Metabolic Regulation by Leucine of Translation Initiation Through the mTOR-Signaling Pathway by Pancreatic β-Cells

Guang Xu, Guim Kwon, Wilhelm S. Cruz, Connie A. Marshall, and Michael L. McDaniel

Recent findings have demonstrated that the branched-chain amino acid leucine can activate the translational regulators, phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I) and p70 S6 kinase ($p70^{s6k}$), in an insulin-independent and rapamycin-sensitive manner through mammalian target of rapamycin (mTOR), although the mechanism for this activation is undefined. It has been previously established that leucine-induced insulin secretion by β-cells involves increased mitochondrial metabolism by oxidative decarboxylation and allosteric activation of glutamate dehydrogenase (GDH). We now show that these same intramitochondrial events that generate signals for leucine-induced insulin exocytosis are required to activate the mTOR mitogenic signaling pathway by β-cells. Thus, a minimal model consisting of leucine and glutamine as substrates for oxidative decarboxylation and an activator of GDH, respectively, confirmed the requirement for these two metabolic components and mimicked closely the synergistic interactions achieved by a complete complement of amino acids to activate $p70^{s6k}$ in a rapamycin-sensitive manner. Studies using various leucine analogs also confirmed the close association of mitochondrial metabolism and the ability of leucine analogs to activate $p70^{s6k}$. Furthermore, selective inhibitors of mitochondrial function blocked this activation in a reversible manner, which was not associated with a global reduction in ATP levels. These findings indicate that leucine at physiological concentrations stimulates $p70^{s6k}$ phosphorylation via the mTOR pathway, in part, by serving both as a mitochondrial fuel and an allosteric activator of GDH. Leucine-mediated activation of protein translation through mTOR may contribute to enhanced β-cell function by stimulating growth-related protein synthesis and proliferation associated with the maintenance of β-cell mass. *Diabetes* 50:353–360, 2001

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Ac-Leu-NH_2, *N*-acetyl leucine amide; Ac-Leu-NHMe, *N*-acetyl leucine *N*-methyl-amide; AOAA, aminooxyacetic acid; BCH, b(±) 2-amino-bicyclo [2,2,1] heptane-2-carboxylic acid; ECL, enhanced chemiluminescence; GDH, glutamate dehydrogenase; H-Leu-NH_2HCl, leucine-amide hydrochloride; KIC, α-ketoisocaproic acid; KRBB, Krebs-Ringer bicarbonate buffer; MEM, minimal essential medium; mTOR, mammalian target of rapamycin; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; $p70^{s6k}$, p70 S6 kinase; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin; PI 3-K, phosphoinositide 3-kinase.

A number of recent studies have described synergistic interactions between insulin and amino acids to regulate protein synthesis by modulating the mRNA binding step in translation initiation. Insulin and amino acids have been shown to exert their effects through phosphorylation of the translational regulators phosphorylated heat-and acid-stable protein regulated by insulin (PHAS-I) and p70 S6 kinase ($p70^{s6k}$) via the serine and threonine protein kinase, mammalian target of rapamycin (mTOR) (1–7). mTOR-mediated activation of $p70^{s6k}$ results in the phosphorylation of ribosomal protein S6, which correlates with the translation of mRNAs that encode both ribosomal proteins and translational elongation factors (8,9). The phosphorylation of PHAS-I through mTOR facilitates the release of eIF-4E and allows its participation in translation initiation, especially of mRNAs with high 5′-UTR secondary structures (10–13). In support of this mechanism, rapamycin, a potent inhibitor of mTOR, blocks the phosphorylation of PHAS-I and $p70^{s6k}$ that results in inhibition of translation initiation in response to insulin and amino acids. This rapamycin-sensitive pathway is believed to preferentially stimulate growth-related protein synthesis, leading to cell-cycle progression and proliferation (14–16).

More recently, the role of amino acids in regulating translation initiation by nutrient signaling rather than in serving solely as precursors for protein synthesis has become an important area of investigation (17,18). Thus, amino acids have been shown to be obligatory for insulin and growth factor–signaling through mTOR (19–26). Furthermore, amino acids, in particular the branched-chain amino acids, leucine, isoleucine, and valine, have been shown to independently activate the mTOR pathway. Of these branched-chain amino acids, leucine has generated significant interest due to its unique ability to regulate PHAS-I and $p70^{s6k}$ at physiological concentrations, stimulate protein synthesis, and inhibit lysosomal autophagy (23,26–30). Leucine has also been reported to enhance pancreatic β-cell replication in the fetal rodent pancreas, although the cellular mechanism for this effect has not been defined (31).

Our previous studies with pancreatic β-cells have demonstrated that amino acids are required for glucose or exogenous insulin to stimulate the phosphorylation of PHAS-I (25). Amino acids alone also dose-dependently stimulate the phosphorylation of PHAS-I, which is enhanced by insulin. We have further shown that branched-chain amino acids retained their ability to induce phosphorylation of PHAS-I and

