REVIEW

Targeting the AMP-regulated kinase family to treat diabetes: a research update

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Practice Points

- AMP-regulated protein kinase (AMPK), an intracellular sensor of energy change, is regulated by AMP:ATP (and ADP:ATP) ratios and is an important regulator of glucose homeostasis in mammals.
- AMPK is emerging as an important regulator of insulin secretion, insulin action and feeding behavior.
- AMPK is responsible for some but not all of the actions of antidiabetic drugs including metformin and thiazolidinediones.
- New mouse knockout models shed light on the roles of the enzymes in different tissues.
- The roles of the separate AMPK catalytic isoforms α1 and α2, as well as of the upstream kinases LKB1 and CaMKK, are frequently distinct.
- AMPK and related kinases including per-arnt sim kinase represent exciting potential targets for new therapeutic drugs.

SUMMARY AMP-activated protein kinase (AMPK) has long been recognized as a master energy sensor. Activation of AMPK in response to metabolic stress preserves energy stores by switching on catabolic pathways, whilst its inhibition consumes the energy by switching on anabolic pathways. Over the past 10–15 years, much attention has been focused on the role of AMPK in mammalian metabolism, and particularly in diabetes. As a consequence, AMPK has emerged as much more than a simple energy regulator and is now recognized as a kinase involved in controlling numerous cellular processes, including cell growth, apoptosis, autophagy and polarity. Using different *in vitro* and *in vivo* tools, AMPK has also been found to play important roles in different glucose-sensing organs and to serve as a key regulator of glucose homeostasis in mammals. Perhaps most importantly, AMPK appears to be the major target for several antidiabetic drugs. Here, we review recent advances in the field and particularly those emerging from the generation of tissue-specific knockout and transgenic mice.

Diabetes & AMP-activated protein kinase

Increases in the prevalence of obesity owing to sedentary lifestyles and the intake of high-calorie food have led to a remarkable and parallel increase in the number of patients with obesity-associated diseases including Type 2 diabetes (T2D), with higher risks in certain ethnic groups [1,2]. With T2D almost reaching pandemic proportions, novel and effective therapies targeting this disease are urgently needed.

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Type 2 diabetes normally begins with blunted responses of peripheral tissues to insulin action with higher fasting blood glucose due to abnormally regulated liver glucose production and moderately impaired glucose tolerance due to the failure of skeletal muscle to take up glucose from the bloodstream [3–8]. This insulin resistance leads, by still unknown mechanisms, to hyperinsulinemia wherein hyperplastic and hypermorphic pancreatic β cells hyper-secrete insulin to compensate. At this stage, hyperinsulinemia is still able to control blood glucose within the normal physiological range (3.6–5.8 mM in humans). It is only when β-cell function deteriorates and/or β-cell mass falls that glucose intolerance and finally the onset of frank T2D occurs with its attendant complications including failure of other important organs such as the kidneys (nephropathy), eyes (retinopathy) and heart (cardiovascular disease) [5–11]. Consequently, different pharmaceutical approaches have been developed that aim to either reduce insulin resistance (e.g., metformin, thiazolidinediones) or to enhance insulin production by β cells (e.g., sulphonylureas, glucagon-like peptide-1 analogs), hence reducing blood glucose and delaying or preventing the onset of frank T2D [12,13].

AMP-activated protein kinase (AMPK), a phylogenetically conserved serine/threonine protein kinase [14], has recently emerged as an interesting potential drug target for treating diabetes [15]. Indeed, several front-line antidiabetic drugs such as metformin and thiazolidinediones appear to act at least in large part through activating AMPK in the liver and adipose tissues [13,16,17]. As an energy sensor, AMPK and the signaling pathways upstream and downstream of this enzyme have been intensively studied for the past decade, indicating that AMPK might play important roles in modulating glucose and insulin sensing in organs such as skeletal muscle, liver, brain, adipose tissues and pancreas [18–21]. However, recent exciting studies, particularly those describing tissue-specific AMPK knockout mice developed with LoxP-*Cre* technology [22] have implied that AMPK activity is regulated differently in different organs in response to glucose or insulin, and that the activation of AMPK may exert different downstream effects in different tissues.

AMPK structure, isoforms, tissue distribution & subcellular localization

Mammalian AMPK exists as a heterotrimer consisting of a catalytic α subunit (α1 or α2) [23,24], a regulatory β subunit (β1 or β2), which is required for the binding of other subunits [25], and an AMP-binding γ subunit (γ1, γ2 or γ3) [26]. Each of the separate subunits and isoforms is encoded by an individual gene [24,27]. The α subunit contains a N-terminal Ser/Thr kinase domain, an auto-inhibitory sequence (AIS) and a C-terminal β-subunit-binding domain [28–30]. The kinase domain includes the critical phosphorylation site, Thr172, which is indispensible for the activation of AMPK by upstream kinases including liver kinase B1 (LKB1; STK 11), a tumor suppressor and Ca^{2+}/calmodulin-dependent protein kinase (CaMKKβ) [31–35]. AMPK can be inactivated by phosphatases including PP2A and PP2C [36], although recent studies suggest that a phosphatase complex containing the catalytic subunit of protein phosphatase-1 and regulatory subunit R6 might be involved in glucose-regulated AMPK inactivation [37]. Single site mutation at Thr172 to aspartic acid (D) reduces AMPK activity by almost twofold. Conversely, a T172D mutation generates a mutated AMPK resistant to dephosphorylation [38]. The (autoinhibitory sequence) AIS domain appears to exert an auto-inhibitory effect on AMPK activity, since truncated AMPK α subunits lacking this domain have an almost fourfold higher AMPK activity than those with an intact AIS [29]. Two isoforms of AMPK catalytic α subunit, α1 and α2, encoded by two distinct genes (*PRKAA1* and *PRKAA2*), exist and share high similarities at the N-terminal of the subunit, and both are capable of being phosphorylated by upstream kinases at the Thr172 site [24]. The distribution of α1 and α2 subunits is distinct in both the subcellular, cellular and whole-body level, with α2 present in both the cytosol and nucleus, suggesting a role in controlling transcription and gene expression. α2-containing complexes are predominately found in the skeletal muscle and heart, whilst α1 complexes, present chiefly in the cytosol, are found in most of the other chief metabolic organs such as the liver, endocrine pancreas and adipose tissues [39,40].

