Amino acid effects on translational repressor 4E-BP1 are mediated primarily by l-leucine in isolated adipocytes

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Recent studies indicated that amino acids may activate the protein kinase activity of the target of rapamycin (TOR) and thereby augment and/or mimic the effects of insulin on protein synthesis, p70S6k phosphorylation, and multicellular clustering in adipocytes. To identify the individual amino acids responsible for these effects, the present study focused on the TOR substrate and translational repressor 4E-BP1. A complete mixture of amino acids stimulated the phosphorylation of 4E-BP1, decreasing its association with eukaryotic initiation factor eIF-4E. Studies on subsets of amino acids and individual amino acids showed that l-leucine was the amino acid responsible for most of the effects on 4E-BP1 phosphorylation; however, the presence of other amino acids was required to observe a maximal effect. The stimulatory effect of leucine was stereospecific and not mimicked by other branched chain amino acids but was mimicked by the leucine metabolite a-ketoisocaproate (a-KIC). The effect of a-KIC, but not leucine, was attenuated by the transaminase inhibitor (amino-oxy)acetate. The latter result indicates that the effects of a-KIC required its conversion to leucine. Half-maximal stimulation of 4E-BP1 phosphorylation occurred at ~430 µM; therefore, the response was linear within the range of circulating concentrations of leucine found in various nutritional states.

Amino acids may mimic or potentiate the actions of insulin on protein metabolism by stimulating cell signaling pathways that are also activated by insulin, such as the FRAP/TOR pathway (15, 40, 49). The FRAP/TOR pathway centers around a proline-directed serine/threonine protein kinase termed TOR, mammalian TOR (mTOR), or FK506- and rapamycin-associated protein (FRAP): TOR is an acronym for the "target of rapamycin," the cellular target for the immunosuppressive drug rapamycin. The receptor for this drug is a small-molecular weight protein called the FK506 binding protein. Interaction of the rapamycin-FK506 binding protein complex with TOR inhibits TOR kinase activity (6). It is currently not known exactly how insulin or amino acids stimulate TOR. However, insulin-stimulated 4E-BP1 phosphorylation appears to require the tyrosine phosphorylation of insulin receptor substrate-1 and may involve the subsequent activation of phosphatidylinositol 3-kinase and protein kinase B (2, 3, 18, 34, 42).

Two downstream targets in a phosphorylation cascade that begins with TOR are the serine/threonine protein kinase p70S6k and the translational repressor 4E-BP1 (also called PHAS-I). Amino acids and insulin increase p70S6k phosphorylation and activity, leading to increased phosphorylation of the ribosomal S6 protein (2, 15, 40). Activation of p70S6k is associated with preferential translation of mRNA containing a polypyrimidine tract at the 5' end of the molecule (22, 35). Both amino acids and insulin also stimulate the phosphorylation of the eukaryotic initiation factor eIF-4E (eIF 4E binding protein 1 (4E-BP1) (5, 12, 23, 40, 49). First identified in adipocytes, 4E-BP1 may play a more immediate role than p70S6k in regulating protein synthesis. In various cell types, including adipocytes, protein synthesis is increased within a few minutes in response to insulin (25). This control of protein synthesis by insulin occurs most notably at the level of initiation of mRNA translation and is due to the phosphorylation of a number of proteins involved in translation initiation. The initiation factor eIF-4E is part of a multimeric eIF-4F complex that is required for recognition and unwinding of secondary structure in the 5'-capped untranslated regions of mRNA (for review, see Ref. 39). In some cells, significant amounts of the initiation factor eIF-4E may be bound to the 4E-BP1 and therefore eIF-4E may be rate limiting for protein synthesis. Because formation of the eIF-4F complex is prevented by the association of eIF-4E with 4E-BP1, 4E-BP1 has been described as a translational repressor protein (5). In response to insulin-mediated phosphorylation of 4E-BP1 at selected sites, eIF-4E dissociates from 4E-BP1. The free eIF-4E is then able to associate with
eIF-4G and eIF-4A, an RNA helicase, and form the active eIF-4F complex required for the unwinding of 5’ mRNA secondary structure. It is thought that regulation of eIF-4E binding proteins as well as eIF-4E may be particularly important in modulating the translation of mRNAs that contain highly structured 5’ untranslated regions, such as ornithine decarboxylase (for review, see Ref. 39). In summary, amino acids are able to regulate protein synthesis at the level of p70S6k and 4E-BP1, two steps that are also regulated by insulin.

Recent studies indicate that p70S6k and 4E-BP1 are on separate branches of a signaling pathway that bifurcates before p70S6k activation (48), most probably at TOR. Although the steps between activation of TOR and the consequent phosphorylation of p70S6k on one branch of this signaling pathway are not known, TOR appears to be a terminal kinase in the branch of this signaling pathway leading to phosphorylation of 4E-BP1 (5). Therefore, examination of the rapamycin-sensitive phosphorylation of 4E-BP1 represents a convenient index of changes in TOR activity.

To further understand the physiological role and the mechanism(s) involved in the effects of amino acids on protein metabolism, TOR activation, and other events such as the multicellular clustering of adipocytes, it is important to identify the regulatory amino acid(s). Therefore, in this study, we have investigated which amino acids are regulatory for 4E-BP1 phosphorylation in adipocytes.

