2005) and that metformin and other AMPK activators (phenformin, A-769662, see below) delay the onset of tumorigenesis in mouse models (Huang et al., 2008). However, AMPK may represent a "double-edged sword" in cancer in that, once a tumor has arisen, it might exert a cell-autonomous effect to protect tumor cells against the actions of cytotoxic agents. AMPK activators might therefore be deleterious in the treatment (as opposed to the prevention) of cancer. Consistent with this idea, in an siRNA screen of protein kinases whose loss was synthetically lethal with c-Myc activation in U2OS cells, two major hits obtained were AMPK-α1 and the AMPK-related kinase ARK5 (NUAK1). These results suggest that these kinases help to protect cells against the metabolic stresses caused by c-Myc activation (Liu et al., 2012). Similarly, it has been suggested that AMPK protects cells against the metabolic stress caused by glucose deprivation and promotes anchorage-independent growth and solid tumor formation in vivo, by reducing NADPH consumption by lipid synthesis and thus preserving the nucleotide for protection against oxidative stress (Jeon et al., 2012).

Activation of AMPK by Drugs and Xenobiotics
In this section we review the mechanisms by which AMPK is activated by pharmacologically active agents. Some of these are drugs in clinical use in conventional medicine, whereas others are natural products present in traditional medicines or derived from food or beverages claimed to have "nutraceutical" properties. The majority of these were in use for these purposes before it was realized that they were activators of AMPK. An important tool in establishing their mechanisms of action was a pair of isogenic cell lines derived from HEK293 cells, which express AMPK complexes containing γ2 with or without a single substitution in site 3 (R531G) that renders the complex insensitive to the effects of ADP and AMP on phosphorylation (Hawley et al., 2010). The mutant cells will be referred to below as RG cells. Agents that act by increasing cellular ADP or AMP fail to activate AMPK in the RG cells, whereas agents that act by other mechanisms (e.g., the Ca^{2+} ionophore A23187, which increases intracellular Ca^{2+} and activates CaMKKβ) activate AMPK in the RG cells to the same extent as in the wild-type cells. Other ancillary approaches used in this study were to measure changes in ADP:ATP ratio by capillary electrophoresis of acid extracts and the use of an extracellular flux analyzer to measure oxygen uptake (Hawley et al., 2010).

5-Aminoimidazole-4-Carboxamide Riboside
5-Aminoimidazole-4-carboxamide riboside (AICA riboside) is an adenosine analog taken up into cells by adenosine transporters and phosphorylated by adenosine kinase to the equivalent ribotide, ZMP (Figure 5), which mimics all three effects of AMP on the AMPK complex (Corton et al., 1995). Because ZMP binds to site 3 on the γ subunit in a similar manner to AMP (Day et al., 2007), AICA riboside does not activate AMPK in RG cells, although, unlike AMPK activators inhibiting mitochondrial function, it does not cause changes in ADP:ATP ratio or oxygen uptake (Hawley et al., 2010). Although ZMP is ≈50-fold less potent as an activator than AMP, AICA riboside activates
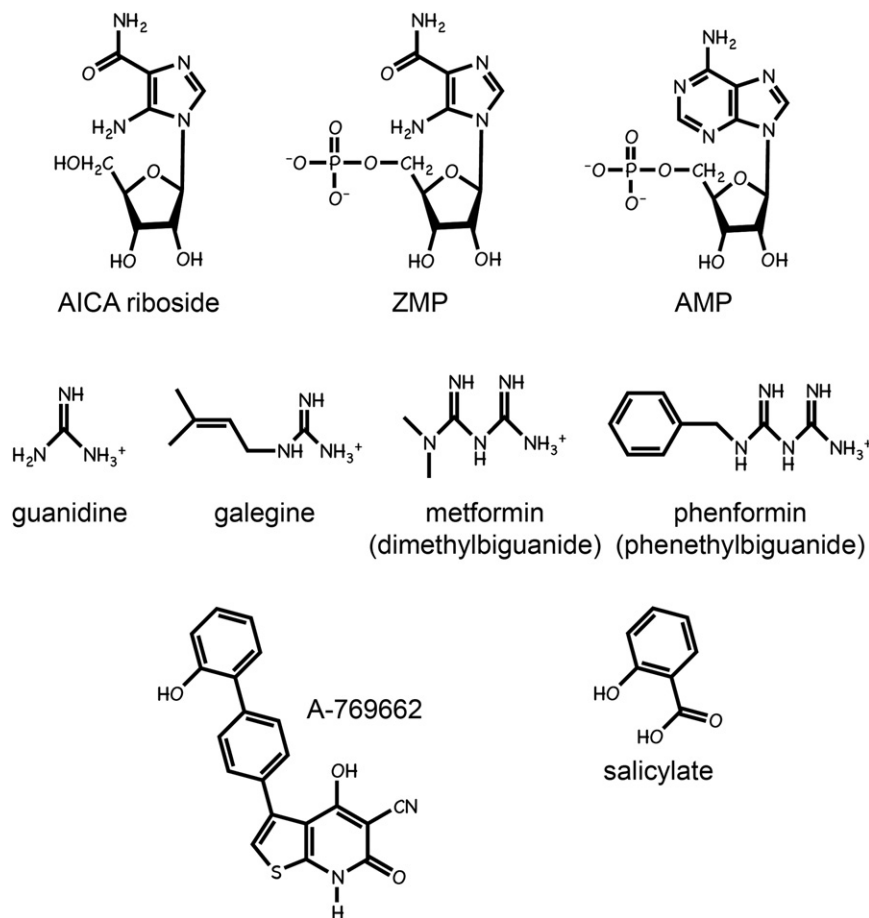
Figure 5. Structures of Some AMPK-Activating Compounds and Related Compounds

