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AMPK: opposing the metabolic changes in both tumour cells and inflammatory cells?

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Abstract

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status that appears to have arisen during early eukaryotic evolution. In the unicellular eukaryote *Saccharomyces cerevisiae*, the AMPK orthologue is activated by glucose starvation and is required for the switch from glycolysis *(fermentation)* to oxidative metabolism when glucose runs low. In mammals, rapidly proliferating cells (including tumour cells) and immune cells involved in inflammation, both tend to utilize rapid glucose uptake and glycolysis (termed the *Warburg effect* or *aerobic glycolysis*) rather than oxidative metabolism to satisfy their high demand for ATP. Since mammalian AMPK, like its yeast orthologue, tends to promote the more energy-efficient oxidative metabolism at the expense of glycolysis, it might be expected that drugs that activate AMPK would inhibit cell proliferation and and hence cancer, as well as exerting anti-inflammatory effects. Evidence supporting this view is discussed, including our recent findings that AMPK is activated by the classical anti-inflammatory drug, salicylate.

Keywords

AMPK; AMP-activated protein kinase; cancer; metabolism; inflammation; innate immunity

Introduction

The AMP-activated protein kinase (AMPK) is a highly conserved sensor of cellular energy status that exists in the form of heterotrimeric complexes containing a catalytic α subunit combined with regulatory β and γ subunits [1, 2]. Genes encoding these three subunits can be readily identified in essentially all eukaryotic genomes, from single-celled protists through to humans [3]. AMPK therefore appears to have arisen early during eukaryotic evolution, and it is instructive to consider the role of the orthologue in a modern day unicellular eukaryote, the yeast *Saccharomyces cerevisiae.* The preferred carbon source for yeast is glucose, and if medium glucose is high the cells grow rapidly, using glycolysis to generate ATP even if oxygen is available. This process (*fermentation)* is closely related to the elevated glycolysis (the *Warburg effect* or *aerobic glycolysis*) exhibited by most rapidly proliferating mammalian cells and by some activated cells of the immune system, except that yeast generate ethanol rather than lactate as the end product. When glucose in the yeast medium becomes depleted their growth temporarily halts, but a second growth phase occurs after a lag. This lag represents the time required for expression of enzymes and transporters needed for mitochondrial oxidative metabolism, which are repressed during growth in high glucose; at the same time expression of glycolytic enzymes is reduced [4]. Once the proteins required for oxidative metabolism are expressed, they can be used for complete oxidation to CO_2 of the ethanol made during the fermentation phase. The yeast AMPK orthologue is activated by glucose deprivation [5, 6] and switches on expression of these oxidative genes. Thus, while it is not necessary for the initial glycolytic phase of growth, it is required for the second phase of growth when the yeast switch to oxidative metabolism [7]. The analogous

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event in mammalian cells would be the switch from the Warburg effect/aerobic glycolysis observed in proliferating tumour cells and some activated immune cells, to the oxidative metabolism that is utilized when these cells revert to a more quiescent state. Interestingly, mammalian AMPK promotes fatty acid oxidation [8], mitochondrial biogenesis [9], and the expression of genes required for oxidative metabolism [10]. This raises the intriguing prospect that AMPK activation would exert "anti-Warburg" or "anti-aerobic glycolysis" effects, and that drugs that activate AMPK might therefore protect against both cancer and inflammation. This idea provides the underlying theme for this review.

Structure and regulation of AMPK

In mammals, all three AMPK subunits occur as multiple isoforms encoded by alternate genes, giving rise to many possible heterotrimeric complexes. The catalytic subunits (α1/α2) contain serine/threonine kinase domains at the N-terminus, whose activity increases >100 fold when phosphorylated at a conserved threonine residue, corresponding to Thr-172 in rat α2. The principal kinase phosphorylating this site is a complex containing liver kinase B1 (LKB1) [11, 12]. This was an exciting discovery because LKB1 had originally been identified as a tumour suppressor; inheritance of one loss-of-function mutant allele of the LKB1 gene causes a cancer susceptibility condition called *Peutz-Jeghers syndrome*. Although this disorder is rare, somatic biallelic mutations in the gene are frequent in spontaneous cancers, especially of the lung, cervix and skin [13].

