The Role of AMPK and mTOR in Nutrient Sensing in Pancreatic β-Cells

Catherine E. Gleason, Danhong Lu, Lee A. Witters, Christopher B. Newgard, and Morris J. Birnbaum

From the 1Howard Hughes Medical Institute and Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, the 2Sarah W. Stedman Nutrition and Metabolism Center and Departments of Pharmacology and Cancer Biology, Medicine and Biochemistry, Medical Center, Duke University, Durham, North Carolina 27704, and the 3Departments of Medicine, Biochemistry, Dartmouth Medical School and the Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

The AMP-activated protein kinase (AMPK) is a central regulator of the energy status of the cell, based on its unique ability to respond directly to fluctuations in the ratio of AMP:ATP. Because glucose and amino acids stimulate insulin release from pancreatic β-cells by the regulation of metabolic intermediates, AMPK represents an attractive candidate for control of β-cell function. Here, we show that inhibition of AMPK in β-cells by high glucose inversely correlates with activation of the mammalian Target of Rapamycin (mTOR) pathway, another cellular sensor for nutritional conditions. Forced activation of AMPK by AICAR, phenformin, or oligomycin significantly blocks phosphorylation of p70S6K, a downstream target of mTOR, in response to the combination of glucose and amino acids. Amino acids also suppressed the activity of AMPK, and this at a minimum required the presence of leucine and glutamine. It is unlikely that the ability of AMPK to sense both glucose and amino acids plays a role in regulation of insulin secretion, as inhibition of AMPK by amino acids did not influence insulin secretion. Moreover, activation of AMPK by AICAR or phenformin did not antagonize glucose-stimulated insulin secretion, and insulin secretion was also unaffected in response to suppression of AMPK activity by expression of a dominant negative AMPK construct (K45R). Taken together, these results suggest that the inhibition of AMPK activity by glucose and amino acids might be an important component of the mechanism for nutrient-stimulated mTOR activity but not insulin secretion in the β-cell.

The β-cell is unique compared with other mammalian cell types in that its primary function to synthesize and secrete insulin is tightly coupled to its metabolic rate. Glucose is the most potent nutrient in stimulating insulin release. Upon entry into the β-cell, glucose is rapidly metabolized, resulting in the generation of mitochondria-derived metabolic intermediates including ATP. This increase in ATP leads to closure of ATP-sensitive K+ (KATP)-channels, depolarization of the plasma membrane and opening of voltage-gated L-type Ca2+- channels. The subsequent increase in intracellular Ca2+ concentration [Ca2+]i, triggers insulin exocytosis (1). The β-cell also utilizes certain key amino acids that, via mitochondrial metabolism, can further generate coupling factors that elicit an insulin secretory response (2, 3). In addition to their role as insulin secretagogues, glucose and other nutrients stimulate protein translation and β-cell growth and proliferation (4, 5). While much is known regarding how the β-cell couples glucose metabolism to insulin secretion, the mechanisms by which β-cells sense metabolism of other fuels, such as amino acids, and augment glucose-stimulated insulin release are less clear. Further, it is unclear how the β-cell coordinates nutrient abundance with enhanced protein translation and cell growth. This aspect of β-cell function is particularly important under conditions of increased insulin demand, such as obesity and/or insulin resistance. Failure of β-cells to compensate for this increase in demand is a critical factor in the development of type 2 diabetes (6).

The AMP-activated protein kinase (AMPK) is a heterotrimERIC serine/threonine protein kinase that is activated by various pathological and physiological stresses that result in a lowered cellular ATP/ADP + AMP ratio (7, 8). The AMPK protein complex consists of a catalytic α-subunit and regulatory β- and γ-subunits. AMPK activity is regulated allosterically by AMP and through phosphorylation at Thr172 in the activation loop of the α-subunit. By phosphorylation of downstream targets, AMPK acts to repress pathways that consume energy and to promote ATP-producing catabolic pathways (7, 8). For example, by phosphorylation of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, AMPK shuts down the ATP-depleting processes of fatty acid and cholesterol biosynthesis, respectively, and replenishes ATP by promoting fatty acid oxidation. AMPK also exerts its effect on cellular energy balance through modification of gene expression and protein translation (9, 10). Because glucose-stimulated insulin secretion from the β-cell is directly tied to

1 A member of the Cox Institute. To whom correspondence should be addressed: University of Pennsylvania, 415 Curie Blvd, CRB Rm. 320, Philadelphia, PA, 19104. Tel.: 215-898-5001; Fax: 215-573-9138; E-mail: birnbaum@mail.med.upenn.edu.

2 The abbreviations used are: AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; BCH, β-(+/-)-2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid; GDH, glutamate dehydrogenase; ACC, acetyl-CoA carboxylase.