AICA riboside

ZMP

AMP

guanidine

galegine

metformin (dimethylbiguanide)

phenformin (phenethylbiguanide)

A-769662

salicylate

AMPK in most cells because ZMP can accumulate to millimolar concentrations within the cell (Corton et al., 1995). Because it is effective in vivo (although it is not orally available and has to be injected), AICA riboside has been much used to identify physiological processes regulated by AMPK in animal models.

Another interesting feature of AICA riboside is that, under the name "acadesine," it was used in small-scale clinical trials in humans undergoing coronary artery bypass graft surgery, where it appeared to provide some benefit (Mangano, 1997). These trials were undertaken before it was realized that AICA riboside activated AMPK, and at the time it was viewed as a means to rapidly replenish the total cellular adenine nucleotide content. Another interesting point is that ZMP is a naturally occurring intermediate in cellular purine nucleotide synthesis and is metabolized by the enzyme AICAR transformylase, which catalyzes the penultimate step in the synthesis of the purine nucleotide IMP. AICA riboside tends to work better as an AMPK-activating agent in quiescent, primary cells than in rapidly proliferating cells, possibly because the latter have a higher rate of purine synthesis, so they more rapidly metabolize ZMP. Consistent with this, the anticancer agent methotrexate (an antifolate drug that inhibits AICAR transformylase) sensitizes tumor cells to the AMPK-activating and growth-inhibitory effects of AICA riboside (Beckers et al., 2006). Pemetrexed, another antifolate drug used in the treatment of lung cancer, is a more selective inhibitor of AICAR transformylase, and it can cause accumula-

tion of ZMP and activation of AMPK even in the absence of exogenous AICA riboside (Racanelli et al., 2009). These findings raise the possibility that some effects of antifolate drugs used to treat cancer might be mediated by AMPK.