The LKB1 complex appears to be constitutively active, but binding of AMP or ADP to the AMPK-γ subunit causes conformational changes that promote net Thr-172 phosphorylation, both by enhancing phosphorylation [14, 15] and inhibiting dephosphorylation [16, 17]. In addition, binding of AMP (but not ADP) causes a further allosteric activation, so that the overall effect (phosphorylation plus allosteric activation) is >1000-fold. Both effects are antagonized by binding of ATP. Thus, any metabolic stress that inhibits the catabolic generation of ATP (e.g. starvation for glucose or oxygen), or that accelerates ATP consumption (e.g. muscle contraction) will activate AMPK due to increases in the cellular ADP:ATP ratio, which are amplified by adenylate kinase (2ADP ↔ ATP + AMP) into even larger increases in AMP:ATP. Many drugs and xenobiotics also activate AMPK; some are used in modern medicine (e.g. metformin, used to treat type 2 diabetes), some derive from traditional Chinese medicine (e.g. berberine), while others are natural products that are promoted as "nutraceuticals" (e.g. resveratrol) [2]. Most of these activate AMPK indirectly by inhibiting mitochondrial ATP synthesis [18].

Metabolism in quiescent versus growing cells – the Warburg effect

Fig. 1 compares the typical pathways of metabolism in quiescent cells compared with growing cells, including tumour cells. In the former, glucose and fatty acids are oxidized to CO_2 using the TCA cycle, which operates as a catabolic pathway generating most cellular ATP. However, in a growing cell the demand for fatty acids for membrane lipid synthesis means that the cell will be synthesizing rather than oxidizing fatty acids. In addition, citrate is withdrawn from the TCA cycle and exported to the cytoplasm, where ATP-citrate lyase generates the acetyl-CoA required as precursor for fatty acid synthesis. In lung adenocarcinoma cells grown in standard culture medium (25 mM glucose, 4 mM glutamine), the estimated flux of carbon out of the cycle via this route is higher than the flux entering it as acetyl-CoA; this is only sustainable because additional carbon enters the cycle as 2-oxoglutarate (2-OG), derived from glutamine by *glutaminolysis* [19]. The steps in the TCA cycle from 2-OG to citrate can actually run in the reverse of the normal direction [19], most likely catalyzed by NADP-linked isoforms of isocitrate dehydrogenase (IDH1, IDH2) rather than the NAD-linked IDH3 isoform [20, 21].

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If the enzymes of the TCA cycle are partly being used for anabolic purposes, it seems obvious that less ATP will be produced by oxidative phosphorylation. Indeed, if the steps from citrate to 2-OG are operating in reverse, they will be oxidizing rather than reducing NAD/NADP. Although the TCA cycle and oxidative phosphorylation will therefore produce less ATP, growing cells will still have a large demand for ATP for biosynthetic reactions. As mentioned above, glucose uptake and glycolysis are usually greatly accelerated in rapidly growing cells (the *Warburg effect)*. One function of this may simply be to satisfy the extra demand for ATP generated by biosynthesis in the face of reduced ATP production by oxidative phosphorylation (although glycolysis and the pentose phosphate pathway do also generate some biosynthetic precursors, such as NADPH for lipid synthesis, ribose for nucleotide synthesis, and serine and glycine for one-carbon metabolism).

The increases in glucose uptake and glycolysis in growing cells appear to be mediated in part via increased expression of the transcription factor hypoxia-inducible factor-1α (HIF-1α), which up-regulates expression of most glycolytic enzymes as well as GLUT1 (catalyzing glucose uptake) and MCT4 (catalyzing lactate export). Translation of HIF-1α mRNA, which contains a 5′-terminal oligopyrimidine (5′-TOP) sequence that otherwise inhibits its translation, is enhanced by activation of the target-of-rapamycin complex-1 (TORC1), a signaling complex that is activated under growth-promoting conditions [22].