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AMPK and Nutrient Sensing in β-Cells

the generation of metabolic intermediates, the unique sensitivity of AMPK to changes in the AMP/ATP ratio makes AMPK an attractive candidate for regulation of β-cell function. Indeed, AMPK has been implicated in the regulation of glucose and amino acid-stimulated insulin release and gene expression in the β-cell, although this is controversial (11–13). More recently, the ability of AMPK to coordinate energy availability with protein synthesis through regulation of the mammalian Target of Rapamycin (mTOR) signaling pathway has received considerable attention in non-β-cell lines (9). Currently, it is not known if AMPK modulates the mTOR signaling pathway similarly in the β-cell.

AMPK regulates protein translation via at least two mechanisms: phosphorylation and activation of the eukaryotic elongation factor 2 kinase (eEF2K) and inhibition of the mTOR signaling pathway (14, 15). Activation of eEF2K leads to phosphorylation of its target eEF2 and inhibition of the elongation step of protein translation. mTOR is a serine/threonine protein kinase that regulates various cellular functions, in particular, the initiation step of protein synthesis. mTOR is activated in response to hormones and growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), and via the phosphatidylinositol 3-kinase-Akt signaling pathway in the presence of amino acids. Amino acids also induce mTOR activity in the absence of additional stimuli. In particular, the branch-chained amino acid leucine is required for the effect of amino acids to activate mTOR. Once activated, mTOR phosphorylates both the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), an inhibitor of translation, and p70 ribosomal S6 kinase (p70S6K) (15).

Because AMPK may represent an alternative mechanism for sensing glucose and amino acids in the β-cell, it is important to understand its downstream targets in this cell type. Moreover, the ongoing development of activators of AMPK for use as drugs in the treatment of type 2 diabetes makes this a particularly relevant issue. In this study, we asked if AMPK regulates glucose-stimulated mTOR activation and insulin secretion in β-cells lines and primary rodent islets. Because amino acids contribute to both stimulation of insulin release and mTOR activation through their metabolic breakdown, we also addressed the question of how amino acids are sensed by AMPK and whether their effect to inhibit AMPK correlates with insulin release.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin/streptomycin solution, sodium pyruvate solution, l-glutamine, Minimum Essential Media (MEM) essential, and non-essential amino acid solutions were obtained from Invitrogen (Carlsbad, CA). 5-Aminomidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (Toronto, On, Canada). Phenformin, oligomycin, rapamycin, and insulin were from Sigma. The phospho-acetyl CoA carboxylase (p-ACC) antibody was from Upstate (Lake Placid, NY). The phospho-rpS6 antibody has been described previously (16). The anti-AMPKα antibody used recognizes the N terminus of both the α1 and α2 subunits of AMPK and has been described previously (17). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Infrared-labeled secondary antibodies for use with the Odyssey Odyssey Infrared Imaging System (LICOR Biosciences, Lincoln, NE) were purchased from Rockland Inc. (Gilbertsville, PA). All other antibodies were from Cell Signaling Technology, Inc (Beverly, MA).

Amino Acid Composition of Buffers—Krebs-Ringer bicarbonate buffer (KRHB: 115 mM NaCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 5 mM KCl, 25 mM NaHCO3, 1.2 mM MgCl2, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin) was supplemented with MEM amino acids solution, MEM non-essential amino acids solution, and l-glutamine. For these experiments, the 1× concentration of amino acids was defined as the following in mM: l-arginine 0.73, l-cystine 0.2, l-glutamine 2.0, l-histidine HCl H2O 0.2, l-isoleucine 0.4, l-leucine 0.4, l-lysine HCl 0.5, l-methionine 0.1, l-valine 0.4, l-phenylalanine 0.2, l-threonine 0.4, l-tryptophan 0.05, l-tyrosine 0.2, l-alanine 0.01, l-asparagine 0.01, l-aspartic acid 0.01, l-glutamic acid 0.01, glycine 0.01, l-proline 0.01, l-serine 0.01. For this study, we have defined physiological concentrations of leucine and glutamine as 0.4 mM and 0.2 mM, respectively, based on reported plasma concentrations of leucine and glutamine (0.119 mM and 0.338 mM, respectively) in fed mice (18).

Cell Culture and Treatment—MIN6 cells (kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University, Osaka, Japan) were used between passages 28 and 40 at ~80% confluence. MIN6 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose supplemented with 15% fetal bovine serum, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 100 µg/ml streptomycin, 100 units/ml penicillin, equilibrated with 5% CO2, 95% air at 37 °C. Prior to treatment, MIN6 cells were cultured overnight in DMEM containing 3 mM glucose and 10% fetal bovine serum. The medium was then removed, and the cells were washed one time with KRHB and incubated in KRHB for 2 h equilibrated at 5% CO2, 95% air at 37 °C. Cells were then stimulated with 3 or 30 mM glucose and/or amino acids with or without drugs for 30 min. For the experiments with insulin (Figs. 3 and 4) 200 mM insulin was added at the same time as glucose, amino acids and/or drugs were added. Medium (500 µl) was removed and assayed for insulin release by radioimmunoassay (Linco Research, St. Charles, MO). The cells were then lysed in ice-cold buffer containing 140 mM NaCl, 10 mM Tris, pH 7.4, 200 mM NaF, 10% glycerol, 1% Nonidet P-40, 1× Complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) and 1× phosphatase inhibitor mixture 1 and 2 (Sigma). 5 µl of lysate was removed for analysis of total protein by BCA assay (Pierce). Some studies were performed with the cell line 832/13, derived as described (19) from INS-1 rat insulinoma cells (20). When using these cells, culture conditions, and insulin secretion assay procedures were as previously described (19).