The regulatory β subunit consists of a glycogen-binding domain (GBD) and α- and γ-subunit-binding domains [41,42]. Recently, much attention has been focused on the functions of the β subunit and the observations that: binding of α1→6-linked branches of glycogen at the GBD during glycogen depletion (e.g., caused by muscle contraction) appears to be necessary for dephosphorylation of glycogen synthase at site 2, promoting glycogen resynthesis [43]; and the

β1 subunit is critical for AMPK activation by the thienopyridone drug A769662 [44,45]. The γ subunit of AMPK has two Bateman domains formed by four tandem cystathionine β-synthase motifs 1–4, three of which are essential for AMP/ATP binding [46]. Recently, the resolution of the crystal structure of the αβγ core complex containing the C-terminal domain of α1 and β2 with full length γ1 confirmed that among these three motifs in the γ subunit, two of them are interchangeable between AMP and ATP binding and the other one tightly occupied by AMP [47].

High AMP:ATP ratios activate AMPK either allosterically [48] or by rendering AMPK a better substrate for upstream kinases [49] and a poorer substrate for protein phosphatases [36]. Owing to the action of adenylate kinase, the AMP:ATP ratio in cells varies approximately as the square of the ADP:ATP ratio, which makes the former ratio a very sensitive indicator of cellular energy changes. Therefore, any activities that deplete ATP such as hypoxia or glucose deprivation are expected to activate AMPK [14,36,50].

Examining the role of AMPK in different tissues

■ AMPK in muscle

The ability of skeletal muscle to take up glucose is essential to maintain normal glucose homeostasis. Aberrant glucose uptake due to insensitivity to insulin has been found in T2D [51,52]. Excessive glucose in the bloodstream promotes insulin secretion from pancreatic β cells, which triggers the PI3K/Akt-AS160-Rab GTPase signaling pathway in the skeletal muscle cells to transport glucose across the cell membrane by delivering glucose transporter 4 (GLUT4) onto the cell surface. After glucose is phosphorylated by hexokinase, it is later converted into glycogen by glycogen synthase and stored in the muscle for use during energy depletion [51]. In contrast to this insulin-dependent glucose uptake signaling pathway, exercise has also been reported to mediate glucose uptake into the muscle cells using an insulin-independent pathway [53].

Does AMPK mediate contraction-induced glucose uptake in muscle?

The activation of AMPK that is observed in skeletal muscle in parallel to increased glucose uptake in response to exercise or metformin administration to Type 2 diabetic patients suggests that AMPK might play a role in controlling glucose uptake [54]. Supporting this view, subcutaneous injection of the AMP analog 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), an AMPK activator, or single-leg arterial infusion of AICAR increased AMPK activity and glucose uptake [55–57]. These changes were also associated with increased hexokinase II transcription in both red and white muscles [55,56], suggesting that AMPK stimulation might serve as an exercise mimetic to enhance glucose uptake. Global deletion of AMPK α2 (AMPK $\alpha2^{-/-}$ mice), but not α1, abolished AICAR- but not contraction-induced glucose uptake in skeletal muscle, probably reflecting activation of α1 activity in skeletal muscle during muscle contraction [57]. Using a transgenic mouse model carrying an inactivating Lys45 to Arg mutation in the AMPK α2 subunit (AMPK α2KD), Mu and colleagues demonstrated that reduction of AMPK activity in these mice completely blocked AICAR-induced glucose uptake in extensor digitorum longus muscle but only partially reduced contraction-induced glucose uptake [58]. Some years later, the groups of Laurie Goodyear and Lynis Dohm [59,60] confirmed that decreases in AMPK α2 activity abolished AICAR, but not contraction- or exercise-induced glucose uptake and translocation to the plasma membrane of the glucose transporter GLUT4. Reduced maximal exercise capacity and glycogen accumulation [61] with a concomitant decrease in hexokinase II protein levels [61,62] were also observed, using transgenic mice overexpressing inactive AMPK α2 subunits selectively in muscle (AMPK α2iTg mice).

Very recently, the GBD of AMPK β subunit has been shown to be necessary for glycogen replenishment during muscle contraction [43]. It is therefore reasonable to suspect that glucose uptake might also be dependent on glycogen binding. However, AMPK β2 whole-body knockout mice with reduced AMPK activity displayed reduced AICAR-induced, but not contraction-induced, muscle glucose uptake [63]. Moreover, no effect of muscle glucose uptake was seen in AMPK β1 knockout mice [44], suggesting, in the short term, that glucose uptake during muscle contraction is probably not modulated by glycogen binding to AMPK β subunits.

The AMPK γ3 subunit is specifically expressed in muscle cells and mutation of this subunit (R225Q) leads to downregulation of AMPK α activity. Using this mouse model (AMPK γ3R225Q), Yu and colleagues confirmed that both basal and AICAR-stimulated glucose uptake in skeletal muscle were significantly

decreased [64]. By contrast, activation of AMPK in skeletal muscle achieved by expression of an active form of AMPK where Arg70 is mutated to Gln in the γ1 subunit in mouse skeletal muscle increased glycogen accumulation [62].

Does AMPK modulate insulin-stimulated glucose uptake?

Recently, AS160/TBC1D4, activated in response to stimulation of the PI3K/Akt/PKB pathway was reported to be phosphorylated by AMPK through activation of α2β2γ1 subunits [65,66]. This leads to the binding of phosphorylated AS160 (inactive) to 14–3–3 proteins and promotes conversion of less active GDP-bound Rab to more active GTP-bound Rab, which then releases GLUT4 from vesicles to the plasma membrane [67]. Another AS160 paralog, TBC1D1, is also phosphorylated by purified AMPK and has been reported to modulate glucose transport [67]. Therefore, the evidence for involvement of AS160 in GLUT4 translocation seems to link AMPK to insulin-dependent glucose uptake.