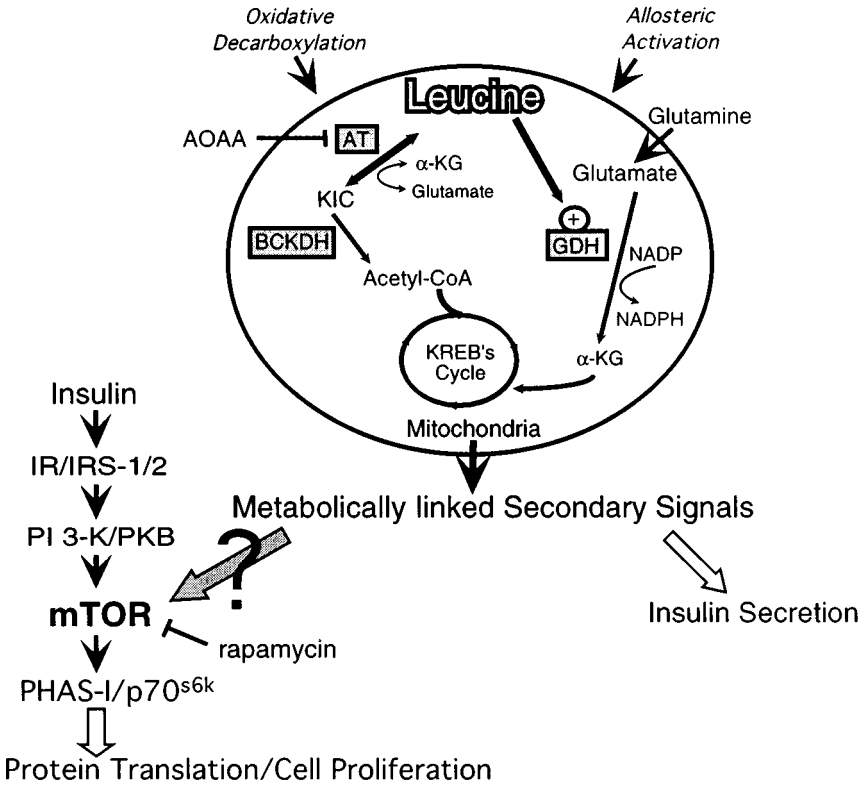

FIG. 1. Proposed model for leucine's role in mitogenic signaling. α-KG, α-ketoglutarate; AOAA, aminooxyacetic acid; AT, aminotransferase; BCKDH, branched-chain α–keto-acid dehydrogenase; GDH, glutamate dehydrogenase; IR, insulin receptor; IRS, insulin receptor substrate; p70^{s6k}, p70 S6 kinase; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin; PKB, protein kinase B.

p70^{s6k} in a rapamycin-sensitive manner under conditions that block insulin secretion by β-cells (26). These findings indicated that branched-chain amino acids modulate protein translation by β-cells via the mTOR-signaling pathway in an insulin-independent manner. Similar findings have been documented with regard to the ability of leucine to activate the mTOR-signaling pathway in other cellular models (20–24).

The cellular mechanisms whereby leucine activates translational regulators through mTOR remain undefined. Studies designed to address the mechanism for the requirement of leucine for mTOR activation have focused on the intracellular metabolism of leucine (23), tRNA aminoacylation (32), structural characteristics of the leucine molecule for a putative recognition site (22,33), kinase(s) (7), or phosphatase(s) (20). It has been previously established that leucine-induced insulin secretion by β-cells is mediated by the metabolism of leucine via the mitochondria by oxidative decarboxylation in combination with the ability of leucine to allosterically activate glutamate dehydrogenase (GDH) as shown in Fig. 1 (34,35). Although the metabolically linked secondary signals generated by the metabolism of leucine to facilitate insulin secretion are unknown, it is probable that these same intramitochondrial events, unique to the metabolism of leucine, result in the generation of other signals independent of insulin that are directed toward enhanced protein translation and cell proliferation. In the present study, we have evaluated the hypothesis that this same metabolic-signaling pathway involving the metabolism of leucine in combination with its allosteric activation of GDH is necessary to activate the mTOR mitogenic–signaling pathway in β-cells.

EXPERIMENTAL PROCEDURES

Materials. CellTiter 96 AQueous One Solution Reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS] reagent) was purchased from Promega (Madison, WI). Rotenone and b(±) 2-amino-bicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) were obtained from Sigma (St. Louis, MO). Leucine analogs, leucine-amide hydrochloride (H-Leu-NH_2HCl), *N*-acetyl leucine *N*-methyl-amide (Ac-Leu-NHMe), α-methyl DL-leucine, and *N*-acetyl leucine amide (Ac-Leu-NH_2), were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). CMRL-1066 and RPMI-1640 tissue culture media, penicillin, streptomycin, L-glutamine, minimum essential medium (MEM) amino acids solution, and MEM nonessential amino acids solution were obtained from Life Technologies (Gaithersburg, MD). Rapamycin was from Biomol (Plymouth Meeting, PA). The antibody for p70^{s6k} was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was peroxidase-conjugated donkey anti–rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagents and L-[U-^{14}C]leucine were from Amersham Pharmacia (Piscataway, NJ). All of the other chemicals were obtained from commercially available sources.

Amino acid composition. Krebs-Ringer bicarbonate buffer (KRBB) (in mmol/l: 25 HEPES, 111 NaCl, 25 $NaHCO_3$,5 KCl, 2.5 $CaCl_2$, and 1 $MgCl_2$) was supplemented with MEM amino acids solution, MEM nonessential amino acids solution, and L-glutamine to make 1×, or complete amino acids, in which composition was described previously (25). Basal amino acids were defined the same as 1×, or complete amino acids, but excluding the branched-chain amino acids (leucine, isoleucine, and valine). Our defined physiological concentrations (mmol/l) of leucine, isoleucine, and valine were 0.4, 0.4, and 0.2, respectively, as in RPMI tissue culture media. This compares with the concentrations (mmol/l) of leucine, isoleucine, and valine (0.20, 0.11, and 0.25, respectively) found in the basal plasma of rats by Mortimore et al. (30) and 0.25, 0.10, and 0.18, respectively, found in the portal vein of fasted rats by Blommaart et al. (18).

Pancreatic β-cell line. RINm5F cells, an insulin-secreting β-cell line (36), were maintained and cultured as described previously (25).