Studies with Isolated Rat Islets of Langerhans—Pancreatic islets of Langerhans were isolated from male Wistar rats (250 – 275 g) by perfusion of the pancreatic duct and in situ collagenase (Liberase RI) digestion, as previously described (21). Islet isolation and assay procedures were as previously described (21). Islet isolation and assay procedures were as described (21) in the presence or absence of 1 mM AICAR and following preincubation of 20 islets/condition in triplicate in HEPES balanced salt.
solution-SAB (HBSS; 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.16 mM MgSO$_4$, 20 mM HEPES, 2.5 mM CaCl$_2$, 25.5 mM NaHCO$_3$, 0.2% bovine serum albumin; pH 7.2) containing 3 mM glucose for 45 min.

**Suppression of AMPK Activity by Adenovirus-mediated Expression of a Mutant Form of AMPK—Ad-AMPK-o2-K45R**, a dominant inhibitory AMPK was prepared as described previously (17, 22). 832/13 cells were grown in 6-well plates to 90% confluence, and Ad-AMPK-o2-K45R or AdCMV-βGAL viruses were added at 50 m.o.i. in 2 ml of medium for 2 h. 24 h after viral treatment, cells were incubated in SAB containing 3 mM glucose for 2 h. The buffer was then removed and replaced with fresh SAB containing either 3 or 12 mM glucose for an additional 2 h. Where indicated, AICAR was added to the SAB/glucose at a concentration of 1 mM for the last 30 min of preincubation time and throughout the 2-h secretion period. Insulin secretion was measured by radioimmunoassay as described (19).

**Immunoblot Analysis**—Cells were washed once with ice-cold PBS and then lysed by the addition of ice-cold buffer containing 140 mM NaCl, 10 mM Tris, pH 7.4, 200 mM NaF, 10% glycerol, 1% Nonidet P-40, 1x Complete protease inhibitor mixture (Roche Applied Science) and 1x phosphatase inhibitor mixtures 1 and 2 (Sigma). The lysates were then centrifuged for 10 min at 10,000 rpm at 4 °C. Protein concentrations were determined using the BCA protein assay kit. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by transfer to nitrocellulose membranes (Whatman, Florham Park, NJ). Detection was performed either using ECL reagents from Amersham Biosciences or a LICOR Odyssey Infrared Imager as described in the figure legends. Blots imaged using the Odyssey Infrared Imager were probed using infrared-labeled secondary antibodies (Rockland, Inc, Gilbertsville, PA) following instructions supplied by the manufacturer (LICOR Biosciences, Lincoln, NE). Quantitation on blots scanned using the Odyssey was performed using the LICOR software.

**AMPK Activity**—AMPK was immunoprecipitated from 100 μg of MIN6 or 832/13 cell lysate by addition of α-AMPK antibody (17) for 1 h, rocking at 4 °C. 20 μl of protein A-agarose beads were added to samples, which were rocked for another 2 h at 4 °C. The beads were pelleted by centrifugation for 1 min at 13,000 rpm, 4 °C, and washed three times with lysis buffer followed by one wash with kinase reaction buffer (50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 0.02% Brij-30). The kinase reaction was conducted at 30 °C for 20 min in buffer containing 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 0.02% Brij-30, 25 mM MgCl$_2$, 0.2 mM SAMS peptide (HMRSAAMSLHLKRR), 0.3 mM AMP, 0.1 mM ATP with 0.25 μCi/μl [γ-32P]ATP. The reactions were stopped by placing samples on ice for 1 min followed by centrifugation at 13,000 rpm for 1 min, and 18 μl of each reaction was spotted onto P81 paper. The P81 paper was washed three times with 1% phosphoric acid and once with acetone. Incorporated [γ-32P]ATP was measured by counting on a Packard liquid scintillation CA16000 counter.

**Statistical Analysis**—Data are expressed as means ± S.E. as indicated in the figure legends. Statistically significant differences between groups were analyzed using analysis of variance (JMP Start Statistics). $p < 0.05$ was considered statistically significant.