Regulation of glucose uptake by kinases downstream of LKB1

Interestingly, muscle-specific knockout of LKB1 (mLKB1 KO) reduced AMPK α2, but not α1 activity and blunted both contraction- and AICAR-induced glucose uptake [68,69]. By contrast, AMPK α2KD or α2iTg [59–61] only partially affected, or had no effect, on contraction-induced glucose uptake. These findings suggest other LKB1 downstream AMPK-related kinases are probably involved. Indeed, targeting of the AMPK-related kinase and LKB1 substrate SNARK by overexpression of mutant SNARK, RNA silencing in C2C12 muscle cells or using skeletal muscle from whole-body SNARK$^{+/-}$ mice led to reduced contraction-induced glucose uptake and also dephosphorylation of AS160 [68]. By contrast, a further potential upstream kinase of AMPK, CaMKKβ [35] has recently been suggested to control glucose uptake in contraction-stimulated mouse skeletal muscle **(Figure 1(2))**. Using STO-609, a CaMKK inhibitor, Jensen and colleagues demonstrated reduced electrical stimulated 2-deoxyglucose uptake in skeletal muscle. However, STO-609 did not have any effects on glucose uptake in muscles from AMPK α2KD mice, suggesting an AMPK-dependent effect of CaMKKβ on glucose uptake [70]. However, the caveat must be borne in mind when interpreting results from experiments using STO-609 that direct inhibition of AMPK by this compound has also been reported [71]. A mouse model in which CaMKKβ is specifically deleted in muscle is needed to fully understand the role of this kinase in muscle glucose uptake.

Thus, results from various AMPK inactive or knockout mouse models seem to argue against a role for AMPK in mediating contraction- or exercise-stimulated glucose uptake and suggest that alternative signaling pathways might be responsible for contraction-induced glucose uptake [57–59,61,64]. As shown in **Figure 1(2)**, the difference of contraction-induced glucose uptake displayed in muscle in mLKB1 KO versus AMPK α2KD mice implies that other AMPK-related protein kinase(s) might be involved in regulation. On the other hand, the emergence of the ability of AMPK to phosphorylate AS160 and TBC1D1 connects AMPK to insulin-dependent glucose uptake and sheds light on the role of AMPK in muscle glucose uptake and whole-body glucose disposal [67].

■ AMPK in the liver

One of the major adverse effects of obesity-induced insulin resistance is elevated fasting hyperglycemia accompanied by increased accumulation of lipid in the liver [72]. Correspondingly, drugs targeting liver glucose output offer powerful diabetes therapies. Indeed, metformin reduces glucose levels in T2D patients by reducing liver glucose production [73]. Since AMPK is strongly activated by metformin in hepatocytes and AICAR infusion reduces glucose output in Zucker obese rats, it has been reasonable to assume, at least until recently, that AMPK regulates liver glucose production [73,74].

AMPK in regulating hepatic glucose output

Foretz and colleagues [75] first demonstrated that short-term activation of AMPK, achieved by injecting streptozotozin-induced diabetic or ob/ob mice with an adenovirus encoding a constitutively active form of AMPK α2, led to reduced blood glucose levels with increased gluconeogenic gene expression, for example phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphase (G6Pase) in the liver. However, liver from mice globally deleted for AMPK β1 subunit, with a more than 50% reduction of AMPK activity in the liver, displayed normal glucose output arguing against the involvement of AMPK in regulating hepatic glucose production [44]. More recently, global AMPK α1 and liver-specific AMPK α2

knockout mice (AMPKa1a2LS$^{-/-}$) were generated and, similar to the findings in AMPK β1-null mice [44], the latter mice showed comparable glucose levels and gluconeogenesis-related gene expression to wild-type controls [76]. By contrast, liver-specific LKB1 knockout mice displayed phenotypes similar to ob/ob mice with elevated blood glucose levels and impaired glucose tolerance (Figure 1(1)) [77]. This was accompanied by decreased phosphorylation of TORC2 (CRTC2) and increased expression of gluconeogenic genes, including peroxisome proliferator-activated receptor-γ coactivator 1-a, G6Pase and PEPCK, and that suggests kinases downstream of LKB1, other than AMPK, might be involved in mediating the effects of LKB1. Indeed, salt-inducible kinase 2 (SIK2) and MARK2 have been suggested to phosphorylate CRTC2, facilitating its binding to 14–3–3 proteins and sequestration in the cytosol [78]. As shown in Figure 1(1), deletion of LKB1, possibly through the loss of SIK2 and MARK2 function, leads to dephosphorylation of CRTC2. This, in turn, relocates CRTC2 to the nucleus where it binds CREB and facilitates CREB-dependent PGC1-α transcription to promote the subsequent expression of gluconeogenic genes such as PEPCK and G6Pase.

