# Metabolic and Autocrine Regulation of the Mammalian Target of Rapamycin by Pancreatic β-Cells

**Michael L. McDaniel, Connie A. Marshall, Kirk L. Pappan, and Guim Kwon**

**Mammalian target of rapamycin (mTOR) is a serine and threonine protein kinase that regulates numerous cellular functions, in particular, the initiation of protein translation. mTOR-mediated phosphorylation of both the translational repressor eukaryotic initiation factor 4E binding protein-1 and p70 S6 kinase are early events that control the translation initiation process. Rapamycin, an inhibitor of mTOR, is a potent immunosuppressant due, in part, to its ability to interfere with T-cell activation at the level of translation, and it has gained a prominent role in preventing the development and progression of rejection in pancreatic islet transplant recipients. The characterization of the insulin signaling cascade that modulates mTOR in insulin-sensitive tissues has been a major focus of investigation. Recently, the ability of nutrients, in particular the branched-chain amino acid leucine, to activate mTOR independent of insulin by a process designated as nutrient signaling has been identified. The β-cell expresses components of the insulin signaling cascade and utilizes the metabolism of nutrients to affect insulin secretion. These combined transduction processes make the β-cell an unique cell to study metabolic and autocrine regulation of mTOR signaling. Our studies have described the ability of insulin and IGFs in concert with the nutrients leucine, glutamine, and glucose to modulate protein translation through mTOR in β-cells. These findings suggest that mitochondria-derived factors, ATP in particular, may be responsible for nutrient signaling. The significance of these findings is that the optimization of mitochondrial function is not only important for insulin secretion but may significantly impact the growth and proliferation of β-cells through these mTOR signaling pathways. *Diabetes* 51:2877–2885, 2002**

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AOAA, aminooxyacetic acid; AT, aminotransferase; ±BCH, β(±) 2-aminobicyclo [2,2,1]heptane-2-carboxylic acid; BP1, binding protein 1; GDH, glutamate dehydrogenase; eIF-4E, eukaryotic initiation factor 4E; IRS, insulin receptor substrate; $K_{ATP}$, ATP-sensitive $K^+$ channel; α-KG, α-ketoglutarate; α-KIC, α-ketoisocaproic acid; KRBB, Krebs-Ringer bicarbonate buffer; mTOR, mammalian target of rapamycin; OAA, oxaloacetic acid; $p70^{s6k}$, p70s6 kinase; PDK1, 3-phosphoinositide–dependent kinase; PHAS, phosphorylated heat- and acid-stable protein; PHAS-I, PHAS regulated by insulin; PI-3 kinase, phosphoinositide-3 kinase; PKB, protein kinase B.

In recent years, there has been an upsurge in the number of people, especially children, developing type 2 diabetes (1). At the same time, there has been increasing success in islet transplantation for the treatment of type 1 diabetes (2,3). These two developments have emphasized the importance of understanding the basic cellular mechanisms that may be responsible for defects in β-cell function, growth, and survival that underlie these disease states. A new aspect of β-cell biology has been the identification of the mammalian target of rapamycin (mTOR) signaling pathway. mTOR is a serine and threonine protein kinase that regulates numerous cellular functions, in particular, the initiation of protein translation (4–7). Rapamycin, an inhibitor of mTOR, is a potent immunosuppressant that interferes with T-cell activation at the level of translation, and it has been, in part, responsible for the success of the Edmonton protocol in islet transplantation in type 1 diabetes (2). Rapamycin has also been shown to be efficacious in kidney transplantation (8) and in the treatment of some carcinomas (9,10). The ability of mTOR to initiate protein translation is a critical event in enhanced protein synthesis that leads to increases in cell cycle progression and proliferation. The focus of this Perspective article is to examine insulin- and nutrient-signaling pathways through mTOR in the β-cell in order to provide new insights to enhance β-cell growth and proliferation that are relevant to both type 1 and type 2 diabetes.

Numerous studies have documented a role for insulin and IGF to modulate translation initiation in a variety of cell lines and primary cells. In the 1970s, Buse and Reid (11) and Li and Jefferson (12) began to describe a role for the branched-chain amino acid leucine in translation. Since then, mTOR and many other factors involved in translation initiation have been identified. More recently, the ability of nutrients, including amino acids and glucose, to exert an essential role in the regulation of protein translation has become an intense area of investigation. Of particular importance, leucine has also been shown to be unique in its ability to activate translation initiation independent of insulin and IGFs by a process designated as nutrient signaling (13–16). Furthermore, the nutrient glucose has been shown to promote the ability of insulin to mediate the phosphorylation of eukaryotic initiation factor 4E–binding protein-1 (eIF-4E–BP1), also designated as phosphorylated heat- and acid-stable protein regulated by

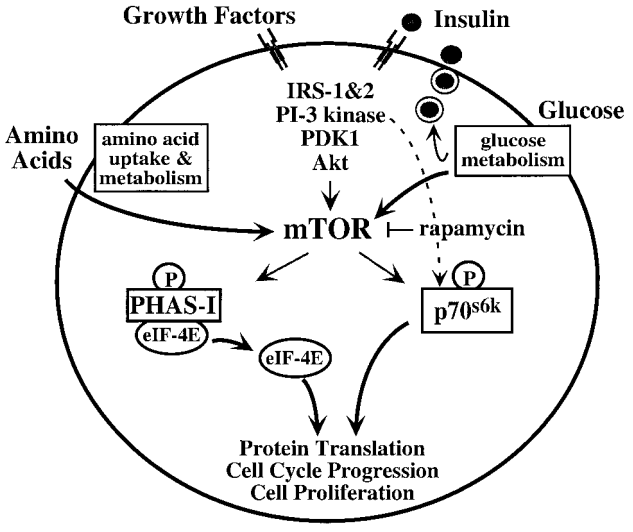


**FIG. 1. Metabolic and autocrine regulation of the mTOR pathway by β-cells.**

insulin (PHAS-I), in CHO cells (17). Glucose-dependent β-cell proliferation also appears to occur by stimulation of the mTOR/p70s6 kinase ($p70^{s6k}$) signaling pathway independent of the activation of 3-phosphoinositide–dependent kinase (PDK1) and Akt, also known as protein kinase B (PKB) (18).