Metformin, Phenformin, and Galegine

Metformin is currently the first choice drug for treatment of type 2 diabetes. Because of the very high prevalence of this disorder, it is currently prescribed to more than 100 million people, or more than 1% of the world population. Metformin is derived from guanidine which, along with galegine (Figure 5), is a natural product found in the plant *Galega officinalis* (also known as Goat's Rue). Goat's Rue was described in the 18th century as an herbal medicine to treat "symptoms such as thirst and frequent urination" (Hill, 1772). Interestingly, it is classed as a noxious weed in the United States because it is poisonous to herbivores, which presumably includes goats! Guanidine is quite toxic, but galegine (an isoprenyl derivative) is a potent AMPK activator (Mooney et al., 2008) and was tested for treatment of diabetes in humans, with some success, in the 1920s (Muller and Reinwein, 1927). Metformin (dimethylbiguanide) is a biguanide derivative that was synthesized in the 1920s and found to be effective in lowering blood glucose in animals (Hesse and Taubmann, 1929). However, the success of insulin as a therapy for diabetes, developed in the same decade, put further studies of the biguanide drugs on hold. Metformin and its sister drug, phenformin (phenethylbiguanide, Figure 5), were finally introduced into clinical practice in the 1960s, by which time the distinction between type 1 and type 2 diabetes had been made. Phenformin was withdrawn from the US market in 1978 due to a rare but life-threatening side effect of lactic acidosis, but use of metformin has greatly increased, particularly when impressive results were reported in the UK Prospective Diabetes Study (UK Prospective Diabetes Study (UKPDS) Group, 1998).

Although the major effect of metformin was known to be a reduction of the high hepatic glucose production in type 2 diabetics (Hundal et al., 2000), its molecular mechanism remained obscure until it was reported that it activated AMPK in isolated hepatocytes (Zhou et al., 2001). The drug did not, however, activate AMPK or affect its phosphorylation by upstream kinases and phosphatases in cell-free systems (Hawley et al., 2002), suggesting that it acted indirectly. Metformin inhibits Complex I of the respiratory chain (El-Mir et al., 2000;

Owen et al., 2000), suggesting that it might activate AMPK by inhibiting ATP synthesis and thus increasing cellular ADP and AMP. This was confirmed when it was found that RG cells were completely resistant to the effects of metformin to activate AMPK; the same was observed for phenformin and galegine. As expected from this mechanism, all three agents also increased the cellular ADP:ATP ratio and inhibited oxygen uptake (Hawley et al., 2010). There remains some controversy as to the extent to which the therapeutic actions of metformin are mediated by AMPK. In favor of this idea, mice with a conditional liver knockout of LKB1, where liver AMPK is no longer activated by metformin, failed to display the antihyperglycemic effects of metformin (Shaw et al., 2005). However, mice with a conditional liver knockout of both AMPK catalytic subunits (α1 and α2) still displayed some responses to metformin in vivo, and acute inhibition of glucose production by metformin in hepatocytes isolated from the mice was still observed (Foretz et al., 2010). A potential explanation of the latter effect is that fructose-1,6-bisphosphatase, one of the key enzymes of gluconeogenesis, is inhibited by AMP. By inhibiting mitochondrial respiration and increasing AMP and ADP, metformin could clearly also modulate AMP- or ADP-sensitive enzymes other than AMPK, such as fructose-1,6-bisphosphatase.

Thiazolidinediones

Representatives of another major class of anti-diabetic drug, the thiazolidinediones, including rosiglitazone (Fryer et al., 2002), pioglitazone (Saha et al., 2004) and troglitazone (LeBrasseur et al., 2006), also activate AMPK in intact cells. Although thiazolidinediones have a known direct target, i.e., the transcription factor PPAR-γ, activation of AMPK in mammalian tissues appears to be too rapid to be explained by effects on transcription (LeBrasseur et al., 2006). One major effect of thiazolidinediones, in this case acting via PPAR-γ, is thought to be the induction and release of the insulin-sensitizing hormone, adiponectin, from adipocytes. Consistent with this, markers of insulin resistance were improved by treatment with pioglitazone (10 mg/kg) in genetically obese, leptin-deficient (ob/ob) mice, but this effect was lost if the adiponectin gene was also knocked out (Kubota et al., 2006). Interestingly, however, some amelioration of disease markers was still seen in the adiponectin null mice at slightly higher doses of pioglitazone (30 mg/kg) (Kubota et al., 2006); it is tempting to speculate that this adiponectin-independent effect was mediated by cell-autonomous activation of AMPK. In any case, the insulin-sensitizing effects of adiponectin are most likely mediated by the ability of the hormone to activate AMPK and stimulate fat oxidation in liver and muscle (Yamauchi et al., 2002). Thus, thiazolidinediones activate AMPK both by a humoral, adiponectin-dependent mechanism and by a cell-autonomous, adiponectin-independent mechanism. It is also clear that the latter is caused (like the effect of metformin) by inhibition of Complex I of the respiratory chain (Brunmair et al., 2004). Thus, thiazolidinediones fail to activate AMPK in RG cells, increase the cellular ADP:ATP ratio, and inhibit oxygen uptake (Fryer et al., 2002; Hawley et al., 2010).