AMPK in liver lipotoxicity

Excessive lipid accumulation in the liver (so-called 'fatty liver') is one of the complications of T2D and results from decreased fatty acid

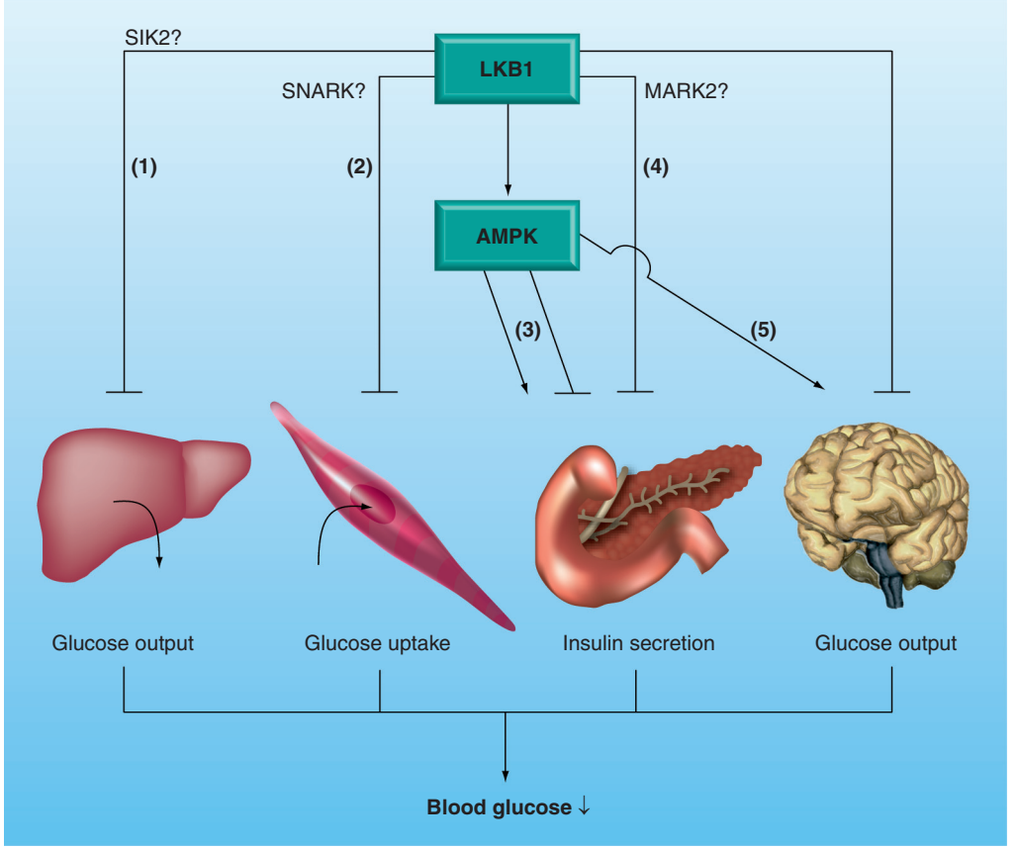

Figure 1. LKB1 and AMP-activated protein kinase regulation of whole-body glucose metabolism. **(1)** LKB1 inhibits gluconeogenesis in the liver, probably through phosphorylation and activation of SIK2. **(2)** LKB1 inhibits glucose uptake into the skeletal muscle via phosphorylation of SNARK. AMPK **(3)** and LKB1 **(4)** play roles in regulating insulin secretion from pancreatic β cells. Note that AMPK activation is likely to exert negative effects in the short term but may have positive consequences in the longer term, for example through the regulation of apoptosis and related pathways. LKB1 action may be mediated via an LKB1-MARK2 signaling pathway. **(5)** Central AMPK activation stimulates hepatic glucose output.
AMPK: AMP-activated protein kinase; LKB1: Liver kinase B1; SIK: Salt-inducible kinase.

oxidation and increased lipogenesis [72]. The consequent 'lipotoxicity' is then thought to lead to insulin resistance probably by affecting the insulin receptor substrate-1/2 PI3K-Akt-GSK3 signaling pathway, hence elevating hepatic glucose output [79,80]. In addition to regulating gluconeogenic gene expression and glucose output, AMPK has also been reported to control lipid deposition in the liver by inhibiting acetyl-CoA (ACC) activity via phosphorylation of ACC and decreases in malonyl-CoA content. These changes subsequently decrease fatty acid synthesis and increase fatty acid oxidation to reduce triglyceride storage [81,82]. Activation of AMPK achieved, for example, by infusion of AICAR into obese Zucker rats, reduced the glycerol turnover rate [74]. A similar effect was seen in hepatocytes treated with metformin or in mice overexpressing constitutively active AMPK α2 (AMPK α2 CA) in the liver by injecting adenovirus carrying AMPK α2 CA through the penis vein; in the latter case fatty acid oxidation was increased whilst there was a decrease in lipogenic gene expression, in other words in FAS and transcription factors related to lipogenesis such as SREBP-1 and ChREBP [73,75]. Conversely, mice with liver-specific deletion of AMPK α2 displayed increased plasma triglyceride levels, suggesting AMPK α2 is an important regulator of fat metabolism in the liver [83]. On the other hand, ablation of LKB1 in the liver reduced ACC phosphorylation and led to increased FAS and SREBP-1 gene expression [77]. However, since lipogenic gene expression was not assessed in mice deleted for both catalytic AMPK isoforms, specifically in the liver, it is still unknown whether the effects of LKB1 on lipogenic gene expression are via AMPK [77].

AMP-activated protein kinase was suggested to regulate liver glucose output in early research studies using AICAR and metformin. However, since AICAR is a mimetic of ZMP and metformin acts on respiratory complex I, the effects of these drugs on liver metabolism might be due to a secondary effect of a change of AMP (ZMP)/ATP levels rather than AMPK itself [84]. With the advent of liver-specific LKB1 or AMPK double catalytic isoform knockout mice, more evidence has been provided to suggest that other AMPK-related kinase lying downstream of LKB1, such as SIK2 or Mark2, might regulate glucose production and gluconeogenic gene expression **(Figure 1)** [1]. Finally, through the phosphorylation and destabilization of hepatocyte nuclear factor-4, AMPK may exert a direct effect on the regulation of lipogenic genes including liver-type pyruvate kinase [19].

■ AMPK in the endocrine pancreas

The endocrine pancreas, and especially pancreatic β cells of the islets of Langerhans, plays a central role in controlling glucose homeostasis via fluctuations in insulin output. Failed first phase insulin secretion in response to glucose stimulation, and reduced β cell, are thus required for the appearance of overt T2D [5–11]. AMPK has recently emerged as a key regulator of insulin secretion in the minute-to-minute timescale and β-cell mass (i.e., survival and proliferation pathways) more chronically [19].