The initial signaling events believed to be responsible for changes in initiating mRNA translation involve the phosphorylation of the translational repressor PHAS-I (19) and $p70^{s6k}$ (4–6) via mTOR. The phosphorylation of PHAS-I results in the release of eIF-4E and allows its participation in the initiation of cap-dependent mRNA translation. mTOR-mediated phosphorylation of $p70^{s6k}$ also results in the phosphorylation of ribosomal protein S6, which correlates with the translation of mRNAs that encode both ribosomal proteins and translational elongation factors. Rapamycin prevents the phosphorylation of these translational regulators (PHAS-I and $p70^{s6k}$), which are believed to be necessary for growth-related protein synthesis, leading to cell cycle progression and proliferation.

As illustrated in Fig. 1, the pancreatic β-cell is unique in its ability to secrete insulin and also express components of the insulin and growth factor signaling cascade; these components include a functional insulin receptor (20–23), the upstream docking proteins insulin receptor substrate (IRS)-1 and -2 (24), and the downstream signaling elements mTOR and translational regulators PHAS-I and $p70^{s6k}$ (15,25–27). Leibiger et al. (28) have proposed two selective insulin signaling pathways in β-cells through the type A and type B insulin receptors, which regulate insulin gene transcription and glucokinase transcription. Thus, β-cells exhibit the necessary components of the insulin signaling cascade to regulate insulin-mediated gene expression and protein translation in an autocrine manner. However, the insulin signaling cascade, as shown in Fig. 1, may not be an exclusively linear pathway from the insulin receptor to mTOR and $p70^{s6k}$. Because $p70^{s6k}$ activity is blocked by the phosphoinositide (PI)-3 kinase inhibitor wortmannin and the mTOR inhibitor rapamycin, it is possible that multiple pathways are required for activation of $p70^{s6k}$ (6,29).

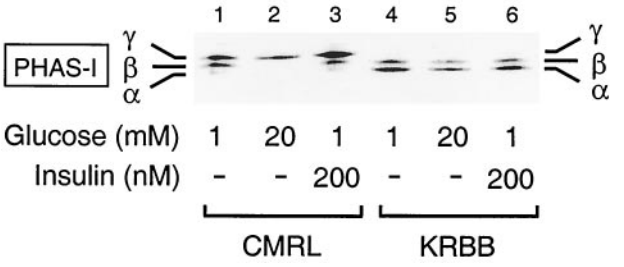


**FIG. 2. Amino acids are essential for phosphorylation of PHAS-I by glucose and insulin in isolated rat islets. Rat islets ($n$ = 200) were serum- and glucose-depleted in 1 ml CMRL-1066 (containing 0.1% BSA) or KRBB (containing 0.1% BSA) for 2 h at 37°C. Islets were then stimulated for 30 min in 1 ml CMRL or KRBB, 1 mmol/l glucose, 20 mmol/l glucose, or 1 mmol/l glucose plus 200 nmol/l insulin. Islets were processed for immunoblotting of PHAS-I. From Xu et al. (26).**

The pancreatic β-cell utilizes metabolically linked signal transduction pathways to affect insulin biosynthesis and secretion. The metabolism of glucose generates an increase in the ATP-to-ADP ratio, which results in inhibition of an ATP-sensitive $K^+$ ($K_{ATP}$) channel localized to the β-cell plasma membrane. This initial event is followed by cellular depolarization, the opening of voltage-dependent calcium channels, and the influx of extracellular calcium, which ultimately culminates in insulin exocytosis by β-cells. The branched-chain amino acid leucine also serves as an insulin secretagogue under appropriate conditions. The ability of leucine to stimulate insulin secretion is coupled to its metabolism by the mitochondria through oxidative decarboxylation and/or the ability of leucine to allosterically activate the enzyme glutamate dehydrogenase (GDH) by β-cells (30). Mitochondria-derived ATP produced by the metabolism of glucose and leucine is believed to exert a critical role in energy-requiring processes such as gene expression, protein translation, ion channel activity, insulin secretory granule movement, and exocytosis by β-cells (31).

**Do glucose, amino acids, and insulin regulate mTOR in β-cells?** Our initial studies, as shown in Fig. 2, were designed to determine whether exposure of isolated rat islets to elevated concentrations of glucose, which increases cellular metabolism and elicits an insulin secretory response, modulates the mTOR signaling pathway (26). In these studies, the hyperphosphorylation of PHAS-I was determined by mobility shift assays. PHAS-I generally appears as three bands when detected by gel shift analysis. These bands represent nonphosphorylated α (faintly visible in this blot), intermediate phosphorylated β, and hyperphosphorylated γ isoforms. In the case of adipocytes, exposure to insulin or growth factors results in a shift of α and β bands to the hyperphosphorylated and slower-migrating PHAS-γ, which is correlated with the release of eIF-4E and enhanced protein translation. As shown in Fig. 2, exposure of isolated islets to an elevated concentration of glucose (*lane 2*) compared with basal conditions (*lane 1*) resulted in increased phosphorylation of PHAS-I, with a shift of the intermediate phosphorylated PHAS-β to the hyperphosphorylated PHAS-γ. Subsequent studies established that glucose-induced formation of PHAS-γ was associated with the release of initiation factor eIF-4E by islets and was prevented by rapamycin. Rapamycin also inhibited glucose-stimulated $^{35}$S-methionine

incorporation into islet protein (26). Although exogenous insulin mimicked the ability of an elevated concentration of glucose to increase the formation of PHAS-γ (*lane 3*), the concentration of insulin required (200 nmol/l) was in the pharmacological range. Our more recent studies indicate that increases in cellular metabolism involving glycolysis and mitochondrial oxidation as a result of exposure of islets to elevated concentrations of glucose dominate this effect, although endogenous insulin secretion by β-cells does further augment the formation of PHAS-γ.