**RESULTS**

Glucose stimulates phosphorylation of p70S6K in MIN6 cells (23). However, the relative contributions of a direct effect of glucose as opposed to the potential autocrine effects of secreted insulin on mTOR activity have not been determined. Therefore, we first examined glucose-stimulated mTOR activity in MIN6 cells and determined what component of that stimulation was caused by glucose alone. MIN6 cells were starved of serum, glucose, and amino acids for 2 h followed by exposure to glucose at increasing concentrations for 30 min. As shown in Fig. 1, glucose in the presence of a 1x concentration of amino acids dose-dependently increased p70S6K phosphorylation with maximal stimulation at 30 mM glucose. The 1x amino acid mixture contains a complete complement of amino acids plus glutamine (2 mM) and is equivalent to the concentration of amino acids present in MEM$_\alpha$ medium. Phosphorylation of ribosomal protein S6 (rpS6), the downstream target of p70S6K, phosphorylated in the downstream target of p70S6K, phosphorylated in the downstream target of p70S6K, phosphorylated in the downstream target of p70S6K, phosphorylated in the downstream target of p70S6K, phosphorylated in the downstream target of p70S6K.

Glucose and amino acids regulate mTOR activity in MIN6 cells. MIN6 cells were serum, glucose, and amino acid starved for 2 h in KRBH buffer. The buffer was then replaced with fresh KRBH containing a 1x amino acid mixture plus glucose at the indicated concentration, and cells were treated as shown for 30 min. Cells were processed for immunoblotting of the indicated proteins as described under “Experimental Procedures.” Results are representative of at least three separate experiments.
Because the direct effect of glucose to activate the mTOR pathway could have been obscured by the simultaneous release of insulin, it was necessary to develop conditions that distinguish the direct contributions of glucose from those of insulin. Therefore, we next asked if stimulating MIN6 cells with glucose in the presence of a saturating concentration of insulin (200 nM) would allow us to discern specifically the direct effect of glucose on activation of the mTOR pathway. As shown in Figs. 2 and 3, increasing the glucose concentration from 3 to 30 mM enhances both p70S6K and rpS6 phosphorylation. The effect of added insulin was indicated by an increase in phosphorylation at serine 473 (Ser473) of Akt, a kinase known to be downstream of the insulin receptor (Fig. 3). In the presence of 200 nM insulin, Akt phosphorylation was maximal and did not increase further with the addition of glucose, indicating that activation of the insulin receptor was saturating. However, in the presence of saturating insulin, increasing the glucose concentration resulted in enhanced phosphorylation of p70S6K and rpS6 (Fig. 3). These results demonstrate the presence of a direct (i.e. without intervening insulin secretion) effect of glucose on mTOR that can be assessed by analysis in the presence of a saturating concentration of insulin.

We next asked whether a reduction in AMPK signaling mediates glucose-stimulated activation of the mTOR pathway in the β-cell. MIN6 cells, preincubated as described previously, were exposed to 3 or 30 mM glucose plus 200 nM insulin with or without a 1/100 amino acid mixture for 30 min. During the 30 min of stimulation, AICAR, phenformin, or oligomycin was added as indicated to activate AMPK (Fig. 4). The drugs chosen to activate AMPK do so via three distinct mechanisms. AICAR is phosphorylated inside the cell to the AMP analogue, ZMP. Phenformin is a biguanide widely used to treat type 2 diabetes. Its method of AMPK activation is unclear but seems to be dependent on changes in the AMP:ATP ratio (25). Lastly, oligomycin depletes cellular ATP by inhibiting mitochondrial ATP synthase. Three surrogate measures of cellular AMPK activity were employed: phosphorylation on threonine 172 of the catalytic subunit; and phosphorylation of its two substrates ACC and eEF2. As expected, pharmacological activation of AMPK led to an increase in these parameters, though the three drugs differed in their relative potencies (Fig. 4A). Again, as
anticipated, incubation in a low concentration of glucose also led to activation of AMPK. Surprisingly however, amino acids completely suppressed the activation of AMPK by glucose depletion. This effect was overcome by exposure of cells to pharmacological activators of AMPK (Fig. 4A). Amino acids not only suppressed AMPK activity, but also activated mTOR signaling, as indicated by increases in phosphorylation of p70S6K and rpS6. AICAR, phenformin or oligomycin antagonized this activation of mTOR and their potencies paralleled their abilities to stimulate AMPK phosphorylation. Activation of AMPK by AICAR partially blocked the phosphorylation of p70S6K and rpS6 at both 3 and 30 mM glucose, whereas phosphorylation of these proteins was completely blocked by oligomycin or phenformin. The ability of three independent activators of AMPK to inhibit mTOR signaling suggests that the latter is a result of AMPK activation.