AMPK regulates β-cell insulin secretion & gene expression

The role of AMPK in controlling insulin secretion from pancreatic β cells was first addressed by Salt and colleagues in a pharmacological study on a tumoral β-cell line and rodent islets [85]. The authors demonstrated that AICAR-treated INS-1 rat β cells displayed increased basal (at 3 mmol/l glucose) but inhibited glucose (16.7 mmol/l) stimulated insulin secretion. Similar results were also obtained by the group of Van de Casteele and Pipeleers [86], and by our own group [87], using MIN6 mouse β cells and primary rat islets. The latter studies showed that AMPK activation by AICAR reduced glucose-stimulated insulin secretion, preproinsulin promoter activity and insulin gene expression [86–88]. Metformin was later shown to activate AMPK in MIN6 β cells and primary human islets [89]. Increases in AMPK activity, achieved by treatment with metformin, were also associated with reduced glucose-stimulated insulin secretion to near basal levels [89,90]. These results were further confirmed by Wheeler's group showing an almost 50% decrease of glucose-stimulated insulin secretion from isolated rat islets treated with AICAR and metformin overnight [91]. However, recent investigations of the involvement of AMPK in controlling insulin secretion by Philipson's group found that AICAR treatment, rather than reducing glucose-stimulated insulin secretion from mouse islets, increased it [90]. Similarly, studies by Birnbaum and Newgard indicated that AICAR and phenformin (a more potent analog of metformin), exerted no effect on glucose-stimulated insulin secretion in MIN6 β cells and primary mouse islets [92,93]. These divergent results highlight the limitations of

using pharmacological approaches in the study of AMPK. AICAR mimics the effect of AMP on AMPK activation, and metformin and phenformin have also been reported to activate AMPK by inhibiting complex I of the respiratory chain, leading to an increase in cellular AMP:ATP ratio; the impact on other AMPK-independent processes and the signaling pathways is likely to be significant [94]. To avoid such complications, a AMPK CA ($\alpha1^{312}$T172D) or a dominant-negative form of the enzyme (DN, α2 D157A) [95] were used in later studies. Whereas overexpression of AMPK CA in MIN6 β cells and rat islets reduced glucose-stimulated insulin secretion and insulin gene expression, AMPK DN-expressing viruses increased basal insulin release and insulin gene expression [87]. The effects of AMPK activation were later shown to reflect reduced insulin vesicle movement in MIN6 cells infected with AMPK CA-expressing virus **(Figure 2(1))** [96].

AMPK regulates β-cell apoptosis

Apart from insulin secretion and insulin gene expression, AMPK has also been reported by several groups to exert pro-apoptotic effects in β cells. Physiologically, this is expected to lower β-cell mass and total insulin output per pancreas [86,97–100]. Prolonged activation of AMPK in MIN6 cells and purified mouse islet β cells, achieved by 24 or 48 h exposure to AICAR or