In these same series of experiments shown in Fig. 2 (*lanes 4–6*), we unexpectedly observed that amino acids were essential for the ability of both elevated glucose concentrations as well as exogenous insulin to mediate the formation of PHAS-γ. Thus, incubation of islets in Krebs-Ringer bicarbonate buffer (KRBB) in the absence of amino acids prevented the ability of glucose or exogenous insulin to enhance the formation of PHAS-γ. This was not caused by inhibition of insulin secretion, because islets incubated in KRBB secrete insulin in response to an elevated glucose concentration in a manner comparable to islets incubated in tissue culture media. These findings indicated that a component normally present in tissue culture media was responsible for this effect. This component was identified as amino acids, and the addition of a complete complement of amino acids to KRBB restored the ability of glucose or exogenous insulin to modulate mTOR signaling (26,27).

In addition to this essential requirement for amino acids to facilitate glucose and insulin signaling through mTOR, our studies demonstrated that amino acids alone were also capable of activating mTOR in the β-cell line RINm5F. Of the complete complement of amino acids normally present in tissue culture media, the branched-chain amino acid leucine was shown to be unique in its ability to activate mTOR signaling at physiological concentrations (27). In these studies, we monitored the phosphorylation of $p70^{s6k}$, another target of mTOR, again using the technique of mobility shift assays. To measure mTOR-mediated phosphorylation of $p70^{s6k}$, RINm5F cells were initially incubated in KRBB in the absence of amino acids for 2 h, a condition that results in the dephosphorylation of these translational regulators to a basal level. As shown in Fig. 3*A* (*lane 2*), the reintroduction of a complete complement of amino acids resulted in a robust phosphorylation of $p70^{s6k}$, as evidenced by the appearance of a slower-migrating upper band. The removal of only the branched-chain amino acids leucine, isoleucine, and valine from the complete complement of amino acids (designated as "basal amino acids") resulted in a return of phosphorylated $p70^{s6k}$ to basal levels (*lane 3*). It was then determined that the addition of only leucine at physiological concentrations (0.4 mmol/l) to the basal amino acids condition restored the phosphorylation of $p70^{s6k}$ (*lane 4*). Although isoleucine and valine failed to mediate the phosphorylation of $p70^{s6k}$ at these low physiological concentrations (*lanes 5* and *6*), both branched-chain amino acids were effective at concentrations as high as 10 mmol/l (15). As shown in Fig. 3*B*, this ability of leucine to mediate the phosphorylation of $p70^{s6k}$ was correlated with increased cellular metabolism, as determined by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (Promega, Madison, WI), which colorimetrically detects the production of NADH or NADPH. This ability of leucine to increase cellular metabolism was dependent on glutamine, which provides glutamate in the β-cell mitochondria. These results suggested that the metabolism of leucine by oxidative decarboxylation and leucine's activation of glutamate metabolism may be related to its unique ability to regulate mTOR by β-cells (15).

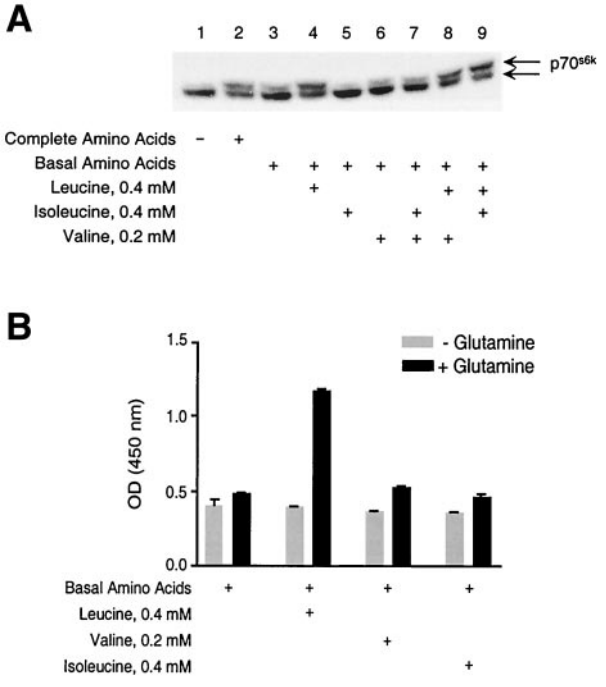


**FIG. 3. Leucine-induced phosphorylation of $p70^{s6k}$ at a physiological concentration in RINm5F cells: possible role of leucine and glutamine-mediated metabolism. *A*: Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Medium was then replaced with KRBB containing either complete amino acids as a positive control or basal amino acids, which excluded leucine, isoleucine, and valine. Leucine, isoleucine, or valine was then added as indicated for 30 min. Cells were processed for immunoblotting of $p70^{s6k}$. *B*: Cells were preincubated in KRBB in the absence of glucose and amino acids for 1 h. Medium was replaced with KRBB containing basal amino acids with or without glutamine (2 mmol/l), leucine, isoleucine, or valine as indicated for 2 h with the MTS reagent. The 450-nm absorbance values were measured at 2 h. From Xu et al. (15).**

**Possible mechanisms to explain the uniqueness of leucine in mTOR signaling.** The identification of the cellular mechanisms responsible for the ability of leucine to activate PHAS-I and $p70^{s6k}$ through mTOR has become an important area of investigation. A complication in this complex area has been the diversity of cell lines and primary cells used in attempting to define the mediators responsible for nutrient signaling. Some of the candidate mechanisms that have emerged include the metabolism of leucine (14,15,32), tRNA aminoacylation (33), leucine recognition sites (34–36), or modulation of protein kinases (37) or phosphatases (13).

Our more recent studies have focused on determining whether the well-established metabolically linked pathways involving the metabolism of leucine by the β-cell mitochondria could provide clues into the mechanism whereby leucine activates mTOR signaling. It has been established previously, as shown schematically in Fig. 4, that leucine serves as an insulin secretagogue due to its