2-Deoxyglucose

2-Deoxyglucose (2DG) is taken up into cells by glucose transporters and converted by hexokinase to 2-deoxyglucose-6-phosphate, which is not metabolized by glycolysis beyond that point. Although usually described as an inhibitor of glycolysis,

it may activate AMPK in part by depleting ATP due to its rapid and uncontrolled phosphorylation by hexokinase. As expected from this mechanism, 2DG caused increases in ADP:ATP and failed to activate AMPK in RG cells, although unlike AMPK activators acting on mitochondrial function it did not inhibit oxygen uptake (Hawley et al., 2010). Interestingly, 2DG can be metabolized to some extent by the pentose phosphate pathway, and it has recently been suggested that the generation of NADPH by this route could represent an AMPK-independent mechanism by which 2DG protects cells against the effects of glucose deprivation (Jeon et al., 2012).

A-769662 and Salicylate

Given that AMPK is an attractive target for drugs aimed at type 2 diabetes, it is not surprising that pharmaceutical companies have conducted high-throughput screens using their compound libraries. Abbott Laboratories developed a thienopyridone compound, A-769662 (Figure 5), which caused allosteric activation of purified AMPK in cell-free assays. Although the oral availability of this compound is poor, when injected intraperitoneally it had metabolic effects expected for an AMPK activator, that is, increased fat oxidation in normal rats and reduced weight gain, decreased plasma glucose and triglycerides, and decreased liver triglycerides in obese (ob/ob) mice (Cool et al., 2006). Interestingly, A-769662 mimics at least two of the effects of AMP on the AMPK system, that is, allosteric activation and protection against Thr-172 dephosphorylation, yet it is clear that it binds at different site(s). Thus, it did not displace [^{3}H] AMP from the AMPK-γ2 subunit in a scintillation proximity assay (Göransson et al., 2007), and did not activate AMPK complexes containing the β2 subunit (Scott et al., 2008), or complexes in which the β1 subunit carried an S108A mutation (Sanders et al., 2007), both of which remain fully sensitive to activation by AMP. Moreover, A-769662 still activates AMPK in RG cells, unlike agents that increase AMP and ADP (Hawley et al., 2010, 2012).

Why should the AMPK complex have a distinct binding site for A-769662, a purely synthetic molecule? It seemed likely there is a natural ligand that binds at that site, and one such natural ligand is salicylate (Figure 5). Salicylate was traditionally extracted from willow bark (Stone, 1763), although it is in fact produced by many plants as a hormone used in a defense response triggered by infection by fungi and other pathogens (Reymond and Farmer, 1998). Acetyl salicylate (ASA, trade name aspirin) is a derivative that is easier to take orally than salicylate, although it is rapidly broken down to salicylate once it enters the circulation (Higgs et al., 1987). Whereas cyclo-oxygenases (COX1 and COX2) are the established targets for aspirin, it has been reported recently that salicylate (although not aspirin) is a direct activator of AMPK at concentrations that are reached in human plasma after oral treatment with high-dose aspirin (Hawley et al., 2012). Although a much smaller molecule and a less potent activator of AMPK than A-769662, salicylate shows some structural resemblance to it (Figure 5). It is clear that salicylate binds at a site that overlaps with that used by A-769662, because (1) both compounds cause allosteric activation, with salicylate antagonizing the larger effect of A-769662, and (2) effects of both compounds are greatly reduced in AMPK complexes containing the β2 rather than the β1 isoform and are blocked by an S108A mutation in β1 (Hawley

et al., 2012). Moreover, both agents clearly have effects in vivo that are mediated by AMPK, because administration of either compound to mice led to an enhanced switch to fat oxidation on withdrawal of food, effects that were abolished in β1 knockout mice (Hawley et al., 2012). Although salicylate is not directly synthesized by humans, it has probably been a component of the diet since humans evolved, especially since at one time humans might have often eaten moldy vegetable matter. It is tempting to speculate that some of the apparent protection against cancer provided by regular aspirin use (Rothwell et al., 2012a, 2012b) might be mediated by AMPK.