Amino acids play an important role in regulating initiation of translation in addition to their function as precursors for protein synthesis (26, 27). The presence of amino acids, in particular, leucine, is essential for activation of mTOR in response to glucose or insulin. Also, amino acid mixtures as well as certain individual amino acids can stimulate insulin release from the β-cell. The effect of amino acids to stimulate both initiation of protein synthesis via mTOR activation and insulin release has been attributed in part to their mitochondrial metabolism and an increase in cellular ATP level (2, 28). As shown in Fig. 4, we observed that the addition of amino acids to the media substantially reduced the effect of glucose to inhibit phosphorylation of AMPK and AMPK targets. As shown in Fig. 5A, MIN6 cells treated with 0.25 mM amino acids for 30 min following a 2-h period of serum, glucose, and amino acid starvation, exhibited a dose-dependent decrease in AMPK phosphorylation as well as phosphorylation of its downstream targets, ACC and eEF2. This inhibition was blocked by addition of oligomycin. Similarly, AMPK kinase activity was significantly decreased by addition of as little as 0.25 mM amino acid mixture (Fig. 5B). Addition of 1 mM amino acids was almost as potent as 30 mM glucose in inhibiting AMPK activity. The decrease in AMPK phosphorylation and activity also correlates with enhanced signaling through mTOR as indicated by the increased phosphorylation of p70S6K.

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**FIGURE 4.** AMPK activity correlates with inhibition of mTOR signaling. MIN6 cells were serum, glucose, and amino acid starved for 2 h. KRBH was then replaced with KRBH containing insulin (200 nM), 1x amino acids, and 3 or 30 mM glucose as indicated. During the 30 min of stimulation, cells were also treated with Vehicle (ethanol), AICAR (2 mM), Phenformin (10 mM), or Oligomycin (1 μM). A, cells were processed for immunoblotting as described previously. The Western blot is representative of three separate experiments. B, the level of p70S6K phosphorylation compared with total p70S6K was quantitated using LICOR software. Error bars indicate ± S.D. *, p < 0.001 for the effect of 3 versus 30 mM glucose.

**FIGURE 5.** Amino acids suppress AMPK and activate mTOR. MIN6 cells were pretreated as in Fig. 1. The pretreatment buffer was replaced with KRBH alone or KRBH containing the indicated concentration of amino acids and/or 30 mM glucose, and cells were stimulated for 30 min. Oligomycin (1 μM) was added as indicated during the 30 min of stimulation. Cell extracts were then processed for AMPK immune complex kinase assay and immunoblotting. A, Western blot representative of three separate experiments. B, AMPK kinase activity measured using the synthetic SAMS peptide as described under “Experimental Procedures.” Kinase activity results show the average ± S.D. of three separate experiments. *, p < 0.01; **, p < 0.001 for the effect of 0 versus AA and/or glucose.
AMPK and Nutrient Sensing in β-Cells

We next sought to address the mechanism by which amino acids suppressed activation of AMPK because of glucose deprivation. Since amino acids stimulate the mTOR signaling pathway in the absence of other factors, we first asked if their effect on AMPK was dependent on activation of mTOR. We treated MIN6 cells with increasing concentrations of amino acids in the absence or presence of rapamycin, which blocks mTOR signaling (Fig. 6). As expected, rapamycin blocked phosphorylation of p70S6K in response to amino acids. However, inhibition of AMPK phosphorylation and activity persisted even in rapamycin-treated cells, indicating that amino acids do not affect AMPK via rapamycin-sensitive mTOR activation.

We next asked if amino acids directly modify AMPK activity or if their effect is through metabolic breakdown and a subsequent decrease in the ratio of AMP:ATP. Glutamine and the branched-chain amino acids leucine, isoleucine and valine, are the only amino acids that are capable of eliciting a partial increase in activation of the mTOR pathway in β-cells. Of these amino acids, only leucine at a physiological concentration in combination with glutamine is sufficient to completely activate mTOR (29). Therefore, we asked if leucine or its non-metabolizable analog, β-(+/-)-2-amino-cyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), alone or in combination with glutamine is sufficient to decrease AMPK activity. MIN6 cells were serum and amino acid-starved for 2 hours and then incubated in buffer containing either a complete complement of amino acids, which contains leucine at 0.4 mM and glutamine at 2 mM, or individual amino acids alone or in combination with glutamine as indicated (Fig. 7). As shown above, complete amino acids decreased AMPK phosphorylation and phosphorylation of AMPK targets, ACC and eEF2. If leucine acts directly to modify AMPK activity, we expected that both leucine and BCH alone should inhibit the enzyme. As shown in Fig. 7, neither BCH nor leucine alone reduced phosphorylation of the AMPK substrates, ACC and eEF2. Both leucine and BCH alone modestly decreased AMPK phosphorylation, however, but not to the same extent as a complete complement of amino acids. Leucine and BCH both activate mitochondrial glutamate dehydrogenase (GDH) allosterically. GDH enhances substrate flux through the tricarboxylic acid cycle via conversion of glutamate to α-ketoglutarate. The effect of leucine or BCH alone is most likely explained by the presence of endogenous glutamate. The combination of leucine plus glutamine or BCH plus glutamine suppressed AMPK and AMPK substrate phosphorylation to the same degree as 1x amino acids, whereas glutamine alone had no suppressive effect. Significantly, glutamine at a physiological concentration (0.2 mM) (18) plus either leucine or BCH potently inhibited AMPK, ACC, and eEF2 phosphorylation. The ability of leucine and glutamine in combination to dramatically reduce AMPK activity suggests that the metabolism of amino acids and a consequent drop in the level of AMP is the mechanism for their effect on AMPK activity. In support of this, pyruvate, which also increases flux through the tricarboxylic acid cycle, reduced AMPK phosphorylation.