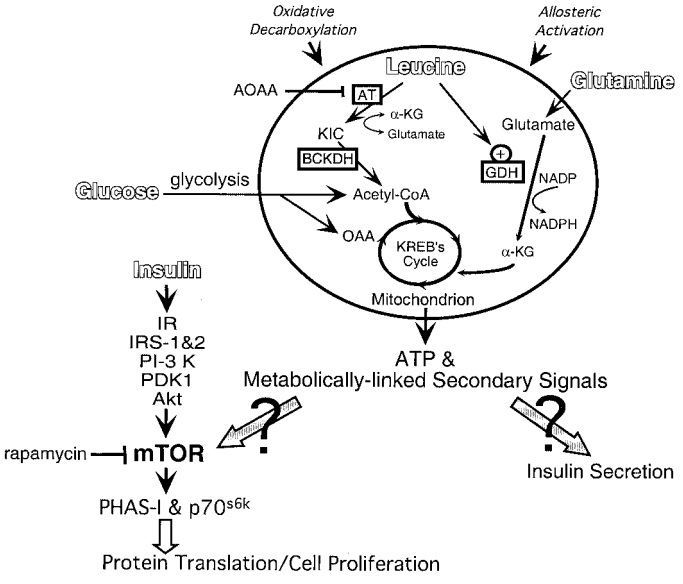


**FIG. 4. Proposed model of nutrient- and growth factor–mediated mitogenic signaling in β-cells. BCKDH, branched-chain α-keto-acid dehydrogenase; IR, insulin receptor.**

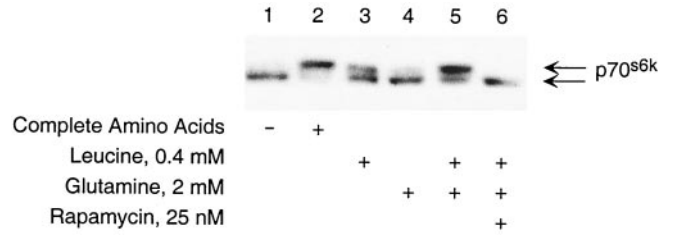


**FIG. 5. Leucine and glutamine-induced phosphorylation of p70$^{s6k}$ in RINm5F cells is rapamycin-sensitive. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last hour of preincubation, rapamycin (25 nmol/l) was added to cells. Medium was replaced with KRBB containing leucine alone, glutamine alone, or both leucine and glutamine with or without rapamycin as indicated for 30 min. Cells were processed for immunoblotting of p70$^{s6k}$. From Xu et al. (15).**

metabolism by oxidative decarboxylation and the generation of acetyl-CoA in combination with the ability of leucine to allosterically activate the enzyme GDH and the production of α-ketoglutarate (α-KG) in the β-cell mitochondria (38,39). In the latter case, glutamine is necessary as a cell-permeable precursor for glutamate, a substrate for GDH. It is generally believed that the combined entry of the Krebs cycle substrates acetyl-CoA and α-KG effectively energizes the β-cell mitochondria. Although the identification of the metabolically linked secondary signals responsible for insulin exocytosis is lacking, mitochondria-derived ATP is a likely candidate. The previous findings illustrated in Fig. 3*B* that demonstrate increased cellular metabolism by leucine in a glutamine-dependent manner reflects the activation of these metabolic pathways. The branched-chain amino acids isoleucine and valine also utilize these same metabolic pathways, although leucine is the most potent allosteric activator of GDH at physiological concentrations.

The overall hypothesis in our studies is that both the oxidative decarboxylation pathway and allosteric activation of GDH by the β-cell mitochondria, as shown in Fig. 4, are required for leucine to activate mTOR signaling. Our evidence in support of a requirement for the oxidative decarboxylation pathway is that aminooxyacetic acid (AOAA), an inhibitor of the reversible aminotransferase (AT) reaction, which converts leucine to α-ketoisocaproic acid (α-KIC), dose-dependently prevented leucine-induced phosphorylation of p70$^{s6k}$ (15). Conversely, AOAA also inhibited α-KIC–induced phosphorylation of p70$^{s6k}$ by preventing the formation of leucine by the AT reaction. This inhibition of the AT reaction by AOAA, as predicted, also prevented the conversion of $^{14}$C-leucine to $^{14}CO_2$ by the β-cell mitochondria. Furthermore, β(±) 2-aminobicyclo [2,2,1]heptane-2-carboxylic acid (±BCH), a nonmetabolized leucine analog that allosterically activates GDH, also failed in the absence of leucine as a substrate for oxidative decarboxylation to stimulate the phosphorylation of p70$^{s6k}$. These findings are consistent with the requirement of the metabolism of leucine by both oxidative decarboxylation and allosteric activation of GDH by the β-cell mitochondria to mediate p70$^{s6k}$ phosphorylation (15).

To further test this hypothesis, we developed a minimal model consisting of only two amino acids, leucine and glutamine, to mimic the effect of a complete complement of amino acids normally present in tissue culture media. In this model, leucine is required as a substrate by the oxidative decarboxylation pathway and also as an allosteric activator of GDH. Glutamine is necessary as a precursor for glutamate, a substrate for GDH. As shown in Fig. 5 (*lane 2*), a complete complement of amino acids stimulates the phosphorylation of p70$^{s6k}$, as indicated by the appearance of the slower-migrating upper band, and leucine or glutamine alone (*lanes 3* and *4*) does not mimic this effect. However, the combination of leucine and glutamine causes the complete phosphorylation of p70$^{s6k}$, which is inhibited by rapamycin. The partial shift to phosphorylated p70$^{s6k}$ in the presence of leucine alone (*lane 3*) is believed to be due to our inability to remove endogenous glutamate (15).

**What metabolically linked mediators are responsible for activation of mTOR signaling?** If the metabolism of leucine is responsible, in part, for the activation of mTOR, the critical question then arises as to the identity of possible metabolically linked secondary signals that result from the metabolism of leucine by the β-cell mitochondria. Our studies have demonstrated that selective inhibitors of mitochondrial function, including azide (an inhibitor of mitochondrial cytochrome c oxidase) and inhibitors of the mitochondrial electron-transfer chain (including rotenone, an inhibitor of complex 1, and antimycin A, an inhibitor of complex 3), produced dose-dependent and reversible inhibition of amino acid–induced activation of p70$^{s6k}$ (15). Collectively, all of these perturbations should, to varying degrees, lower ATP levels in β-cells. Basal free ATP concentrations have been estimated to be in the range of 1 mmol/l in both the cytosol and mitochondrial matrix and beneath the plasma membrane of MIN6 and primary β-cells, and these concentrations increase in a time- and glucose-dependent manner, as determined by a luciferase and photon imaging technique (40). In another approach, glucose was reported to dose-dependently increase ATP and decrease ADP in dispersed islet cells and purified β-cells (41). An increase in glucose concentration from 1 to 10 mmol/l resulted in a ~75% increase in ATP levels in