Phenobarbital

Phenobarbital is another drug that was in clinical use long before it was known to activate AMPK. It is a particularly effective inducer of cytochrome P450 enzymes that catalyze oxidation of hydrophobic drugs, a key initial step in the process that renders them more water soluble for excretion. The gene *CYP2B6*, encoding one cytochrome P450 in humans, is induced by the nuclear hormone receptor CAR (constitutive androstane receptor). It has been reported that phenobarbital activates AMPK and that other AMPK activators (AICA riboside, metformin) also induce *CYP2B6* in a human hepatoma cell line, whereas in hepatocytes from AMPK null mice phenobarbital failed to induce *Cyp2b10*, the mouse ortholog (Rencurel et al., 2005, 2006). Thus, induction of this P450 enzyme by phenobarbital is mediated by AMPK activation. Experiments with wild-type and RG mutant HEK293 cells confirmed that phenobarbital activates AMPK, like biguanides and thiazolidinediones, because it is an inhibitor of the respiratory chain (Hawley et al., 2010). Mitochondria contain several very large multiprotein complexes, including the four respiratory chain complexes as well as the ATP synthase. It seems likely that there might be many binding sites within these complexes where hydrophobic, xenobiotic compounds could bind and cause some inhibition of ATP production. An interesting speculation is that AMPK is being used in this case as a general sensor of mitochondrial poisons that might need to be eliminated by cytochrome P450 metabolism.

Nutraceuticals and Traditional Medicines

Over recent years, a bewildering variety of natural plant products, some of which are present in foods and beverages used by humans, have been reported to activate AMPK in intact cells. Many of these are reputed to have health benefits and are being touted as "nutraceuticals." Equally, there have been several reports of AMPK activation by plant products that are components of traditional medicines. As well as galegine and salicylate (discussed above), these plant products include resveratrol from grapes and red wine (Baur et al., 2006), epigallocatechin-3-gallate from green tea and capsaicin from Chili peppers (Hwang et al., 2005), curcumin from turmeric (Lim et al., 2009), and garlic oil from *Allium sativum* (Ki et al., 2007), as well as two compounds derived from traditional Chinese medicine, berberine from Chinese Goldthread (Turner et al., 2008) and hispidulin from Snow Lotus (Lin et al., 2010). Although some of these can be classed as polyphenols, they have very varied structures and it was at first difficult to see how they could all be AMPK activators. However, it has now been shown that several inhibit mitochondrial function, either inhibiting the respiratory chain (berberine; Turner et al., 2008) or the F1 ATP

synthase (resveratrol and EGCG; Zheng and Ramirez, 2000), or acting as uncouplers (curcumin; Lim et al., 2009). Consistent with the idea that this is how they activate AMPK, berberine and resveratrol decreased the ATP:ADP ratio in cultured cells and failed to activate AMPK in cells expressing the AMP/ADP-insensitive RG mutant (Hawley et al., 2010).

Why should so many plants produce compounds that are mitochondrial inhibitors and hence AMPK activators? These compounds are generally secondary metabolites, and some appear to have roles in defense against pathogens or in deterring grazing by insect or animal herbivores. For example, resveratrol is produced in grapes in response to fungal infection (Romero-Pérez et al., 2001), whereas (as discussed above) Goat's Rue (a source of galegine) is classed as a noxious weed in the United States because it is poisonous to herbivores. As discussed in the previous section, the respiratory chain and ATP synthase might have many potential binding sites for hydrophobic, xenobiotic compounds, and the production of mitochondrial poisons might be a good general mechanism for plants to deter infection by pathogens or grazing by herbivores. However, a side effect of this is that many of these compounds would also be AMPK activators and hence may have medicinal uses.