MTS assay. RINm5F cells were cultured in 96-well plates at a density of 2.0 × 10^5 cells/200 μl culture media. To achieve a quiescent state, cells were washed

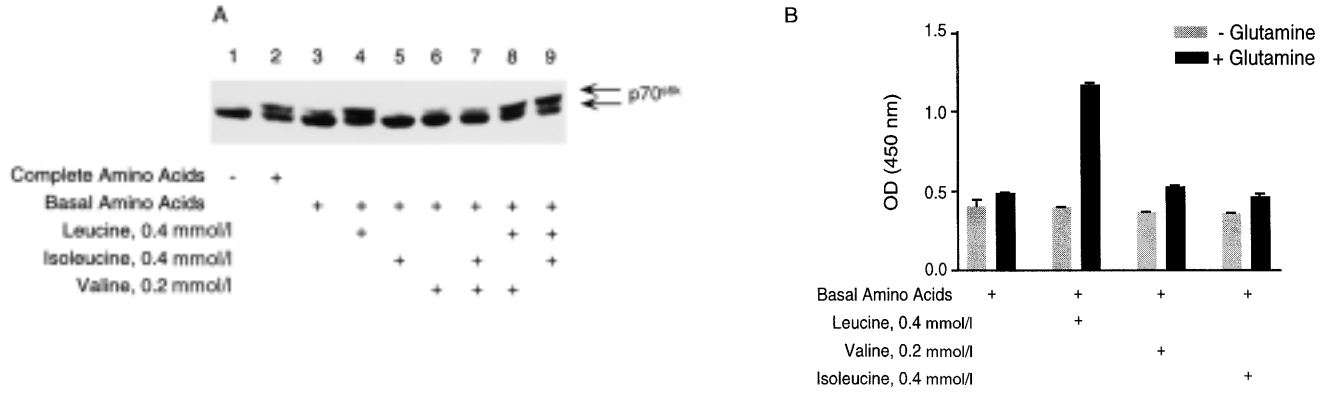

FIG. 2. *A*: Leucine-induced phosphorylation of p70^{s6k} at a physiological concentration in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Media were then replaced with KRBB containing either complete amino acids as a positive control or basal amino acids, which excluded leucine, isoleucine, or valine. Leucine, isoleucine, or valine was then added as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of four separate experiments. The multisite phosphorylation of p70^{s6k} in this and all subsequent immunoblots is detected by a slower migrating or upper band compared with nonphosphorylated p70^{s6k} as determined by gel-shift mobility assays. *B*: Effects of glutamine on branched-chain amino acid–mediated metabolism by RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 1 h. Media were replaced with KRBB containing basal amino acids ± glutamine (2 mmol/l), leucine, isoleucine, or valine as indicated for 2 h with the MTS reagent. Results are representative of five separate experiments.

free of culture media and fetal bovine serum three times with KRBB and preincubated for 1 h at 37°C in KRBB in the absence of glucose and amino acids. KRBB was replaced as described in the figure legends. The MTS assay reagent (20 µl/well) and treatment solution (100 µl/well) were added simultaneously to the cells and mixed well by gentle shaking. Cells were further incubated for 2 h at 37°C. The 450-nm absorbance values were measured at 2 h.

p70^{s6k} Assay. RINm5F cells (1.0×10^6cells/ml) were preincubated for 2 h at 37°C in KRBB in the absence of glucose and amino acids. KRBB was replaced as described in the figure legends. After experimental treatments, cells were processed for SDS-PAGE and Western blotting of p70^{s6k} (26). Detection was performed using ECL reagents. The activation of p70^{s6k} occurs through multisite phosphorylation that results in a slower migrating band compared with nonphosphorylated p70^{s6k}, which is detected by gel shift mobility assays (37). The insulin in the culture media was assayed by the radioimmunoassay core facility of the Washington University Diabetes Research Training Center.

L-[U-^{14}C]leucine oxidation assay. RINm5F cells (2.0×10^7) were preincubated in T-25 culture flasks in KRBB (5 ml) in the absence of glucose and amino acids for 2 h. Buffer was then replaced with KRBB (5 ml) containing basal amino acids, 0.4 mmol/l leucine ± aminooxyacetic acid (AOAA) (5 mmol/l) ± BCH (5mmol/l) + 1 µCi L-[U-^{14}C]leucine for 2 h. The amount of $^{14}CO_2$ produced was trapped and quantitated by scintillation counting.

RESULTS

In the present experimental design, RINm5F cells are initially incubated for 2 h in the complete absence of amino acids in KRBB to dephosphorylate p70^{s6k} to basal levels. Subsequently, β-cells were incubated under the following conditions: *1*) complete amino acids, including the branched-chain amino acids, as a positive control; *2*) basal amino acids, which are devoid of leucine, isoleucine, and valine; and *3*) amino acids in the presence of either leucine, isoleucine, or valine as a test condition to evaluate the ability of each branched-chain amino acid separately and/or in combination to mediate p70^{s6k} phosphorylation. The multisite phosphorylation of p70^{s6k} is detected by the appearance of a slower migrating or upper band compared with nonphosphorylated p70^{s6k} as determined by gel shift mobility assays.

Effects of leucine, isoleucine, and valine on p70^{s6k} activation. As shown in Fig. 2*A*, leucine at 0.4 mmol/l promotes the phosphorylation of p70^{s6k} (*lane 4*), with the appearance of a slower migrating band (upper band) compared with basal conditions, but isoleucine (0.4 mmol/l), valine (0.2 mmol/l), or the combination of isoleucine and valine (*lanes 5–7*) do not mimic this effect. However, leucine in the presence of valine or isoleucine (*lanes 8* and *9*) again restores phosphorylation of p70^{s6k}. To determine if leucine under these conditions also increases cellular metabolism by β-cells, the MTS assay was used as a measure of NAD(P)H production. As shown in Fig. 2*B*, only leucine (0.4 mmol/l) in the presence of glutamine (2 mmol/l), as a precursor for glutamate that serves as a substrate for glutamate dehydrogenase (GDH), significantly enhances β-cell metabolism, suggesting that mitochondrial metabolism is important for leucine to activate p70^{s6k}.