islet cells and slightly lower increases in purified β-cells. These quantitative approaches have been complicated by the heterogeneous cell population of islets, the large sequestered ATP pools present in insulin secretory granules, and the numerous ATP-consuming processes associated with insulin exocytosis (42). However, it is reasonable to assume that mitochondrial metabolism generates the majority of ATP synthesis, and that mitochondrial-derived ATP resulting from the metabolism of leucine by β-cells may in some manner regulate mTOR signaling. Consistent with this possibility, Dennis et al. (43) have recently reported that intracellular concentrations of ATP in the physiological range of 1–5 mmol/l directly regulate mTOR activity with an apparent Michaelis constant ($K_m$) of slightly greater than 1 mmol/l in HEK293 cells. Because most protein kinases demonstrate an apparent $K_m$ for ATP in the range of 10–15 μmol/l, these authors proposed that mTOR actually serves as a homeostatic sensor of ATP. It was also demonstrated in this study that amino acid deprivation of HEK293 cells had no effect on concentrations of ATP, suggesting that the regulation of mTOR by ATP is independent of amino acid pools, although amino acids were shown to be essential for insulin-induced phosphorylation of $p70^{s6k}$ and PHAS-I. It is important to note that the HEK293 cells were heavily dependent on glycolysis for generation of ATP. Thus, in this study it appears that ATP directly regulates mTOR, whereas the requirement of amino acids for insulin and growth factors to activate mTOR uses a separate mechanism. Our findings suggest that the products of the metabolism of glucose and leucine generate ATP in the β-cell mitochondria and may directly regulate mTOR. In addition, leucine also functions, in part, as a requirement for insulin and IGF signaling through $p70^{s6k}$ and PHAS-I independent of ATP.

**Are there differences in the regulation of mTOR signaling in primary β-cells and β-cell lines?** An important issue in the regulation of mTOR is whether insulin and nutrient signaling mechanisms as determined in β-cell lines are applicable to primary β-cells (44). To address this question, a series of comparisons have been performed with isolated rat and human islets. Our initial approach has been to determine whether the minimal requirements of leucine and glutamine to activate mTOR signaling in the β-cell line RINm5F is mimicked by primary β-cells. As shown in Fig. 6*A*, exposure of human islets to an elevated glucose concentration in KRBB containing a complete complement of amino acids results in full activation of $p70^{s6k}$ (*lane 2*). In contrast, exposure of human islets to an elevated glucose concentration in KRBB containing only glutamine (*lane 3*) or glutamine in combination with leucine (0.4 mmol/l) (*lane 4*) fails to significantly increase the phosphorylation of $p70^{s6k}$. However, leucine dose-dependently increases the phosphorylation of $p70^{s6k}$, with maximal effects observed at concentrations of 4–6 mmol/l (*lanes 5–7*).

In Fig. 6*B*, it is observed that exposure of human islets to KRBB containing leucine (4 mmol/l) and glutamine (2 mmol/l) allows glucose to increase the phosphorylation of $p70^{s6k}$ in a dose-dependent manner, with maximum effects observed in the physiological range of 8–10 mmol/l (*lanes 5–7*). Overall, these results suggest that the minimal nutrient requirements for activation of mTOR in human islets

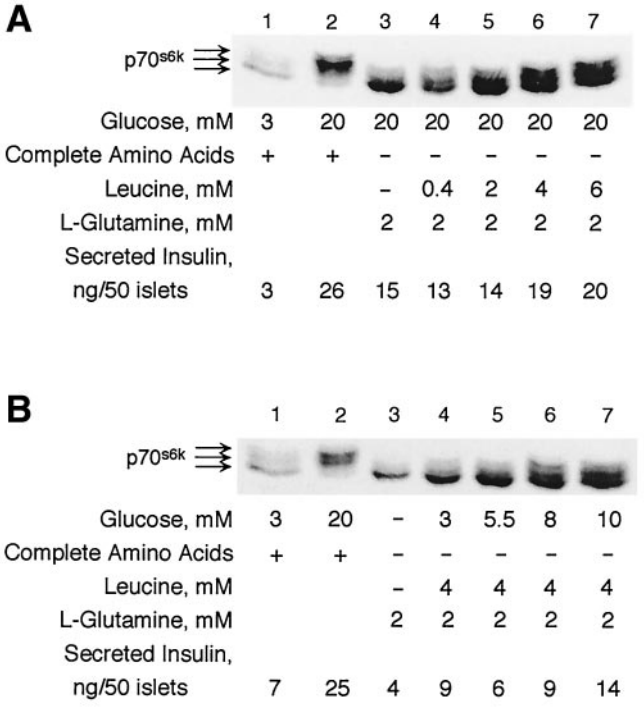


**FIG. 6. Dose-response of glucose- and amino acid–mediated $p70^{s6k}$ phosphorylation in isolated human islets. *A*: Islets ($n$ = 50) were preincubated in KRBB (containing 0.1% BSA) in the absence of glucose and amino acids for 2 h. Medium was replaced with KRBB containing 3 or 20 mmol/l glucose plus complete amino acids or glutamine alone or with increasing concentrations of leucine for 2 h. Cells were processed for immunoblotting of $p70^{s6k}$, and supernatants were saved for insulin radioimmunoassay. *B*: Islets ($n$ = 50) were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Medium was replaced with KRBB containing the indicated concentrations of glucose plus complete amino acids or glutamine alone or with 4 mmol/l leucine for 2 h. Cells were processed for immunoblotting of $p70^{s6k}$ and supernatants were saved for insulin radioimmunoassay.**

consist of glucose (8–10 mmol/l), leucine (4–6 mmol/l), and glutamine. However, these minimal amino acid requirements do not achieve the maximum activation of mTOR as observed with a complete complement of amino acids (*lane 2*). An autocrine stimulation of mTOR may partially account for this latter effect, because exposure of islets to glucose in KRBB containing a complete complement of amino acids also results in increased insulin secretion, compared with conditions using only glucose, leucine, and glutamine. The reason for the additional synergy observed with the complete complement of amino acids is unknown.