Metabolism in pro-inflammatory cells: aerobic glycolysis

It is now becoming clear that unstimulated or naïve immune cells, including dendritic cells, neutrophils, macrophages and T cells, utilize mainly oxidative metabolism, including fatty acid oxidation, to generate ATP. However, when activated by pro-inflammatory cytokines, ligands for Toll-like receptors (TLRs), or antigen presentation, they switch to the use of aerobic glycolysis instead. In the case of T cells this may partly be because of increased biosynthetic demands, as in tumour cells. However, activation of dendritic cells does not lead to rapid proliferation, yet when stimulated by bacterial lipopolysaccharide (LPS, a ligand for TLR4), fatty acid oxidation and oxygen consumption decline, while glucose uptake, glycolysis and lactate output increase [23]. The decline in oxygen uptake appears to be due to the production of inducible NO synthase (iNOS) [24], whose product NO competes with oxygen at the terminal enzyme of the electron transport chain, cytochrome c oxidase [25]. Increased glycolysis may be necessary not only to replace the consequent loss of ATP production via the respiratory chain, but also to avoid apoptosis by maintaining mitochondrial membrane potential, which can be achieved by reversal of the adenine nucleotide translocase and ATP synthase reactions [26].

Turning now to macrophages, when stimulated with interferon-γ, LPS and certain other TLR ligands they acquire the “classical” or M1 phenotype, releasing pro-inflammatory cytokines and becoming effective at killing bacteria due to production of reactive oxygen species (ROS). By contrast, macrophages stimulated by IL4 and IL13, or IL10, acquire the “alternative” M2 phenotype; they are more concerned with wound healing and tissue repair, release anti-inflammatory cytokines and produce less ROS. Differentiation into the M1 but not the M2 phenotype is associated with a switch from oxidative metabolism to aerobic glycolysis. M1 macrophages are now thought to produce some of the ROS required for bacterial killing from complex I of the respiratory chain [27], and increased glycolysis may be necessary due to a switch of mitochondrial function away from ATP production and towards ROS production instead.

Whereas quiescent or memory T cells utilize mainly oxidative metabolism to generate ATP, activated T cells use aerobic glycolysis, with oxidative pathways such as fatty acid oxidation being suppressed [28]. Of the various lineages of $CD4^+$ T cells, Th17 cells promote

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inflammation, whereas Tregs are anti-inflammatory and help to inhibit autoimmunity. Th17 cells exhibit high rates of glycolysis dependent upon expression of HIF-1α, while Tregs are more oxidative. Intriguingly, the glycolytic inhibitor 2-deoxyglucose causes a switch away from the Th17 lineage and towards the Treg lineage. This suggests that metabolic cues partly determine this switch, with glycolysis favoring the pro-inflammatory Th17 cell and oxidative metabolism favoring the anti-inflammatory Treg cell [29].

Regulation of metabolism by AMPK: exerting an anti-Warburg effect?

Once activated by a metabolic stress, drug or xenobiotic that increases cellular ADP:ATP and AMP:ATP ratios, AMPK acts to restore energy homeostasis by switching on alternative catabolic pathways that generate ATP, while switching off anabolic pathways and other processes consuming ATP, such as progress through the cell cycle [1]. Catabolic processes that are switched on include fatty acid oxidation, mitochondrial biogenesis and expression of enzymes of oxidative metabolism [1]. At the same time, AMPK activation switches off most anabolic processes, including synthesis of lipids, carbohydrates, ribosomal RNA and proteins [1]. AMPK down-regulates protein synthesis by inhibiting TORC1 [30, 31], promoting translation of mRNAs containing 5′-TOP sequences, including those encoding ribosomal proteins and HIF-1α. By inhibiting TORC1, AMPK would not only down-regulate expression of ribosomal proteins (complementing its ability to inhibit ribosomal RNA synthesis [32]), but also reduce expression of HIF-1α and thus expression of the glycolytic enzymes and transporters required for the Warburg effect. Consistent with this, expression of HIF-1α and many of its target genes is markedly up-regulated in mouse embryo fibroblasts deficient in either LKB1 or AMPK [33].