Although leucine activates p70^{s6k} at a physiological concentration of 0.4 mmol/l, isoleucine and valine require greater than physiological concentrations to produce this same effect. As shown in Fig. 3, leucine at 2, 5, and 10 mmol/l causes complete activation of p70^{s6k} (*lanes 4–6*), whereas isoleucine and valine mimic these effects only at 10 mmol/l (*lanes 9* and *12*). Leucine is metabolized in β-cells exclusively by the mitochondria, initially by its transamination to α-ketoisocaproic acid (KIC) and subsequently by oxidative decarboxylation of KIC to acetyl-CoA (see Fig. 1). KIC also dose-dependently activates p70^{s6k} over a concentration range of 0.4–4 mmol/l (data not shown).

As shown in Fig. 4, the ability of leucine to promote phosphorylation of p70^{s6k} is blocked by rapamycin (25 nmol/l), an inhibitor of mTOR, and wortmannin (100 nmol/l), an inhibitor of phosphoinositide 3-kinase (PI 3-K). Because PI 3-K activity is not required for amino acid stimulation of mTOR (23), inhibition of p70^{s6k} by wortmannin may be due to decreased amino acid transport, a direct effect on mTOR, or nonspecific inhibition of other phosphatidylinositol kinases (23,38). Rapamycin (25 nmol/l) also did not inhibit leucine-induced cellular metabolism as determined by the MTS assay or the metabolism of L-[U-^{14}C]leucine by oxidative decarboxylation to $^{14}CO_2$ (data not shown).

Role for leucine metabolism by oxidative decarboxylation and allosteric activation of GDH to promote p70^{s6k} phosphorylation. To determine if both oxidative decarboxylation and GDH activation are essential in leucine-

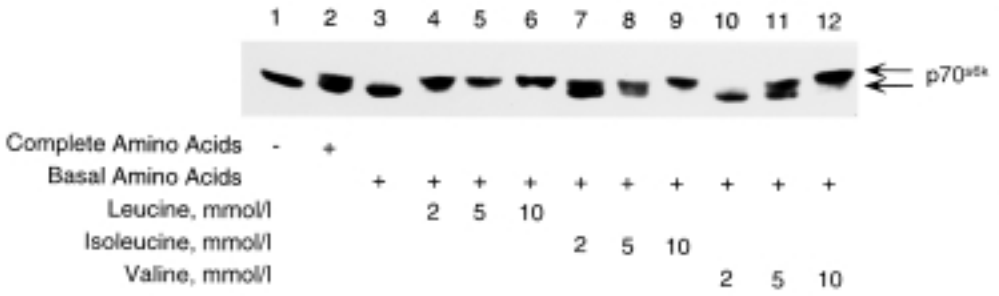

FIG. 3. Isoleucine and valine-induced phosphorylation of p70^{s6k} at greater than physiological concentrations in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Media were then replaced with KRBB containing either complete amino acids as a positive control 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}. Results are representative of five separate experiments.

mediated activation of p70^{s6k}, experiments were performed in the absence of glutamine and glutamate as shown in Fig. 5 (*lanes 3–10*). This removal did not prevent the ability of leucine to promote activation of p70^{s6k} (*lane 4*). This unexpected result may be explained by the endogenous production of glutamate, which is mediated, in part, by the availability of α-ketoglutarate as an acceptor for the aminotransferase reaction that results in glutamate formation as shown in Fig. 1. In fact, removing glutamine and glutamate from the complete amino acids resulted in a 2.8-fold increase in the conversion of ^{14}C-leucine to $^{14}CO_2$ by the oxidative decarboxylation pathway (data not shown), which would increase the generation of glutamate from α-ketoglutarate by this same aminotransferase reaction. Thus, removing glutamine and glutamate from the incubation media stimulates the catabolism of leucine by enhancing the transamination of leucine to KIC with the concomittant generation of endogenous glutamate (39).

To block the formation of endogenous glutamate and to evaluate a role for the metabolism of leucine via oxidative decarboxylation to mediate p70^{s6k} phosphorylation, we next used AOAA, an inhibitor of the transamination of leucine to KIC (22,40). As shown in Fig. 5 (*lanes 5–8*), leucine-induced p70^{s6k} phosphorylation is dose-dependently inhibited by AOAA (1–10 mmol/l). In a similar manner, the ability of KIC to activate p70^{s6k} (*lane 9*) is also prevented by AOAA (*lane 10*). This latter effect is explained both by the metabolism of KIC by oxidative decarboxylation to acetyl-CoA and also by the rapid conversion of KIC to leucine through the reversible aminotransferase reaction, which allows leucine to also allosterically activate GDH (34,35).

Minimal model including only leucine and glutamate. Our next approach was to determine if leucine and glutamine are sufficient to promote phosphorylation of p70^{s6k} in the absence of all other amino acids. In this minimal model, leucine is required to provide substrate for its metabolism by the oxidative decarboxylation pathway and also serve as an allosteric activator of GDH. Glutamine is necessary to provide a source of glutamate for the GDH-mediated production of α-ketoglutarate and its subsequent metabolism by the mitochondria. In addition, glutamine is extensively converted to glutamate, whereas glutamate is poorly transported into β-cells (34). As shown in Fig. 6*A*, leucine (0.4 mmol/l) or glutamine (2 mmol/l) (*lanes 3* and *4*) alone produced a small increase in p70^{s6k} phosphorylation above basal values, whereas a combination of leucine and glutamine markedly activated p70^{s6k} (*lane 5*). This full activation of p70^{s6k} is comparable to that produced by a complete complement of amino acids (*lane 2*). This ability of a combination of leucine and glutamine to mediate full activation of p70^{s6k} was also dose-dependently inhibited by AOAA (*lanes 6–8*). Figure 6*B* also illustrates that this same minimal model consisting of leucine and glutamine that results in full activation of p70^{s6k} (*lane 5*) is blocked by rapamycin (*lane 6*).