**Do primary β-cells display autocrine regulation of mTOR by insulin?** Additional studies were performed to address this synergy between insulin and the nutrients amino acids and glucose in primary rat islets by assessing the effects of both endogenous and exogenous insulin. As shown in Fig. 7*A*, islets exposed to elevated glucose concentrations in the presence of a complete complement of amino acids in KRBB (*lanes 2* and *3*) or CMRL-1066 (*lanes 7* and *8*) resulted in enhanced phosphorylation of $p70^{s6k}$ and also increases in insulin secretion. Inhibition of insulin secretion with diazoxide, an activator of the $K_{ATP}$ channel that prevents β-cell depolarization, partially blocked this effect in both conditions (*lanes 5* vs. *3* and *10* vs. *8*). As shown in Fig. 7*B*, exogenous insulin at high concentrations (200 nmol/l) resulted in only a small degree

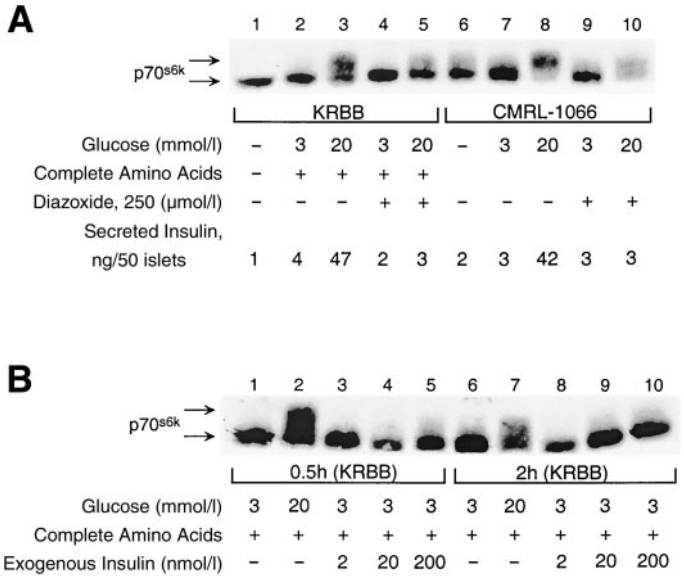


**FIG. 7. Glucose and amino acid metabolism and insulin secretion synergize to enhance p70$^{s6k}$ phosphorylation in isolated rat islets. *A*: Islets ($n$ = 50) were preincubated in KRBB (containing 0.1% BSA) in the absence of glucose and amino acids or in CMRL in the absence of serum and glucose for 2 h. During the last 30 min of preincubation, diazoxide (250 μmol/l) was added where indicated. Medium was replaced with KRBB containing glucose, complete amino acids, and diazoxide as indicated for 2 h. Cells were processed for immunoblotting of p70$^{s6k}$, and supernatants were saved for insulin radioimmunoassay. *B*: Islets ($n$ = 50) were preincubated in KRBB (containing 0.1% BSA) in the absence of glucose and amino acids for 2 h. Medium was replaced with KRBB containing glucose and complete amino acids with or without 2, 20, or 200 nmol/l insulin as indicated for 2 h. Cells were processed for immunoblotting of p70$^{s6k}$, and supernatants were saved for insulin radioimmunoassay.**

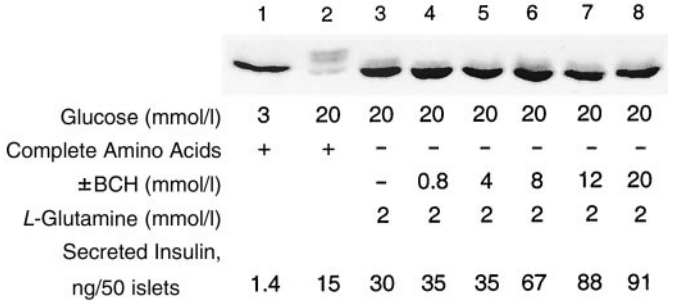


**FIG. 8. Activation of GDH stimulates insulin secretion but not p70$^{s6k}$ phosphorylation in isolated rat islets. Islets ($n$ = 50) were preincubated in KRBB (0.1% BSA) in the absence of glucose and amino acids for 2 h. Medium was replaced with KRBB containing glucose, complete amino acids, ±BCH, and L-glutamine as indicated for 2 h. Cells were processed for immunoblotting of p70$^{s6k}$, and supernatants were saved for insulin radioimmunoassay.**

of phosphorylation of p70$^{s6k}$ after incubations of 0.5 and 2 h (*lanes 5* and *10*). Our interpretation of these results is that the combination of glucose and amino acids with endogenous insulin secretion produces full activation of p70$^{s6k}$ by β-cells. This is believed to reflect autocrine regulation of mTOR by the β-cell through endogenous insulin secretion, which is not mimicked by exogenous insulin in the absence of amino acids and a stimulatory concentration of glucose. This may be caused by the inability of the β-cell to generate the necessary metabolic signals, such as ATP, to activate mTOR because of the presence of only basal glucose concentrations.

**Are the metabolically linked secondary signals identical for insulin exocytosis and mTOR activation?** Our current studies with primary β-cells indicate an essential requirement for glucose in addition to the amino acids leucine and glutamine to activate the mTOR signaling pathway, whereas insulin secretion requires only glucose and a balanced salt solution to provide $Ca^{2+}$ and can be potentiated by leucine and glutamine. To further establish that metabolically linked secondary signals are not identical for insulin exocytosis and mTOR activation, additional experiments have been performed. In this experimental approach, islets were exposed to glucose, glutamine, and increased concentrations of BCH, a nonmetabolized analog of leucine that is a potent activator of GDH (38,45). In this design, leucine was absent, which precluded its metabolism by the oxidative decarboxylation pathway. As shown in Fig. 8, the active enantiomer of BCH (0.4–10 mmol/l) activates GDH, as evidenced by increases in insulin secretion above that of glucose (20 mmol/l) alone. However, BCH fails to increase the phosphorylation of p70$^{s6k}$. This inability of BCH to mediate the phosphorylation of p70$^{s6k}$ in the absence of leucine was observed in both rat and human islets. These results clearly indicate that the metabolically linked secondary signals that mediate insulin secretion differ from those required for mTOR activation by β-cells. These findings combined with our previous data using the transaminase inhibitor AOAA (15) suggest that the metabolism of leucine by oxidative decarboxylation along with the activation of GDH generate important signals necessary for mTOR activation. Furthermore, rapamycin does not inhibit insulin secretion, at least up to 48 h of exposure, yet it completely inhibits p70$^{s6k}$ phosphorylation.