AMPK-activating drugs as anti-cancer agents

Although AMPK has not yet been formally demonstrated to be a tumour suppressor, there are many indications that this is the case: (i) AMPK activation inhibits cell growth and proliferation [1]; (ii) AMPK exerts many effects of LKB1, a known tumour suppressor [11, 12]; (iii) as discussed in the previous section, AMPK up-regulates oxidative metabolism and would therefore be expected to mediate an “anti-Warburg” effect; (iv) like other known tumour suppressors, AMPK activation is down-regulated in many tumour cells, by loss of LKB1 and other mechanisms [13, 34, 35]. These considerations led to studies investigating whether the use of AMPK-activating drugs in humans might affect the incidence of cancer. Indeed, type 2 diabetics taking the AMPK-activating drug metformin have a significantly reduced incidence of cancer compared with those on other medications [36]. This is based on retrospective studies, which can only test for association rather than causation, although randomized controlled trials of metformin for cancer treatment are now under way. It should be emphasized that it remains uncertain whether these apparent anti-cancer effects of metformin are mediated by AMPK, or even whether they are direct effects on the tumours themselves [37].

It is also possible that the anti-cancer effects of other, well-established anti-cancer drugs might be mediated in part by AMPK. The folate analogues methotrexate and pemetrexed, which are used for cancer treatment, were thought to act primarily by inhibiting dihydrofolate reductase and thus preventing recycling of N^5, N^{10}-methylene tetrahydrofolate, an essential cofactor for thymidylate synthase and hence DNA synthesis. The nucleotide 5-aminoimidazole-4-carboxamide ribotide (ZMP) is an intermediate in purine nucleotide synthesis, but also acts as an AMP mimetic that mimics all of the effects of AMP on the AMPK system [38]. Incubation of most cells with the equivalent riboside (AICA riboside) causes accumulation of ZMP, because its uptake and phosphorylation to ZMP is rapid compared with subsequent metabolism [38]; it has therefore been widely used

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to activate AMPK in intact cells and in vivo. ZMP is metabolized in two steps to the purine nucleotide IMP (Fig. 2), the first being catalyzed by an enzyme that transfers a formyl group from N^{10}-formyltetrahydrofolate to ZMP. This enzyme is inhibited by pemetrexed [39] and by polyglutamate derivatives formed by intracellular metabolism of methotrexate [40]. Interestingly, methotrexate greatly potentiated AMPK activation when breast cancer cells were incubated with AICA riboside [41], while pemetrexed causes AMPK activation in a leukaemia cell line even in its absence [39]. However, the extent to which AMPK activation explains the anti-cancer actions of antifolate drugs remains to be determined.

AMPK-activating drugs as anti-inflammatory agents

As mentioned earlier, activated immune cells involved in inflammatory responses tend to utilize aerobic glycolysis, while their unactivated or anti-inflammatory counterparts tend to utilize oxidative metabolism. This suggests that AMPK-activating drugs, which down-regulate glycolysis and promote oxidative metabolism, might have anti-inflammatory effects. Evidence supporting this idea has recently begun to appear. In dendritic cells, phosphorylation of AMPK at Thr-172 was reduced when they were activated with LPS, while the maturation and increased glucose consumption induced by LPS were enhanced by AMPK knockdown and repressed by AICA riboside [23]. In macrophages, stimulation with LPS or fatty acids caused Thr-172 dephosphorylation and AMPK inactivation, whereas anti-inflammatory cytokines had the opposite effects. Moreover, down-regulation of AMPK increased expression of pro-inflammatory cytokines in response to LPS or fatty acids, while expression of an activated AMPK mutant had the opposite effects [42, 43]. In another study, the AMPK-activating drug berberine suppressed pro-inflammatory responses in macrophages, the effect being reduced by expression of a dominant negative AMPK mutant [44]. Thus, AMPK appears to promote macrophage polarization towards the anti-inflammatory M2 phenotype, rather than the pro-inflammatory M1 phenotype. Studies using bone-marrow-derived macrophages from AMPK-$\beta1^{-/-}$ mice, in which total AMPK activity was reduced by 85%, support the idea that these effects may be mediated, at least in part, by changes in metabolism [45]. Thus, expression of mitochondrial proteins was greatly reduced in the knockout cells, and they displayed reduced fatty acid oxidation and increased expression of pro-inflammatory markers, particularly when stimulated with fatty acids. In wild type macrophages, activation of JNK (a pro-inflammatory response) was reduced by the AMPK activator A-769662, and this effect was blocked by inhibition of fatty acid oxidation. Finally, when bone marrow from $\beta1^{-/-}$ mice was transplanted into irradiated wild type mice that were then challenged with a high-fat diet, there was increased infiltration of inflammatory macrophages into adipose tissue and liver, which was associated with hepatic insulin resistance [45].