Leucine and glutamine in combination stimulate insulin secretion. Because leucine and glutamine together also potently inhibit AMPK phosphorylation and activity, we considered the possibility that inhibition of AMPK could be part of the mechanism whereby amino acids stimulate insulin secretion. Indeed, a recent study by Leclerc et al. suggests that inhibition of AMPK in MIN6 cells by amino acids correlates with their ability to stimulate insulin secretion (30). Therefore, to determine whether the decrease in AMPK activity is caused by amino acids correlates with amino acid-stimulated insulin release, we incubated MIN6 cells with 0.4 mM leucine plus increasing concentrations of glutamine (Fig. 8). Maximal inhibition of AMPK phosphorylation and activity was achieved with addition of 0.2 mM glutamine. Increasing the glutamine concentration above 0.2 mM did not decrease AMPK phosphorylation further. However, insulin secretion was not affected until addition of either 1 or 2 mM glutamine (Fig. 8C). These results indicate that AMPK inhibition in response to physiological concentrations of leucine and glutamine does not correlate with stimulation of insulin secretion. Whereas all concentrations of glutamine added significantly inhibit AMPK phosphorylation and activity, insulin release was only stimulated by glutamine concentrations equal to or greater than 1 mM.
Because our data suggesting a lack of correlation between AMPK activity and insulin secretion conflicts with published reports (12, 31), we used several additional strategies and cellular models to investigate this issue further. First, incubation of MIN6 cells with phenformin or AICAR significantly increased AMPK phosphorylation at 30 mM glucose. (Fig. 9A). In the same experiments, these drugs did not suppress insulin secretion at stimulatory glucose levels as has been reported by others. In contrast, phenformin augmented insulin release at low glucose (Fig. 9B). Second, we performed a series of experiments in an alternative β-cell model system, the robustly glucose-responsive INS-1-derived cell line 832/13 (19). In these cells treated with a control recombinant adenovirus, AdCMV-βGAL, 12 mM glucose suppressed AMPK activity by 40% compared with cells at 3 mM glucose, and this suppression was prevented in the presence of AICAR (Fig. 10A). Consistent with our findings in MIN6 cells, AICAR had no effect on insulin secretion from AdCMV-βGAL-treated 832/13 cells at stimulatory glucose levels, and caused an increase rather than a decrease of insulin secretion at lower glucose concentrations (Fig. 10B). Third, we manipulated AMPK activity in 832/13 cells by a molecular approach, involving expression of an AMPKα subunit with a point mutation that causes the enzyme to function as a dominant negative suppressor of endogenous AMPK activity (K45R). Overexpression of this construct in 832/13 cells had the anticipated effect of decreasing AMPK activity (Fig. 10A), but this suppression of AMPK activity had no impact on glucose-stimulated insulin secretion (GSIS) (Fig. 10B). Finally, we treated freshly isolated rat islets with AICAR in the presence of various glucose concentrations (Fig. 11). In rat islets, AICAR increased rather than suppressed insulin secretion at low and intermediate glucose concentrations (5.5 and 8.3 mM), and had no effect on secretion at a maximally stimulatory glucose concentration (16.7 mM). Thus, contrary to prior reports (12, 31), pharmacologic and molecular manipulations that increased AMPK activity in three different β-cell

**FIGURE 8.** AMPK activity in response to amino acids does not correlate with insulin secretion. MIN6 cells were pretreated as described in the legend to Fig. 1. Cells were then stimulated with KRBB plus vehicle (1 N HCl) or containing leucine (0.4 mM) plus the indicated concentrations of glutamine for 30 min. A, cells were processed for immunoblotting. The Western blot is representative of three separate experiments. B, quantitation of AMPK phosphorylation compared with total AMPK using LICOR software. Error bars indicate ± S.D. C, insulin released during the 30 min of stimulation. Insulin data show the average ± S.D. of three separate experiments. B, ***, p < 0.001; *, p < 0.01 versus vehicle; C, *, p < 0.001 versus vehicle.