**Evidence for insulin and nutrient signaling in vivo.** Our data indicate that insulin and the branched-chain amino acid leucine activate mTOR by different pathways involving the insulin cascade and nutrient signaling in β-cell lines and primary β-cells. Indicators of mTOR signaling in these studies have been the phosphorylation of the two regulatory proteins p70$^{s6k}$ and PHAS-I in a rapamycin-dependent manner. In an attempt to expand these findings to an in vivo setting, we have recently determined in collaboration with Clay Semenkovich at Washington University whether insulin and leucine can activate p70$^{s6k}$ through these pathways in human skeletal muscle biopsies (46). To assess whether insulin and leucine mediate these effects by different pathways, we also monitored the phosphorylated form of Akt, an immediate upstream signaling component in the insulin signaling pathway.

Subjects in this study were healthy young men and women who, after an overnight fast, received a 2-h infusion of leucine alone, insulin alone, or leucine and insulin. Muscle biopsies were obtained before the start of the infusion and immediately after the infusion in the contralateral leg. Plasma leucine concentrations were ~120 μmol/l at baseline fasting conditions and increased threefold during the infusion. Importantly, the threefold increase in leucine was not accompanied by any significant changes in basal insulin concentrations. Insulin concentrations were maintained at a high physiological concentration of 400 pmol/l under euglycemic conditions of 90 mg/dl.

Our hypothesis is that increasing insulin alone should

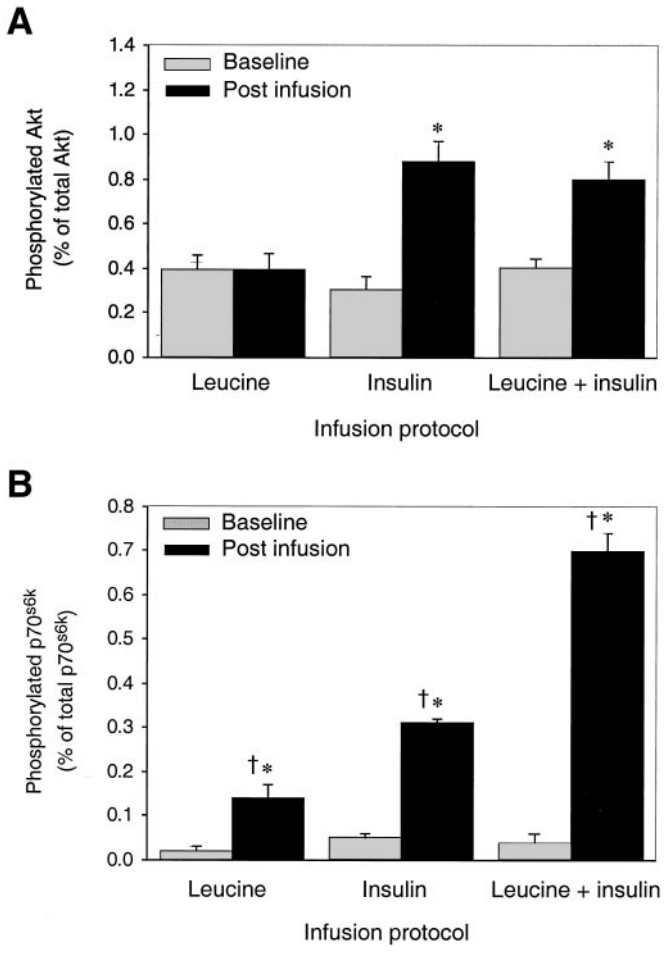


**FIG. 9. Effects of leucine and insulin on activation of Akt and p70$^{s6k}$ in human skeletal muscle in vivo. *A*: Phosphorylation of serine$^{473}$ (Ser$^{473}$) of Akt (Ser$^{473}$ Akt) before (■) and immediately after (■) a 2-h infusion of leucine alone (left columns), insulin alone (middle columns), or leucine plus insulin (right columns). *Significant difference between baseline and postinfusion samples ($P < 0.05$). Values represent arbitrary optical density units of phosphorylated Ser$^{473}$ Akt and are expressed as means ± SE ($n = 6$) for each condition. *B*: Phosphorylated ribosomal protein p70 S6 kinase (p70$^{s6k}$) before (■) and immediately after (■) a 2-h infusion of leucine alone (left columns), insulin alone (middle columns), or leucine plus insulin (right columns). *Significant difference between baseline and postinfusion samples ($P \leq 0.01$); †significant difference compared with either of the remaining two solid columns ($P < 0.01$). Data are expressed as the percentage of total p70$^{s6k}$ in the phosphorylated state and represent means ± SE ($n = 6$) for each condition. From Greiwe et al. (46).**

mediate the phosphorylation of the translational regulator p70$^{s6k}$ by the insulin signaling cascade, as determined by activation of Akt. In contrast, increasing leucine concentrations alone should activate p70$^{s6k}$ through mTOR by the nutrient signaling mechanism without exerting any effects on Akt activation. In addition, increasing both insulin and leucine should produce a synergistic effect by converging on mTOR by both the insulin cascade and nutrient signaling pathway, which results in increased phosphorylation of p70$^{s6k}$. As shown in Fig. 9*A*, a threefold increase in leucine alone had no effect on the activation of Akt, whereas insulin alone or insulin in combination with leucine caused an approximately twofold increase in Akt as an indicator of the insulin signaling cascade. In contrast, as shown in Fig. 9*B*, the phosphorylation of p70$^{s6k}$ increased ~4-fold with the infusion of leucine alone and 8-fold with insulin alone, and the combination of leucine and insulin synergized to produce an ~18- to 20-fold increase in p70$^{s6k}$ phosphorylation.