Indirectly supporting the idea that AMPK activation has anti-inflammatory effects, our laboratory has recently reported that AMPK is activated by the classical anti-inflammatory drug salicylate [46]. Salicylate is a natural plant product abundant in white willow *(Salix alba)*, whose medicinal use was documented in ancient times. It is usually now administered in the form of aspirin (acetyl salicylic acid), which is rapidly broken down to salicylate following adsorption. Aspirin, but not salicylate, is a potent inhibitor of the cyclo-oxygenases COX1 and COX2, thus inhibiting prostanoid biosynthesis [47]. However, following oral administration of aspirin, the plasma half-life and concentration of aspirin are very low compared with those of its breakdown product, salicylate [48]. Interestingly, at doses that generate serum salicylate concentrations of 2-5 mM, the anti-inflammatory effects of aspirin or salicylate in a murine air pouch model were COX-independent [49]. Our laboratory found [46] that salicylate, but not aspirin, activated AMPK in cultured cells at concentrations within this range, which was due to direct binding of salicylate at the same site that binds A-769662, a synthetic AMPK activator [50]. Both agents allosterically

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activate AMPK and promote Thr-172 phosphorylation, and their effects are not observed in AMPK complexes containing the β2 isoform. We made use of the latter observation by showing that the effects of A-769662 or salicylate to stimulate fatty acid oxidation in isolated hepatocytes, and to accelerate the switch from carbohydrate to fat oxidation triggered by food withdrawal in vivo, were abolished in AMPK-β1 knockout mice [46]. Thus, at least one effect of salicylate *in vivo*, the stimulation of fatty acid oxidation, is mediated by AMPK. Although further work is required to determine whether any anti-inflammatory actions of salicylate are mediated by AMPK, the fact that naïve or anti-inflammatory cells often use fatty acid oxidation to generate ATP, whereas activated pro-inflammatory cells tends to use aerobic glycolysis instead, supports the idea that activation of AMPK by salicylate might have anti-inflammatory effects.

Interestingly, although methotrexate has been used for many years to treat the inflammatory disease rheumatoid arthritis, its mechanism of action in this context is not fully elucidated. As discussed above, methotrexate can activate AMPK under certain circumstances [41]. Although this does not appear to have been studied in cells of the immune system, it is tempting to speculate that AMPK may also be involved in some of the anti-inflammatory actions of methotrexate.

Conclusions and perspectives

The metabolic profiles of rapidly proliferating tumour cells and activated pro-inflammatory cells of the immune system are strikingly similar. Both utilize rapid glucose uptake and glycolysis to generate much of their ATP. These metabolic changes are expensive in terms of glucose consumption, because of the small amount of ATP that can be generated by aerobic glycolysis compared with that generated by complete oxidation of glucose or fatty acids. It is perhaps not surprising that the AMPK pathway, which is normally activated in response to glucose starvation and/or decreases in cellular energy status, should oppose these changes and thus restrain the growth of tumour cells, as well as the pro-inflammatory responses of immune cells. This is completely consistent with the role of the AMPK orthologue in a unicellular eukaryote such as yeast, which is required for the switch from fermentation to oxidative metabolism when glucose in the medium runs low.