Effects of structural analogs of leucine on p70^{s6k} activation. A recent report by Shigemitsu et al. (33) evaluated the structural requirements of leucine that are necessary to activate p70^{s6k} by a rat hepatoma cell line, H4IIE. In this

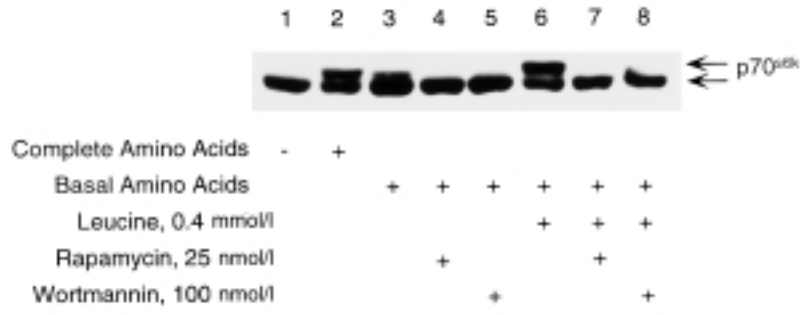

FIG. 4. Effects of rapamycin and wortmannin on leucine-induced phosphorylation of p70^{s6k} in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last hour of preincubation, rapamycin or wortmannin was added to cells. Media were then replaced with KRBB containing either complete amino acids as a positive control or basal amino acids, which excluded leucine, isoleucine, or valine. Leucine ± inhibitors were then added as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of three separate experiments.

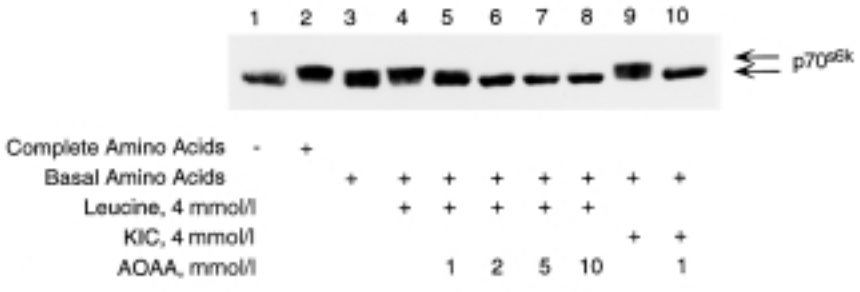

FIG. 5. Phosphorylation of p70^{s6k} induced by leucine requires its metabolism and allosteric activation of GDH in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last 30 min of preincubation, AOAA was added to cells. Media were then replaced with KRBB containing either complete amino acids as a positive control or basal amino acids, which excluded leucine, isoleucine, and valine. *Lanes 3–10* do not contain glutamine and glutamate. Leucine or KIC ± AOAA was added as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of three separate experiments.

structural analysis, it was found that leucine with a modification of its carboxyl group, H-Leu-NH_2HCl, and leucine with a modified α-hydrogen atom, H-α-Me-DL-Leu-OH, stimulated p70^{s6k} activity. In contrast, leucine with modifications of its amino and carboxyl groups, Ac-Leu-NH_2 and Ac-Leu-NHMe, lacked activity and also inhibited the ability of leucine to activate p70^{s6k}. As shown in Fig. 7*A*, we also observed that H-Leu-NH_2HCl (*lane 6*) and H-α-Me-DL-Leu-OH (*lane 7*) activated p70^{s6k}, whereas Ac-Leu-NH_2 (*lane 5*) and Ac-Leu-NHMe (*lane 8*) are inactive. In our experimental model, the ability of the most active leucine analog, H-Leu-NH_2HCl, correlated with its ability to stimulate β-cell metabolism in the presence of glutamine as determined by the MTS assay. As shown in Fig. 7*B*, H-Leu-NH_2HCl stimulates an increase in β-cell metabolism similar to leucine, whereas the partial agonist, H-α-Me-DL-Leu-OH, resulted in no detectable increase. It is presumed that our inability to demonstrate an enhancement of β-cell metabolism by the partial agonist, H-α-Me-DL-Leu-OH (Fig. 7*B*), is due to the limits of detection by the MTS assay.

Effects of the nonmetabolized leucine analog BCH. It has been demonstrated previously that leucine and a nonmetabolized leucine analog, BCH, activate GDH in pancreatic β-cells (34,35). The ability of this nonmetabolized leucine analog to activate p70^{s6k} in our model system was next evaluated. As shown in Fig. 8*A*, BCH over a concentration range of 0.2–10 mmol/l failed to activate p70^{s6k}. Because leucine-induced p70^{s6k} activation is proposed based on our studies to require the metabolism of leucine by oxidative decarboxylation and allosteric activation of GDH, we evaluated the effects of BCH and AOAA on the metabolism of L-[U-^{14}C]leucine by oxidative decarboxylation to $^{14}CO_2$ by the β-cell mitochondria (Fig. 8*B*). As anticipated, AOAA, an inhibitor of the aminotransferase reaction that converts leucine to KIC, completely blocked the metabolism of ^{14}C-leucine to $^{14}CO_2$. Unexpectedly, BCH also almost completely blocked the metabolism of ^{14}C-leucine by oxidative decarboxylation to $^{14}CO_2$. Although the failure of BCH alone to activate p70^{s6k} is consistent with our hypothesis, the inhibitory effects produced by BCH on the oxidative decarboxylation pathway of leucine by β-cells limited its use in elucidating the mechanism responsible for leucine-induced activation of p70^{s6k}.