PT1

Another small molecule activator of AMPK, termed PT1, was isolated via a screen of compounds that activated an AMPK-α1 construct containing just the KD and the AID (Pang et al., 2008). The compound activated the KD-AID construct and a complete α1β1γ1 heterotrimer but failed to activate a construct containing the KD alone, suggesting that its binding site was in the cleft between the KD and the AID. Consistent with this, PT1 caused phosphorylation of ACC in L6 myotubes without altering the AMP:ATP ratio and also promoted ACC phosphorylation and lowered lipid content in HepG2 cells (Pang et al., 2008).

Inhibition of AMP-Metabolizing Enzymes

An alternative approach to AMPK activation would be to inhibit the downstream metabolism of ADP and AMP, analogous to using inhibitors of phosphodiesterases, such as sildenafil, to increase cyclic nucleotides. Indeed, downregulation of expression of 5′-nucleotidases that convert AMP to adenosine, that is, NT5C2 in cultured human myotubes or NT5C1A in mouse muscle, activated AMPK and triggered ACC phosphorylation (Kulkarni et al., 2011). The validity of this approach has also been examined by overexpressing the 5′-nucleotidase cN-IA and the AMP deaminases AMPD1 and AMPD2 (which convert AMP to IMP) in HEK293 cells. Overexpression of all three enzymes decreased the activation of AMPK by oligomycin in the cells, although overexpression of cN-IA had the largest effect (Plaideau et al., 2012). Taken together, these results suggest that 5′-nucleotidase inhibitors might be useful as AMPK activators.

Conclusions

The evidence presented in this review suggests that AMPK-activating drugs are not only effective in treating type 2 diabetes but might also be effective in providing protection against cancer. If this appears "too good to be true," it does not stop there because it has recently also been suggested that AMPK activators reduce the accumulation of the Aβ peptides in cell

culture models of Alzheimer's disease and thus might be useful for treatment of that disease (Salminen et al., 2011). One caveat is that, although there is growing evidence that AMPK provides protection against the development of cancer, it is perhaps an unjustified leap of faith to say that AMPK activators would also be useful in the treatment of cancer once it has arisen. Indeed, there is now evidence that AMPK protect cells against the metabolic stress caused by overexpression of c-Myc (Liu et al., 2012), whereas the LKB1-AMPK pathway also protects cultured cells against metabolic stress caused by glucose starvation (Jeon et al., 2012). Thus, AMPK activators might be both a "friend and foe" in cancer, providing protection against its development, while at the same time making tumor cells harder to kill using cytotoxic agents once tumors have arisen.

Although it is generally easier to develop agents that are enzyme inhibitors rather than enzyme activators (as most indications for AMPK demand), the wide variety of agents that do activate AMPK when applied to intact cells show that development of AMPK activators is an achievable objective. Most of the existing agents activate AMPK indirectly by inhibiting ATP production, whether by inhibiting oxidative phosphorylation (metformin, phenformin, galegine, thiazolidinediones, resveratrol, berberine) or glycolysis (2-deoxyglucose), thus increasing cellular ADP:ATP and AMP:ATP ratios. Exceptions to this are (1) AICA riboside (acadesine), a prodrug that is converted to the active derivative, ZMP, which binds at the adenine nucleotide-binding sites; (2) A-769662 and salicylate, which bind to a common site that, although not fully defined, is distinct from the adenine nucleotide-binding sites; and (3) PT1, which appears to bind between the KD and the AID on the α subunit (Pang et al., 2008). Some of these, including acadesine and A-769662, have poor oral availability, but salicylate is orally available, and the best bet to develop novel AMPK activators may be to target the salicylate-binding site. Finally, one should not discount the utility of AMPK inhibitors, although the only chemical inhibitor targeting the kinase domain reported to date (compound C; Zhou et al., 2001) has very poor selectivity for AMPK (Bain et al., 2007). If AMPK does indeed protect tumor cells against cell death induced by cytotoxic agents, then combinations of AMPK inhibitors with cytotoxic drugs might be worth considering.

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