Overall, these data are supportive of our hypothesis that insulin and leucine utilize these same distinct pathways to stimulate the phosphorylation of the translational regulator p70$^{s6k}$ in human skeletal muscle in vivo, as described in pancreatic β-cells in vitro. However, a number of issues remain unanswered. In this experimental approach, we have used human skeletal muscle as a surrogate for human β-cells. Based on our in vitro studies with primary β-cells, it would be anticipated that an elevated concentration of glucose in addition to leucine would be required to activate p70$^{s6k}$ by the nutrient signaling pathway in β-cells in vivo. In addition, our findings in skeletal muscle biopsies of healthy subjects precluded the use of rapamycin to establish that the phosphorylation of p70$^{s6k}$ was mediated primarily through mTOR. Furthermore, these studies have not demonstrated whether insulin or leucine via these different pathways involving p70$^{s6k}$ or PHAS-I results in increased protein synthesis in human skeletal muscle.

Recently, several other studies have evaluated the effects of hyperinsulinemia or branched-chain amino acids on the activation of the translational regulators PHAS-I or p70$^{s6k}$ in human skeletal muscle in vivo. Hillier et al. (47) reported that physiological hyperinsulinemia increased the phosphorylation of p70$^{s6k}$ but did not affect the phosphorylation of PHAS-I in human skeletal muscle. Our human muscle studies did not measure the phosphorylation of PHAS-I. The explanation for this dissociation between the phosphorylation of p70$^{s6k}$ and PHAS-I by activation of the insulin signaling cascade by Hillier et al. is unknown. However, a possible explanation for these findings may be the lack of stimulatory levels of the nutrients glucose or leucine to provide the necessary levels of cellular ATP to regulate mTOR. In this case, activation of the insulin signaling cascade in the presence of basal glucose and amino acid concentrations may be sufficient to phosphorylate p70$^{s6k}$, perhaps by an mTOR-independent signaling pathway. In a subsequent study, Liu et al. (48) demonstrated that infusion of the branched-chain amino acids leucine, isoleucine, and valine increased the phosphorylation of PHAS-I and p70$^{s6k}$ independent of insulin secretion by a nutrient signaling mechanism and potentiated protein synthesis. Furthermore, muscle mass in both humans and rodents decreases with age. In two studies using aging rats, Dardevet et al. (49,50) have shown that the sensitivity of muscle protein synthesis to leucine stimulation was decreased and that this response could be restored by feeding a leucine-supplemented meal.

**Proposed model for coupling metabolically linked Krebs cycle fluxes in the β-cell mitochondria.** The β-cell mitochondria is recognized as the major fuel sensor that produces factors that couple the metabolism of nutrients to insulin secretion. As recently reviewed by Maechler and Wollheim (31), ATP generation in the mitochondria by provision of NADH and $FADH_2$ for oxidative phosphorylation appears to be the main coupling messenger in insulin secretion. As illustrated in Fig. 10, glucose metabolism by the β-cell mitochondria generates both oxaloacetic acid (OAA) and acetyl-CoA to ensure anaplerosis or the provision of carbons to the Krebs cycle for the production of ATP (51). The increase in the ratio of

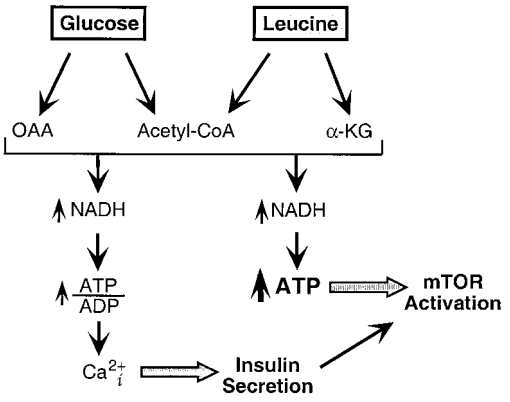


**FIG. 10. Model for coupling metabolically linked Krebs cycle fluxes in β-cell mitochondria to insulin secretion and mTOR activation. ATP/ADP, ratio of ATP to ADP; $Ca^{2+}_i$, intracellular $Ca^{2+}$ concentration.**

ATP to ADP inhibits the $K_{ATP}$ channel, which results in cell depolarization due to the opening of voltage-dependent $Ca^{2+}$ channels, increases in cytosolic $Ca^{2+}$ concentrations, and ultimately insulin secretion. The branched-chain amino acid leucine, which is believed to be exclusively metabolized by the mitochondria, also produces Krebs cycle substrates by different mechanisms that increase the production of NADH and $FADH_2$. In essence, the metabolism of leucine by oxidative decarboxylation generates acetyl-CoA, and the ability of leucine to allosterically activate GDH produces α-ketoglutarate (α-KG), which also ensures anaplerosis to the Krebs cycle.

Although mitochondria-derived ATP appears to be an important coupling factor in both nutrient-mediated insulin secretion and the activation of mTOR signaling, these processes can be dissociated. For example, diazoxide, an activator of the $K_{ATP}$ channel, completely inhibits glucose-induced insulin secretion by islets. In contrast, diazoxide only partially inhibits glucose-induced activation of mTOR, which is believed to be due to the loss of autocrine regulation of mTOR by insulin. These effects suggest major differences in $Ca^{2+}$ sensitivities between nutrient-mediated insulin secretion and mTOR activation. In addition, it also appears that insulin secretion is more dependent on the ratio of ATP to ADP, whereas the regulation of mTOR activity depends on the intracellular concentration of ATP in the physiological range of 1–5 mmol/l.

## CONCLUSIONS

The β-cell is unique in its ability to generate metabolically linked factors that are derived from the metabolism of glucose and amino acids and that stimulate insulin exocytosis. These factors include increases in the production of ATP by the β-cell mitochondria, changes in the ATP-to-ADP ratio, and intracellular $Ca^{2+}$. Our studies suggest that further increases in mitochondria-derived ATP, through enhanced substrate flux, may also be responsible for the ability of glucose, glutamine, and leucine to activate mTOR signaling in β-cells. This may be affected by mTOR serving as a homeostatic ATP sensor, as recently proposed (43). Furthermore, the singular ability of β-cells to sense nutrient levels through these metabolically linked pathways and respond by secreting insulin allows for the autoregulation of mTOR. The significance of these findings is that the optimization of mitochondria function is not only important for insulin secretion but may significantly impact the growth and proliferation of β-cells.

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