Role for the mitochondria in mediating leucine-induced activation of p70^{s6k}. To further confirm a role for the β-cell mitochondria to mediate leucine-induced activation of p70^{s6k}, a series of studies were performed to inhibit mitochondrial function. In this experimental design, β-cells were exposed to a complete complement of amino acids to stimulate p70^{s6k} activation in the presence of 3 mmol/l glucose. As shown in Fig. 9*A*, exposure of β-cells to azide, an inhibitor of mitochondrial cytochrome c oxidase (41) dose-dependently (1–10 mmol/l) inhibited the ability of a complete complement of amino acids to promote activation of p70^{s6k} (*lanes 3–5*). This ability of azide to block amino acid–induced activation of p70^{s6k} was not the result of cytotoxicity because its

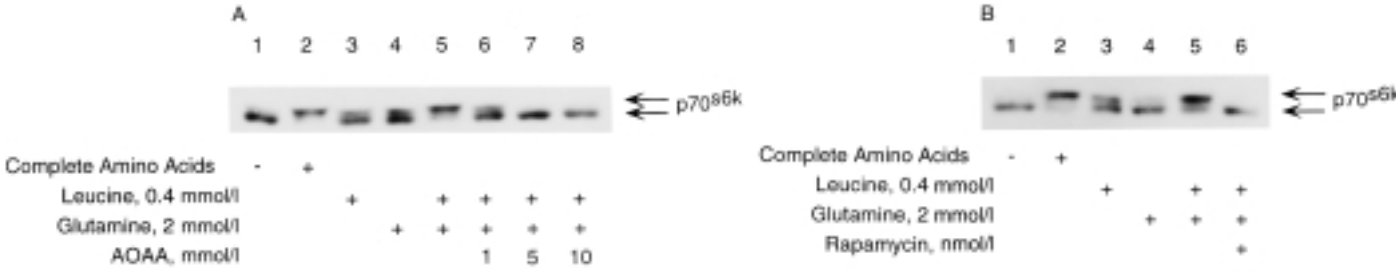

FIG. 6. *A*: Leucine and glutamine are sufficient to induce phosphorylation of p70^{s6k} in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last 30 min of preincubation, AOAA was added to cells. Media were then replaced with KRBB containing either complete amino acids as a positive control or leucine alone, glutamine alone, or both ± AOAA, as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of five separate experiments. *B*: Leucine and glutamine-induced phosphorylation of p70^{s6k} 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. Media were then replaced with KRBB containing leucine alone, glutamine alone, or both ± rapamycin as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of four separate experiments.

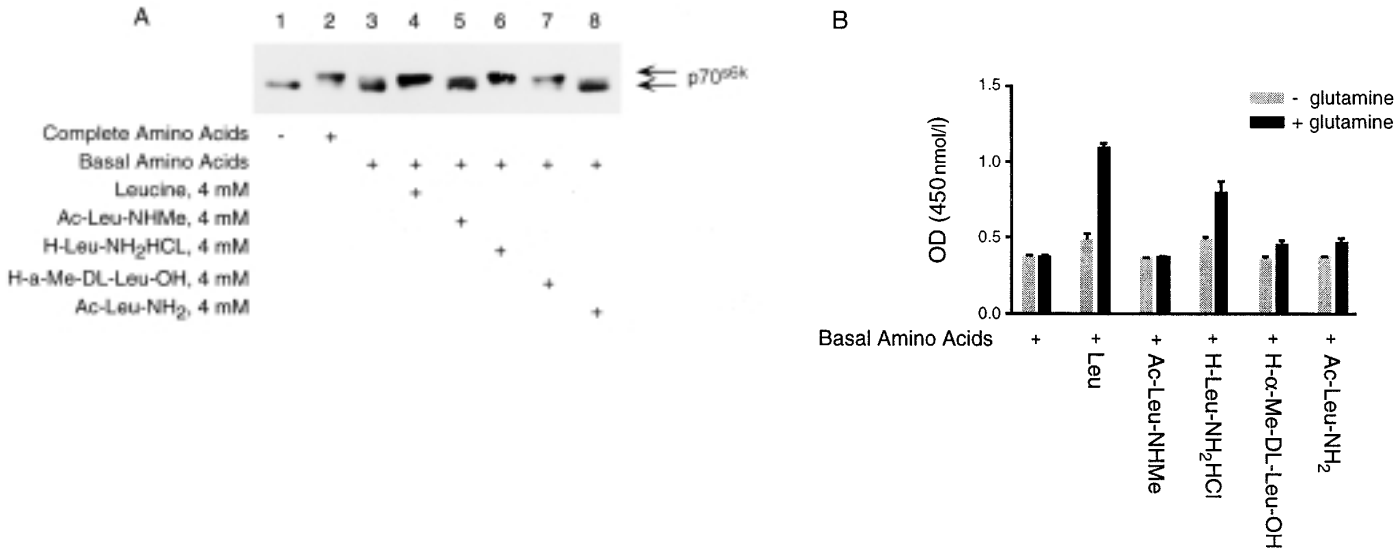

FIG. 7. *A*: Phosphorylation of p70^{s6k} induced by leucine and leucine analogs. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Media were then replaced with KRBB containing either complete amino acids as a positive control or basal amino acids, which excluded leucine, isoleucine, or valine. Leucine or leucine analogs were added as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of four separate experiments. *B*: Effects of leucine and leucine analogs on RINm5F cell metabolism. Cells were preincubated in KRBB in the absence of glucose and amino acids for 1 h. Media were then replaced with KRBB containing basal amino acids ± glutamine (2 mmol/l), ± leucine or leucine analogs (4 mmol/l) as indicated for 2 h with the MTS reagent. Results are means ± SE of three separate experiments.

inhibitory effect was readily reversed, as shown in *lanes 6–8*. Also, as shown in Fig. 9*B*, azide, under these identical conditions, did not inhibit glucose metabolism through glycolysis indicating that a global reduction in cellular ATP is not responsible for its inhibitory effects on p70^{s6k} activation. Furthermore, additional inhibitors of the mitochondrial electron–transfer chain including rotenone, an inhibitor of complex 1, and antimycin A, an inhibitor of complex 3 (42), produced similar dose-dependent and reversible inhibition of amino acid-induced activation of p70^{s6k} (data not shown).