**FIGURE 9.** Activation of AMPK by AICAR or phenformin does not impair glucose-stimulated insulin release. MIN6 cells were pretreated in KRBB without glucose for 1 h. The cells were then stimulated with 3 or 30 mM glucose and the indicated drug for 1 h. A, cells were processed for immunoblotting as described under “Experimental Procedures”. The Western blot is representative of two separate experiments. B, insulin released during the 1 h of stimulation. Insulin data show the mean ± S.D. of two separate experiments each performed in triplicate. *, p < 0.001 for 3 mM glucose compared with 30 mM glucose ± drug; **, p < 0.005 for 3 mM glucose compared with 3 mM + 10 mM phenformin.
model systems, including primary islets, failed to suppress glucose-stimulated insulin secretion. Furthermore, suppression of AMPK activity by a molecular approach in 832/13 cells failed to enhance GSIS. Thus, these studies provide multiple lines of evidence for a lack of correlation between AMPK activity and regulation of insulin secretion by glucose and other metabolic fuels.

DISCUSSION

A number of recent studies has emphasized the ability of the sensor of nutritional stress, AMPK, to regulate proliferation, growth, and protein synthesis. In this study, we have examined the AMPK pathway in β-cells, in particular focusing on the regulation of mTOR by AMPK and the relationship of AMPK regulation to insulin secretion. A connection between AMPK and mTOR in the β-cell is of particular relevance, since this organ is uniquely designed to sense and respond to the availability of simple nutrients. The mTOR signaling pathway has been implicated in regulation of glucose-stimulated protein synthesis and cell growth in the β-cell. In this report, we show that the β-cell does indeed possess a mechanism by which low glucose can suppress mTOR activity via AMPK; in addition, we present the surprising result that amino acids are equally potent in regulating this pathway. However, multiple lines of evidence presented in this study in several different β-cell model systems demonstrate that regulation of AMPK does not influence insulin secretion at stimulatory glucose concentrations.

Glucose and amino acids stimulate phosphorylation of the mTOR target proteins p70S6K and 4E-BP1 in various pancreatic β-cell lines and rodent islets (23, 29, 32, 33). The regulation of mTOR by nutrients, however, is particularly complex in the β-cell, since exposure to glucose can lead to activation of the insulin signaling pathway by an autocrine mechanism. Thus, glucose can lead to mTOR activation through stimulation of insulin secretion and activation of the insulin receptor or directly through an unknown mechanism. In agreement with studies in other β-cell lines, our results show that a predominant portion of the response to glucose is mediated by autocrine activation of the insulin receptor in MIN6 cells (33). However, in this study we demonstrate clearly the existence of a glucose-dependent effect on mTOR activation independent of enhanced insulin secretion. This was demonstrated in two
ways: by pharmacologically inhibiting insulin release and by incubating cells under conditions in which insulin signaling is maximally activated, i.e. in the presence of 200 nm insulin. It is possible that a certain level of insulin signaling is required for optimal mTOR activation by glucose. It is difficult to estimate the relative contribution of these two pathways in vivo.

Another issue addressed in this study is the regulation of mTOR and AMPK in β-cells by nutrients. In β-cells, both glucose and amino acids stimulate signaling through mTOR, although the mechanism(s) for their action is not completely understood. Using HEK293 cells, Dennis et al. (34) suggested that mTOR itself might sense cellular ATP levels based on its high \( K_m (~1 \text{ mM}) \) for ATP. However, recent studies have proposed an alternative mechanism providing substantial evidence that mTOR is a downstream component of an energy-sensing pathway with AMPK as the primary cellular energy sensor. 2-deoxyglucose and AICAR, both activators of AMPK, inhibit p70S6K activity (35, 36). Inoki et al. (37) provided direct evidence that AMPK regulates signaling through mTOR by demonstrating that AMPK can phosphorylate TSC2. TSC2 is part of the TSC1/TSC2 complex that lies upstream of mTOR, and phosphorylation of TSC2 by AMPK presumably leads to its activation and the subsequent inhibition of mTOR activity. Additional studies in more physiological cell types have since demonstrated that activation of AMPK in response to both physiological (i.e. exercise) and pathological (i.e. glucose deprivation, mitochondrial dysfunction) stresses leads to inhibition of the mTOR signaling pathway (38 – 40). In the β-cell, glucose and the amino acids leucine and glutamine activate mTOR, but the role of AMPK in this process has not been studied (28, 29). Nonetheless, the importance of this pathway is demonstrated by mice lacking p70S6K1, a downstream target of mTOR (41). These mice exhibit hypoinsulinemia and glucose intolerance as a result of diminished β-cell size. Our finding that mTOR activation is inversely related to AMPK activity and that AMPK activation inhibits the mTOR signaling pathway suggests that one component of the mechanism whereby glucose and other nutrients stimulate protein synthesis in the β-cell is through the inhibition of AMPK.