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Abbreviations

AMPK	AMP-activated protein kinase
LKB1	liver kinase B1
iNOS	inducible isoform of nitric oxide synthase
LPS	bacterial lipopolysaccharide
ME	malic enzyme
MDH	malate dehydrogenase
OAA	oxaloacetate
2-OG	2-oxoglutarate
3-PG	3-phosphoglycerate

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TLR Toll-like receptor

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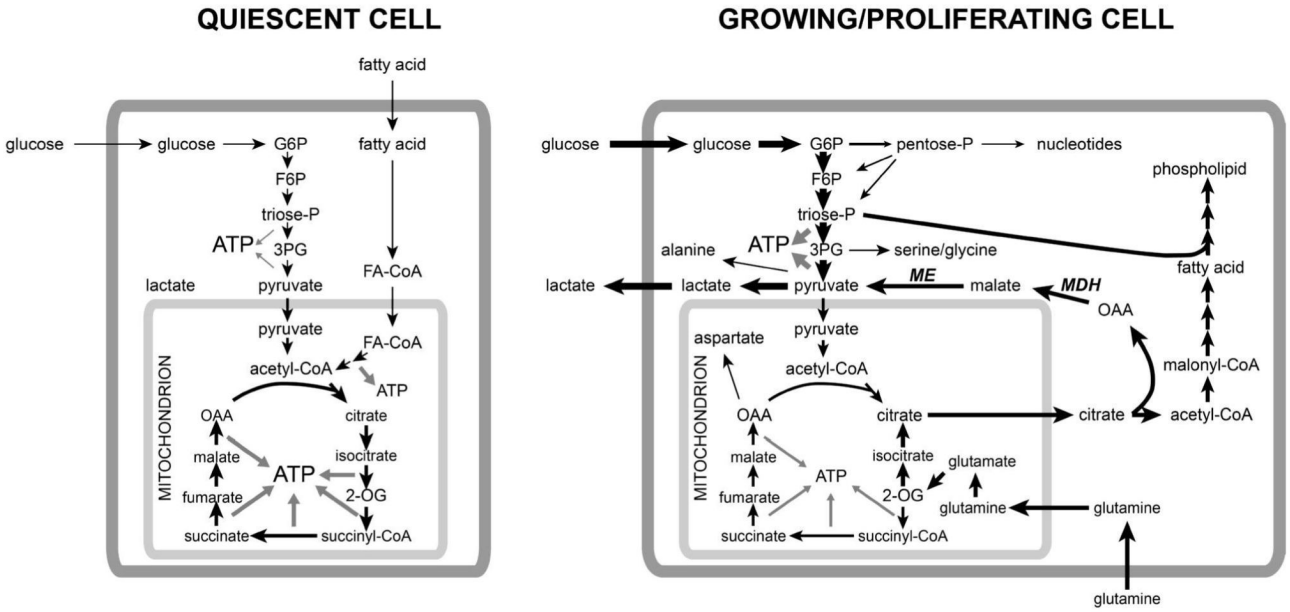

Figure 1.

Summary of central pathways of carbohydrate and lipid metabolism in a quiescent cell (left) and in a growing or proliferating cell (right). See the text for a full discussion. Abbreviations used: ME, malic enzyme; MDH, malate dehydrogenase; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; 3-PG, 3-phosphoglycerate.