DISCUSSION

In the present study, we have attempted to determine the mechanism responsible for the unique ability of leucine to activate the mTOR-signaling pathway relative to other amino acids. These studies have focused on the ability of leucine to be metabolized exclusively by the mitochondria in combination with its unique ability to allosterically activate GDH that further energizes the mitochondria by the production of the Krebs Cycle intermediate, α-ketoglutarate. The removal of leucine from a complete complement of amino acids inhibits

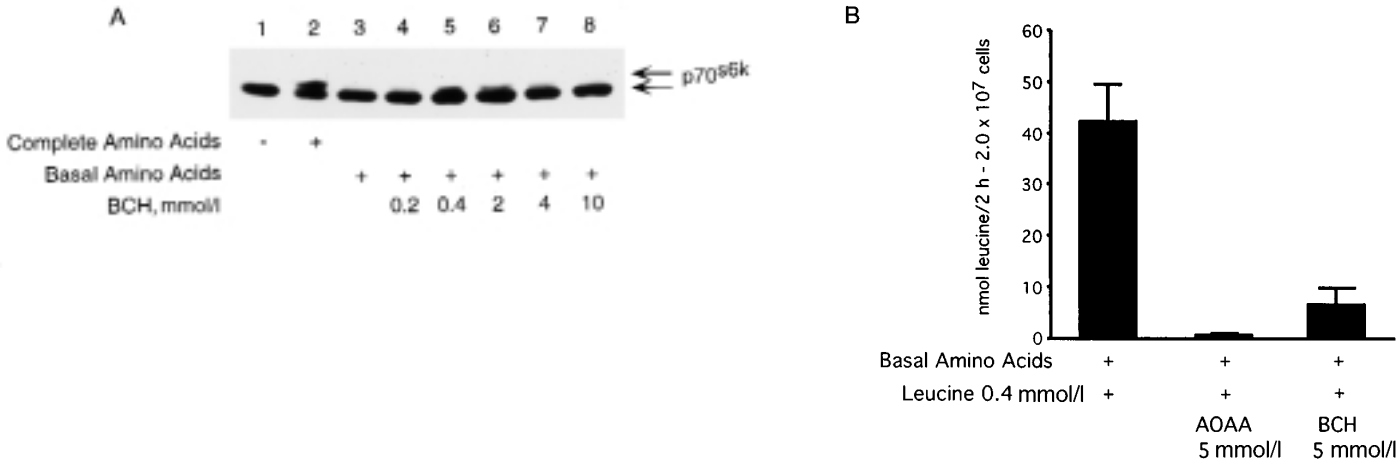

FIG. 8. *A*: Activation of GDH by nonmetabolized BCH does not induce phosphorylation of p70^{s6k} in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Media were then replaced with KRBB containing either complete amino acids as a positive control, basal amino acids, which excluded leucine, isoleucine, or valine, or basal amino acids + BCH as indicated for 30 min. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of three separate experiments. *B*: Inhibition of L-[U-^{14}C]leucine decarboxylation by AOAA and BCH. Cells (2.0×10^7) were preincubated in KRBB in the absence of glucose and amino acids for 2 h. Media were then replaced with KRBB containing basal amino acids supplemented with 0.4 mmol/l leucine + 1 μCi L-[U-^{14}C]leucine. AOAA or BCH was added as indicated for 2 h. The amount of $^{14}CO_2$ produced was trapped and quantitated by scintillation counting. Results are means ± SE of three separate experiments.