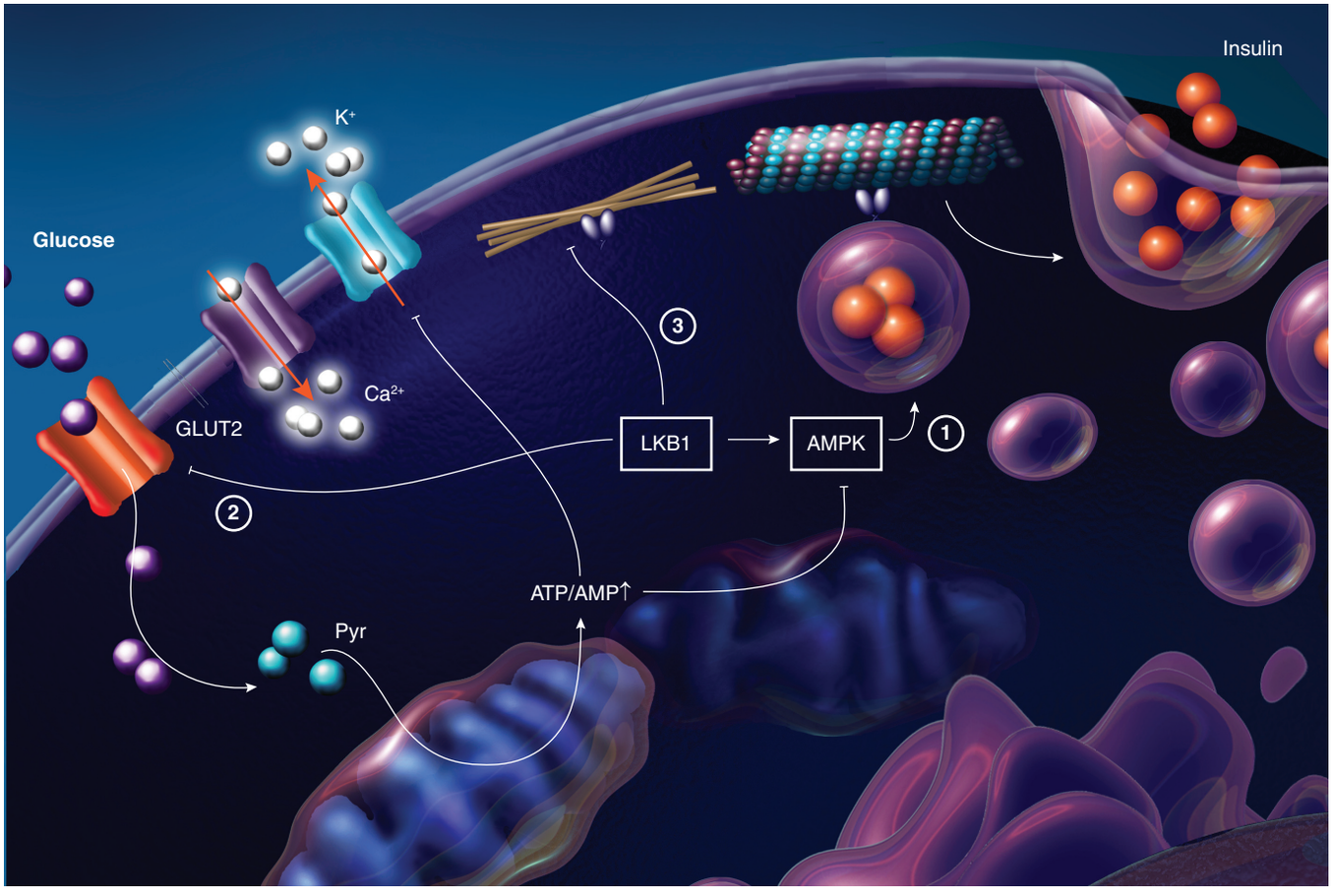

Figure 2. Likely mechanisms through which LKB1 and AMP-activated protein kinase regulate insulin secretion and polarity in pancreatic β cells. In pancreatic β cells, membrane glucose transporters (GLUT2) transport glucose across the plasma membrane. Glucose is metabolized via glycolysis to form pyruvate, which enters the citrate cycle in mitochondria to generate ATP. Increased ATP triggers closure of KATP channels, depolarization and opening of voltage-gated Ca^{2+} channels. Influx of Ca^{2+} promotes insulin granules to move to the cell surface prior to insulin release events. **(1)** Increased ATP:AMP ratios inhibit AMPK activity and increase the number of docked insulin granules beneath the plasma membrane. This is probably achieved through an inhibitory effect of AMPK on kinesin light chain-1 phosphorylation. **(2)** LKB1 inhibits glucose-stimulated insulin secretion in part by lowering GLUT2 expression. **(3)** LKB1 affects pancreatic β-cell polarity by reorganizing microtubules, actin filaments and tight junctions.
AMPK: AMP-activated protein kinase; GLUT2: Glucose transporter 2; LKB1: Liver kinase B1.

metformin, led to an increase in the number of apoptotic cells assessed by DNA fragmentation [86,97,98,101,102]. This change was proposed to be due to increased oxygen radical formation and mitochondrial dysfunction. The latter was then proposed to activate JNK and caspase 3-dependent death pathways, eventually leading to the onset of apoptosis [86,99]. Using an adenovirus to express AMPK CA, we confirmed that activation of AMPK in MIN6 cells and dissociated mouse islet β cells significantly increased levels of cleaved-caspase 3, a key marker of apoptosis [103]. Conversely, AMPK DN virus-infected cells showed decreased levels of active caspase 3 after cytokine treatment [103]. Correspondingly, in pancreatic β cells isolated from AMPK α2-deficient mice, AICAR-induced apoptosis was significantly reduced [101].

β-cell AMPK & LKB1 regulate whole-body glucose homeostasis in mice

A first attempt to identify the role of AMPK specifically in pancreatic β cells in controlling whole-body glucose metabolism was achieved by our group using streptozotocin-induced diabetic mice, which were transplanted with islets infected with AMPK CA or DN viruses. Mice receiving AMPK CA-infected islets demonstrated poorer glycemic index over 20 days post-transplantation and poorer glucose tolerance, whereas those receiving AMPK DN-infected islets had improved glycemic control and better glucose tolerance [104]. More recent studies by our group [105] and that of Michael Ashford [106] using RIP2-Cre transgenic mice to delete both AMPK α1 and α2 subunits in pancreatic β cells and a subpopulation of 'RIP2 neurons' in the brain revealed severe glucose intolerance. In the 'double knockout' mice insulin secretion *in vivo* was sharply reduced [105,106], despite increased insulin sensitivity of peripheral tissues [105]. It seems likely that this reflects central deletion of AMPK, since our further studies revealed that stereotactic injection of AMPK DN into the hypothalamus of rats decreased glucose output, presumably reflecting increased insulin sensitivity [107,108]. However, as shown in **Figure 1** [3], although both studies reported that AMPK deletion abolishes insulin secretion *in vivo*, glucose-stimulated insulin secretion from islets *in vitro* revealed important differences between the two studies. Thus, insulin secretion from double AMPK (α1, α2; dKO) mouse islets was reduced and β cells were hyperpoloarized in the study by Beall *et al.* [106]. By contrast our own data [105], consistent with earlier studies [87,96], revealed increased granule number beneath the plasma membrane and enhanced insulin release **(Figure 2)** [1]. These important apparent discrepancies are likely to reflect differences in the protocols used for the culture of islets (e.g., glucose concentrations), and the selection of islets for studies (size, 'condition'). A definitive answer is likely to come only from studies using perfused pancreas (the most physiological *ex vivo* preparation for the study of insulin secretion), more β-cell selective knockout, achieved, for example, using RIP-*Cre* or Pdx1-*Cre*ER deleter strains [109,110], or a more complete understanding of the effects of hypothalamic AMPK deletion on insulin secretion.

In addition to the effects of modulation of AMPK on insulin secretion, we have also demonstrated that pancreatic β cells from AMPK dKO mice are smaller than those from control islets [105] and show enhanced levels of apoptosis and autophagy [Sun G, Marchetti P, Tooze S, Leclerc I, Rutter GA, Unpublished Data]. These observations would appear to argue against the involvement of mTOR signaling pathways, which have been widely believed to lie downstream of AMPK, controlling cell growth in many other cell types [111]. Further examination of isolated islets using transmission electron microscopy demonstrated increased apoptosis with enlarged mitochondria, indicating the importance of the presence of AMPK for cell survival [Sun G, Marchetti P, Tooze S, Leclerc I, Rutter GA, Unpublished Data]. Perhaps the most surprising discovery from three recent studies on mice with pancreatic β-cell LKB1 deletion using RIP2-Cre (our group) (βLKB1 KO) [112] or Pdx1-CreER [113,114] transgenes is the extent to which these mice fail to phenocopy βAMPK dKO mice. Thus, mice lacking LKB1 in β cells display increased insulin secretion and improved glucose tolerance largely due to enlarged β-cell mass and insulin synthesis. This is associated with dephosphorylation of MARK2 and upregulation of mTOR signaling pathways, suggesting the involvement of LKB1–MARK2–mTOR signaling pathways in controlling β-cell mass **(Figure 1(4))**.