Our findings that amino acids, specifically leucine and glutamine, inhibit AMPK phosphorylation and activity also support the hypothesis that AMPK is a critical factor in nutritional regulation of mTOR signaling. The branched chain amino acids are known for their unique ability to stimulate mTOR activity independently of other amino acids (42). Leucine is the most potent of the branched chain amino acids and has been shown to stimulate protein synthesis, regulate 4E-BP1 and p70S6K phosphorylation and inhibit autophagy at physiological concentrations (43 – 46). In the β-cell, only leucine in combination with glutamine at a physiological concentration is capable of eliciting complete activation of p70S6K. This effect of leucine has been attributed to its metabolic breakdown via oxidative decarboxylation and allosteric activation of GDH in the mitochondria (29). Consistent with this model, we found that only leucine or its nonmetabolizable analog, BCH, in combination with glutamine was able to inhibit AMPK to the same extent as glucose or a full complement of amino acids. Leucine and BCH did decrease AMPK phosphorylation and phosphorylation of its downstream targets to a small extent. However, this effect was most likely caused by endogenous glutamate and/or the production of glutamate from α-ketoglutarate during transamination of leucine to α-ketosuccinopropionic acid (KIC) (47). Surprisingly, the combination of BCH plus glutamine also significantly inhibited AMPK suggesting that oxidative decarboxylation of leucine is not required for the combined effect of leucine and glutamine. Further, KIC, a product of leucine metabolism, only modestly inhibited phosphorylation of the AMPK targets, ACC and eEF2 (Fig. 7). Thus, our results suggest that the mechanism for amino acid inhibition of AMPK involves elevated cellular ATP via enhanced flux through the tricarboxylic acid cycle. Importantly, this provides evidence for a secondary mechanism by which amino acids regulate mTOR activity, as proposed in several recent review articles (28, 48).

In addition to their role in regulation of protein translation, in the β-cell, amino acids also act as potent fuel stimulants for insulin release. To be effective stimulants, physiological amino acid mixtures generally require the presence of permissive levels of glucose. Certain amino acids, such as leucine, can elicit insulin release in the absence of glucose. Leucine by itself is approximately one-third as potent as glucose at stimulating insulin release. However, in the presence of glucose, leucine is nearly as potent as glucose (49). As discussed above, the combined effect of glutamine and leucine on stimulation of insulin release is also thought to be due to both oxidative decarboxylation of leucine and allosteric activation of GDH by leucine (2). Our finding that leucine in combination with glutamine is sufficient to completely inhibit AMPK activity could imply that inhibition of AMPK is important for amino acid-stimulated insulin release. However, our data indicate that the inhibition of AMPK by these amino acids does not correlate with insulin secretion, and therefore, is unlikely to be an important signaling intermediary. During the course of this study, Leclerc et al. (30) reported their observations that amino acids inhibit AMPK activity. Similar to our findings, they also attributed the effect of amino acids on AMPK to the elevation in cellular ATP content. Inconsistent with our results, they found that the effects of pharmacological concentrations of individual amino acids on insulin release correlates well with the inhibition of AMPK activity. Our results, using physiological concentrations of amino acids, demonstrate that AMPK is significantly inhibited at concentrations of amino acids that are below the threshold required for stimulation of insulin release.

At a more general level, the role of AMPK in regulation of insulin release is controversial. AMPK was initially implicated in regulation of insulin release based on studies reporting that elevated glucose concentrations inhibited AMPK activity and that AMPK activity is inversely correlated with insulin release (11, 50). In addition, forced activation of AMPK either by treatment with AICAR or metformin, or via overexpression of constitutively active AMPK inhibited glucose-stimulated insulin release (12, 31). However, other previous reports suggested that activation of AMPK with AICAR actually potentiates insulin release (51, 52), whereas insulin release from isolated islets is normal in mice lacking either the α1 or α2 catalytic subunit of AMPK (53, 54). Most recently, Hurley et al. demonstrated in the INS-1 β-cell line that AMPK activity is inhibited following
AMPK and Nutrient Sensing in β-Cells

glucose-dependent insulino tropic polypeptide-induced elevation in cAMP (55). The question of whether or not AMPK regulates insulin release in the β-cell is clinically important since AMPK has recently been considered a promising candidate for drug treatment of type 2 diabetes. Metformin, a drug already in use for the treatment of type 2 diabetes, is a potent activator of AMPK (56). Here, we show that in both MIN6 and INS-1-derived 832/13 cells, treatment with AICAR does not inhibit glucose-stimulated insulin release. We found that AICAR potenti
gulates insulin release in the β-cell. The question of whether or not AMPK reg-
ulated rat islets with AICAR caused an increase rather than a no effect on insulin secretion. Finally, treatment of freshly iso-
stimulated by high glucose. Moreover, suppression of AMPK
component of a mechanism allowing coordination between energy
availability, protein synthesis, and cell growth independent of effects on protein synthesis (57). Future
interestingly, it has been suggested that mTOR regulates cell
growth independent of effects on protein synthesis (57). Future
studies are necessary to fully elucidate the contribution of
AMPK to regulation of protein synthesis and/or cell growth in the
β-cell.

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