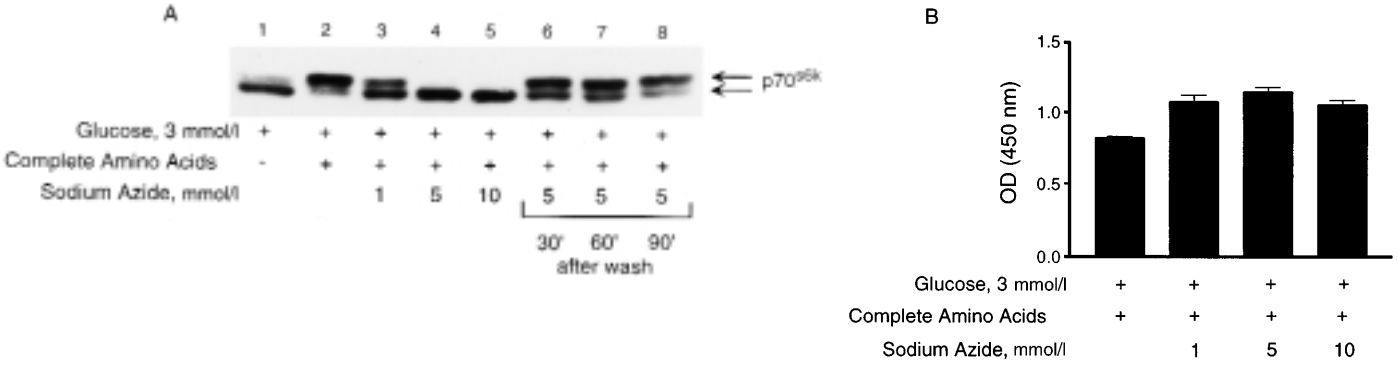

FIG. 9. *A*: Phosphorylation of p70^{s6k} induced by amino acids requires mitochondrial oxidation in RINm5F cells. Cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last hour of preincubation, azide was added to cells. Media were then replaced with KRBB containing 3 mmol/l glucose and complete amino acids ± azide for 30 min. In addition, cells in *lanes 6–8* were washed three times with PBS and buffer was replaced with KRBB containing complete amino acids and 3 mmol/l glucose without azide for 30, 60, or 90 min as indicated for recovery. Cells were processed for immunoblotting of p70^{s6k}. Results are representative of three separate experiments. *B*: Effects of azide on RINm5F cell metabolism. Cells were preincubated in KRBB in the absence of glucose and amino acids for 1 h. During the preincubation, azide was added to cells. Media were then replaced with KRBB containing complete amino acids + glucose (3 mmol/l) ± azide as indicated for 2 h with the MTS assay reagent. Results are means ± SE of three separate experiments.

p70^{s6k} activation and, at corresponding concentrations from fasting to postprandial, dose-dependently restores the ability of the complete complement of amino acids to activate p70^{s6k}.

Although isoleucine and valine can activate p70^{s6k}, this is only achieved at higher concentrations as compared with leucine. This large difference in the concentration dependency of isoleucine and valine to activate p70^{s6k} may relate to the reduced ability of isoleucine and valine to activate GDH compared with leucine because similar pathways involving oxidative decarboxylation also metabolize isoleucine and valine (34).

The specific mechanisms responsible for leucine activation of these translational regulators are unknown. Both the intracellular metabolism of leucine and its interaction with a recognition site or other unknown proteins requiring the unique structure of leucine have been proposed (7,20,22,23,32,33). The ability of KIC to activate p70^{s6k} has been used to support the hypothesis that leucine metabolism is required for p70^{s6k} activation (23). However, the observation that AOAA, an inhibitor of the amino transaminase that blocks the rapid conversion of KIC to leucine, prevents KIC-mediated p70^{s6k} activation has suggested that the specific structure of leucine is important (22). In the present study, we show that both leucine metabolism and its allosteric interaction with GDH requiring its specific structure or recognition site are important in p70^{s6k} activation. Studies using various leucine analogs also support the close association of mitochondrial metabolism and the ability of leucine to activate p70^{s6k}. The unique ability of leucine to stimulate insulin secretion by its metabolism and its interaction with GDH has been previously documented (34,35,43).

The unique ability of leucine to activate p70^{s6k} via its metabolism and its interaction with GDH has been further studied using a minimal model system consisting of only leucine and glutamine. This minimal model mimicked closely the synergistic interactions achieved by a complete complement of amino acids and provided full activation of p70^{s6k} in a rapamycin and AOAA-inhibited manner (Fig. 6*A*). The small increases in basal p70^{s6k} observed with leucine alone in this minimal model are believed to be mediated, in part, by endogenous glutamate formation as a consequence of the conversion of leucine to KIC by the aminotransferase reaction. An additional factor believed to contribute to elevated basal levels of p70^{s6k} activation in this in vitro model is our inability to regulate the endogenous production of leucine by β-cells under these nutrient-deprived culture conditions.

Our attempts to assess the effects of activation of GDH with the nonmetabolized leucine analog, BCH, were problematic. BCH was clearly ineffective in activating p70^{s6k} over a concentration range of 0.2–10 mmol/l in the absence of branched amino acids, which is consistent with our overall hypothesis. However, it was determined subsequently that BCH blocked L-[U-^{14}C]leucine metabolism by oxidative decarboxylation to $^{14}CO_2$. Plausible explanations for this latter effect are that BCH inhibits the conversion of leucine to KIC by the aminotransferase reaction and/or that BCH competes with leucine entry into β-cells at the level of the L-system amino acid transporter due to its structural similarity to leucine. In either case, our data support the hypothesis that the ability of leucine to activate the mTOR pathway is not only structural but also requires the metabolism of leucine.

Because our studies have indicated that leucine's ability to activate mTOR is linked to its metabolism by the β-cell mitochondria, attempts were made to further establish this correlation by perturbing mitochondrial function. As demonstrated with azide, its ability to inhibit leucine activation of p70^{s6k} was rapidly reversed after its removal and was not associated with a global reduction in ATP levels because glucose metabolism by glycolysis was unaltered (Fig. 9*B*). It has also been recently proposed that increases in mitochondrial-derived ATP by β-cells may provide localized or privileged elevations of ATP essential for sustained closure of K_{ATP} channels (44). In addition, a β-cell line depleted of mitochondrial DNA displayed defects in cytochrome c oxidase activity, glucose, and leucine-induced increases in cellular ATP content and respiratory chain–driven ATP synthesis (45).

In summary, as illustrated schematically in Fig. 1, leucine has been previously shown to stimulate insulin secretion by β-cells due to its metabolism by oxidative decarboxylation and the ability of leucine to allosterically activate GDH by the β-cell mitochondria. Both acetyl-CoA and α-ketoglutarate appear to be necessary as Kreb's Cycle substrates to fully acti-

vate the β-cell mitochondria. Although these metabolically linked secondary signals originate from the mitochondria, the identity of these mediators and the mechanism leading to insulin exocytosis are unknown. Our findings show that leucine also uses these same metabolic pathways to activate mTOR mitogenic signaling in β-cells. This mechanism appears to involve other metabolically linked secondary signals because leucine-induced activation of protein translation is rapamycin sensitive and insulin independent. Future studies will explore the metabolic links between the metabolism of leucine by the β-cell mitochondria and mTOR activation.

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