Of the 12 other AMPK-related kinases [115], Snf-related kinase, NUAK1/2 and MARK1–3 were found to be highly expressed in pancreatic islets at the mRNA level [112]. Only a very low level of expression of SIK1/2 was apparent whilst BRSK1/2 (also-called SAD-A/B in the central nervous system) mRNA was undetectable [112].

Interestingly, Snf-related kinase, NUAK and MARK have been implicated in the control of cell polarity and growth in other cell types [116]. Studies of these kinases in pancreatic β cells, for example using β-cell-specific knockout mouse models, may reveal the possible function of these kinases in controlling pancreatic β-cell function and growth downstream of LKB1.

In summary, careful studies are still needed to distinguish between the effects of AMPK deletion in the brain and in β cells, and to determine the involvement of other AMPK-related kinases in the control of insulin secretion.

AMPK regulates glucagon secretion & gene expression in pancreatic α cells

Glucagon, secreted by pancreatic α cells in response to hypoglycemia [117], is a critical counter-regulatory hormone to insulin and thus in the maintenance of glucose homeostasis in mammals. Glucagon acts on liver glucagon receptors to promote hepatic glucose production, and downregulation of glucagon signaling, achieved for example by whole-body deletion of glucagon receptors ($GCR^{-/-}$ mice), leads to severe hypoglycemia during fasting [118]. On the other hand, both recurrent hypoglycemia in Type 1 diabetic patients and insulin treatment-induced hypoglycemia in Type 2 diabetic patients are associated with hyposecretion of glucagon. Both AMPK α1 and α2 subunits are expressed in pancreatic α cells [112]. However, little information has been obtained to date regarding the role of AMPK in pancreatic α cells. Interestingly, silencing of Pas (per-arnt-sim) domain-containing protein kinase (PASK), which is distantly related to AMPK, in mouse αTC1–9 cells leads to increased AMPK α2 gene expression accompanied by increased glucagon secretion and pre-proglucagon gene expression. Conversely, overexpression of PASK in αTC1–9 cells or human islets activates glucagon release, suggesting that PASK might regulate glucagon signaling partially through AMPK [119]. Recent approaches to regulate AMPK activity in the mouse αTC 1–9 cell line have included using AMPK activators, such as metformin, phenformin or A769662 [120], or viruses carrying AMPKα2 CA [120]. In the same study AMPK was inhibited in α cells using compound C or a virus carrying AMPK α1 DN. These studies revealed a potentially important role for AMPK in regulating glucagon release. Thus, activation of AMPK increased glucagon secretion whilst inhibition of AMPK led to decreased glucagon secretion with blunted low glucose-stimulated intracellular Ca^{2+} oscillations. By contrast, activation of AMPK specifically in pancreatic α cells in intact islets by pre-proglucagon promoter-driven AMPK CA expression demonstrated the stimulatory effect of AMPK on glucagon secretion [120]. Thus, these observations indicate that AMPK is a critical regulator of glucagon release from the α cell, and is likely to be involved in counter-regulatory responses to hypoglycemia.

Key remaining questions are thus whether regulation of glucagon secretion via AMPK-dependent pathways will affect total glucose metabolism in the long term? Which isoforms of AMPK catalytic subunits are involved? What are the mechanisms through which AMPK exerts diametrically opposite effects on hormone secretion in α cells (stimulation) versus β cells (inhibition)? Studies with AMPK inactivated specifically in pancreatic α cells will be needed to fully address these questions.

■ AMPK in the ventromedial hypothalamus

Hypothalamic AMPK in regulating food intake

The basomedial hypothalamic area of the brain is a key compartment for energy sensing and satiety regulation. Hypothalamic AMPK activity is regulated by feeding status and various hormones and nutrients including insulin, ghrelin, leptin, adiponectin and cannabinoids, and these changes have been suggested to control bodyweight and food intake [121–126]. Increases in AMPK activity, achieved by expressing AMPK CA in the mediobasal hypothalamus (including arcuate and paraventricular areas) or intracerebroventricular injection of AICAR, increased food intake and bodyweight, concomitant with increased expression of the orexigenic peptides neuropeptide and agouti-related protein (AgRP) [127]. Conversely, introduction of adenoviral AMPK DN or compound C decreased the expression of both peptides and lowered food intake [127]. It was proposed that two intracellular signaling pathways might be involved in the satiety-controlling effects of AMPK. One was through hypothalamic lipid metabolism, that is, AMPK-mediated inhibition of ACC carboxylase and malonyl-CoA activation leading to enhanced carnitine palmitoyltransferase (CPT)-1 activity [128]. An alternative mTOR-dependent signaling pathway in the mediobasal hypothalamus was recently suggested to mediate AMPK-regulated increase in food intake. Here,

AMPK was proposed to inhibit phosphorylation of its downstream target, S6K1 [129,130]. To clarify exactly which areas of the hypothalamus, and which neuron types, were involved in these effects, Wither's group generated proopiomelanocortin and AgRP-specific AMPK α2 knockout mice. Mice lacking AMPK α2 in proopiomelanocortin neurons displayed increased food intake and became obese, while mice inactivated for AMPK α2 in AgRP neurons showed the opposite phenotype, with decreased food intake and bodyweight [131]. Glucose-inhibited neurons isolated from the basomedial hypothalamus responded to decreased glucose concentrations in a similar way to the treatment of neurons with AICAR. The stimulatory effects of AICAR on these neurons were reversed by the AMPK inhibitor, compound C. These results indicated that activation of AMPK in these neurons might lead to increased bodyweight [125].