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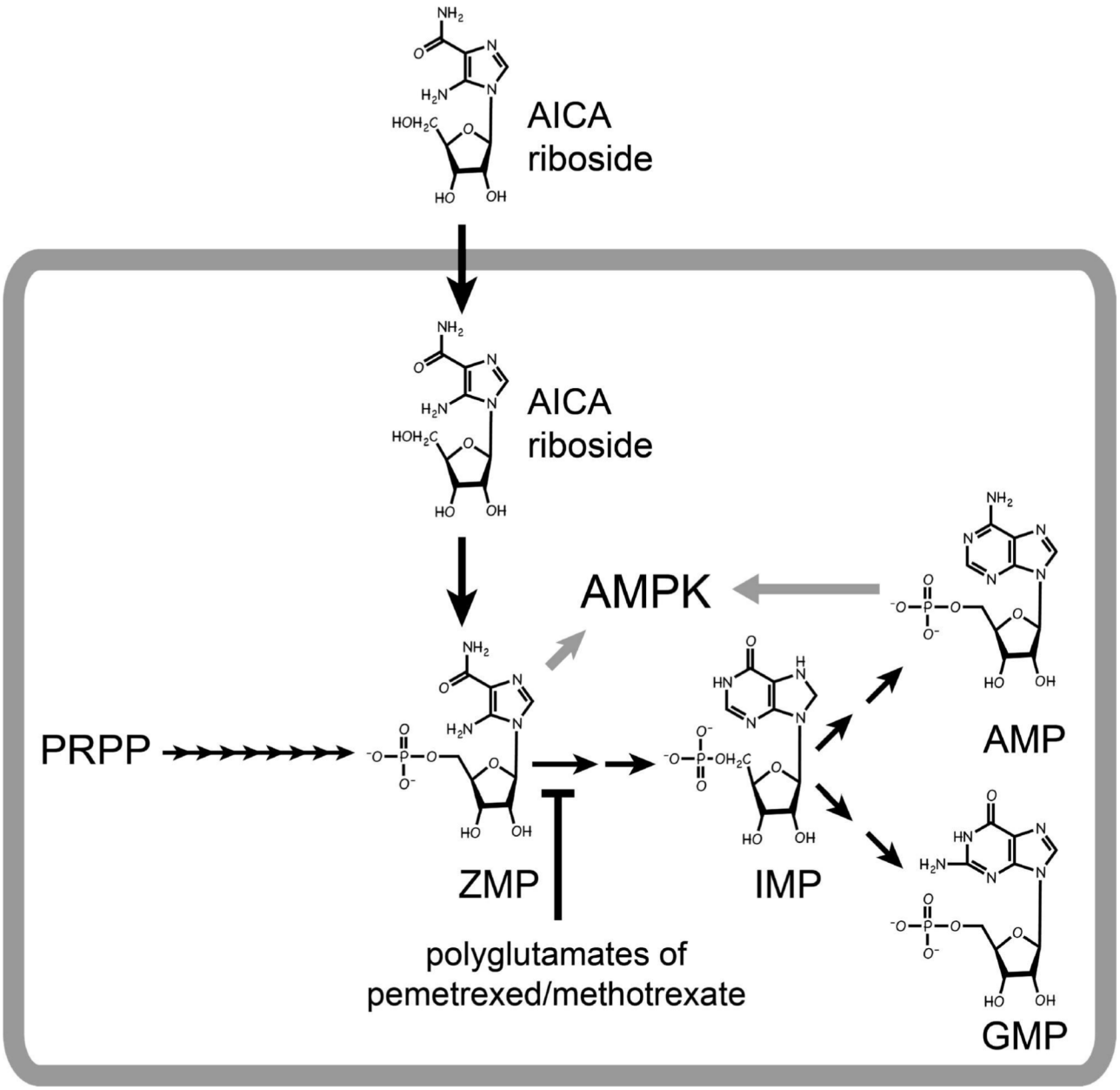

Figure 2.

Purine nucleotide synthesis and its role in regulation of AMPK. ZMP (5-aminoimidazole-4-carboxamide ribonucleotide, AICA ribotide) is a natural intermediate in the biosynthetic pathway leading from phosphoribosyl pyrophosphate (PRPP) to the purine nucleotides IMP, AMP and GMP. Incubation of many cells with AICA riboside leads to the accumulation of ZMP because uptake and phosphorylation of the riboside is more rapid than its subsequent metabolism to IMP. Depending on the flux through the purine nucleotide synthesis pathway, the antifolate drugs pemetrexed [39] and methotrexate [41] or their polyglutamate derivatives can also cause accumulation of ZMP and lead to activation of AMPK.