Hypothalamic AMPK & the regulation of hepatic glucose production

In addition to regulating satiety and bodyweight, the hypothalamus has also been implicated in integrating energy signaling to control hepatic glucose production. In light of the reduced glucose output in mice with decreased CPT-1 levels in the hypothalamus [132], it is plausible that AMPK, whose phosphorylation increases CPT-1 activity through decreasing ACC and malonyl-CoA levels, might also increase hepatic glucose production. Most recently, using rats infused into the mediobasal hypothalamus with virus carrying AMPK DN or compound C, Yang and colleagues demonstrated that inhibition of AMPK in this brain region reduced glucose production without changing peripheral glucose disposal [107]. Thus, central AMPK might be an important regulator of hepatic glucose production **(Figure 1(5))**.

Hypothalamic AMPK in controlling β-cell function & β-cell mass

As well as modulating hepatic glucose production, the hypothalamus may be able to control β-cell function and β-cell mass by controlling central hormone secretion such as resistin [133]. Thus, intracerebroventricular infusion of resistin into rats for 4 weeks led to a remarkably elevated first phase insulin secretion, concomitant with increased β-cell mass. Decreased AMPK phosphorylation in response to leptin was also alleviated by resistin infusion, implying that the modulation of AMPK activity in the hypothalamus by resistin might regulate β-cell function and β-cell growth [133]. As mentioned above, deletion of both AMPK catalytic isoforms using the RIP2-Cre transgene also expressed in the hypothalamus [109] led to opposing insulin secretion patterns *in vivo* and *in vitro*, implicating the involvement of central AMPK in controlling insulin secretion *in vivo* [105]. Careful analysis of which hypothalamic neuronal cell types and which AMPK isoforms are involved in controlling β-cell function or mass needs to be further addressed.

Upstream kinases for AMPK in the hypothalamus

Mice deleted for LKB1 in RIP2-expressing cells (βLKB1 KO), including those in the hypothalamus [109], showed a hypophagic phenotype and decreased bodyweight [112]. Neither β-cell selective-AMPK double knockout mice (generated using the same RIP2-Cre deleter mice as for LKB1 elimination in these cells) or mice in which LKB1 was inactivated in the β-cell of adult mice using a regulatable Pdx1 CreERT mouse strain, showed any changes in satiety or bodyweight. These findings suggest that the observed decrease of food intake observed as a result of hypothalamic LKB1 elimination (a consequence of the expression of the RIP2.Cre transgene in parts of the central nervous system, as well as in the pancreatic β-cell) was independent of AMPK [105,113,114]. Further studies of LKB1, or possibly other AMPK-related kinases, in RIP2 neurons are needed to fully understand the mechanisms by which LKB1 controls satiety in RIP2-Cre neurons.

In summary, AMPK is clearly a key regulator of energy signals in the hypothalamus, integrating signals from peripheral tissues to control satiety, glucose production and insulin secretion. However, caution needs to be taken when interpreting data from intracerebroventricular infusion studies that affect large areas of the mediobasal hypothalamus. Thus, the identity of the neurons involved, and the role played by AMPK in such neurons, remains only partly clarified. Total ablation of AMPK using neuron-specific Cre transgenes will be a useful tool to answer these questions.

Conclusion & future perspective

In this article we have focused on recent studies in which gene inactivation has been used as a tool to study the role and regulation of AMPK

in diabetes-relevant tissues in mice. However, it is important to point out two caveats when interpreting such studies [1]:

- The possibility exists that changing the expression of a gene might unmask a pathway whose role in normal physiology is, due to redundancy, usually minimal or zero. Such redundancies mean that the absence (or mildness) of a phenotype apparent after the inactivation of a particular gene cannot be taken as proof that the gene and gene product in question are unimportant [2];
- That the considerable differences between the physiology of mice (mass ~25 g; lifespan <3 years) versus man (mass ~50–90 kg; lifespan ~75 years) mean that extrapolation of results from the former to the latter must be made with caution.

Nonetheless, at least for the time being, mice do provide the most suitable *in vivo* system to analyze the role of enzymes such as AMPK and the role they play in health and in disease settings.

Because AMPK balances ATP generation and consumption, it is natural to think of it as acting as a gauge to control energy status [15,50]. Thus, manipulation of AMPK might restore the imbalance of energy usage in metabolic disorders such as T2D where AMPK activities and ATP/AMP levels can be affected. In fact, the most widely prescribed antidiabetic drug, metformin, strongly activates AMPK in many insulin-sensitive and glucose-regulating organs, such as the liver, muscle, hypothalamus and pancreas [134,135]. Early studies using metformin also demonstrated the beneficial effects of AMPK in restoring energy balance, such as decreasing hepatic glucose output, elevating skeletal muscle glucose uptake and promoting satiety in the hypothalamus [134]. However, with increased usage of tissue-specific AMPK and LKB1 knockout mouse models, it is gradually emerging that the effects of metformin are probably not, or only partially, mediated via AMPK. Moreover, discrepancies between the phenotypes of mice inactivated for AMPK or LKB1 in the same tissue suggest that other AMPK-related kinases downstream of LKB1 might be associated with the control of whole-body glucose metabolism. Notably, in the cases of muscle contraction-induced glucose uptake and hepatic glucose production, it seems very likely that SNARK and SIK2 or MARK2 are involved. Likewise, the discrepancies in the phenotypes of RIP2-AMPK dKO and RIP2-LKB1 KO mice in terms of food intake and insulin secretion also argue against the universal control of satiety by a simple linear 'LKB1-AMPK' signaling pathway. Therefore, the concept of AMPK as a master energy sensor in different glucose- and insulin-sensing organs may need careful re-evaluation. In particular, further investigation of the role of the other 12 AMPK-related kinases [115] using tissue-specific knockout or transgenic mice model may help to understand how these kinases regulate glucose levels. Importantly, drugs acting specifically on certain kinases or downstream targets may provide the promise of new therapies for T2D.

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