**EXPERIMENTAL PROCEDURES**

**Materials.** Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). BioMag goat anti-mouse IgG magnetic beads were obtained from Perseptive Biosystems (Framingham, MA), and the magnetic sample rack was from Promega (Madison, WI). Amino acids were purchased from Sigma (St. Louis, MO) and United States Biochemical (Cleveland, OH). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), disodium EDTA, and benzamidine were all purchased from Sigma. Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad (Hercules, CA), and NitoBind nitrocellulose membrane was from MSI (Westborough, MA). Horseradish peroxidase-linked sheep anti-mouse IgG secondary antibody, horseradish peroxidase-linked goat anti-rabbit IgG secondary antibody, and the enhanced chemiluminescence Western blotting detection kit were all obtained from Amersham (Arlington Heights, IL).

**Isolation of adipocytes.** Adipocytes were isolated from 7- to 8-wk-old male Sprague-Dawley rats by collagenase digestion as previously described (15). The cells were washed in Krebs-Ringer-HEPES (KRH) buffer three times and allowed to incubate at 37°C in BSA-free KRH buffer for 20 min before the start of an experiment. After this “resting” period, the underlying buffer was removed from beneath the cells with a syringe. This resulted in a cell suspension with a 60–80% cytocrit, allowing the cells to be more readily aliquoted into other tubes. The cells were then further incubated under various experimental conditions as indicated.

4E-BP1 associated with eIF-4E. The amount of 4E-BP1 associated with eIF-4E in cells was determined as previously described (23). Briefly, the eIF-4E·4E-BP1 complex was immunoprecipitated from adipocyte homogenates using a monoclonal antibody against eIF-4E, and the amount of eIF-4E and 4E-BP1 in the immunoprecipitates was quantitated by protein immunoblot analysis.

**RESULTS**

The regulation of protein synthesis by insulin in adipocytes and other cells is accomplished through multiple mechanisms, including the phosphorylation of 4E-BP1. 4E-BP1 is a translational repressor that binds to eIF-4E and thereby prevents its association with eIF-4G and the formation of the eIF-4F complex. Phosphorylation of 4E-BP1 in response to insulin decreases its association with eIF-4E and thereby allows translation to proceed (26). To investigate the effect of amino acids on the association of 4E-BP1 with eIF-4E, adipocytes were incubated in the absence or presence of a complete mixture of amino acids at a concentration of 4x [where 1x is the approximate plasma concentration found in the postabsorptive or fasting state of rats as previously defined (33)]. Incubation of adipocytes with amino acids resulted in a 74% reduction in the amount of 4E-BP1 associated with eIF-4E (Fig. 1). Thus amino acids, like insulin (e.g., Ref. 23), are able to regulate the binding activity of 4E-BP1 in adipocytes.

There are at least five potential phosphorylation sites on 4E-BP1, resulting in the formation of three bands, which can be resolved by SDS-PAGE (12). These are termed the α (least phosphorylated and fastest migrating), β (intermediate), and γ (most phosphorylated and most slowly migrating) forms (Fig. 2A). We focused on the formation of the γ-form in the present...
Adipocytes were incubated in absence (Control) or presence of 4× concentrations of amino acids as indicated for 10 min before buffer was withdrawn and cells were frozen in liquid nitrogen. Amount of 4E-BP1 associated with eIF-4E was measured in 500-µl aliquots of postmitochondrial supernatant and was corrected for amount of immunoprecipitated eIF-4E. Results are means ± SE of separate immunoprecipitations from 3 different aliquots of adipocyte suspension in a single experiment that are representative of 2 such experiments.

The reason for the focus on the γ-form is that formation of the γ-form is associated with release of eIF-4E from the eIF-4E·4E-BP1 complex, whereas both the α- and β-forms bind to eIF-4E (27, 41).

In Fig. 2, the effects of insulin and amino acids on the phosphorylation of 4E-BP1 and the formation of the γ-form of 4E-BP1 are shown. Both amino acids and insulin increased 4E-BP1 phosphorylation; however, 4× amino acids were significantly more effective than 100 nM insulin in stimulating phosphorylation of 4E-BP1 (Fig. 2B). It was noted, however, that the absolute percentage of the overall 4E-BP1 immunoreactivity found in the γ-form in response to 4× amino acids or insulin was consistent between replicates within individual experiments but was less consistent among experiments. For example, in 20 separate cell preparations, the amount of the γ-form observed in response to 4× amino acids ranged from 15 to 62%. The reasons for these differences are not known. However, Mortimore’s group (37) noted that stimulatory responses to amino acids in liver were also variable between experiments but could be made more consistent by feeding animals a casein diet within a timed, synchronous feeding regimen and by making measurements 42 h after the last feeding. Further studies will be required to determine whether fasting, type of diet, or ad libitum feeding is responsible for the range of stimulatory responses to 4× amino acids observed in adipocytes.

4E-BP1 phosphorylation was also examined to delineate the individual amino acids responsible for the effects seen with the complete mixture of amino acids. Figure 2 shows that when leucine was removed from the complete mixture of 4× amino acids, the amount of 4E-BP1 in the γ-form was not significantly different from control. In other experiments, amino acids were divided into small groups, and their effects on 4E-BP1 phosphorylation were compared with those of insulin or a complete mixture of amino acids (data not shown). Only amino acid groups containing leucine showed a significant effect of the formation of the γ-form. None of the other amino acids was capable of generating the γ-form even at supraphysiological concentrations (data not shown). This result would seem to indicate that leucine was solely responsible for the amino acid-stimulated phosphorylation of 4E-BP1. However, when the effects of 4× leucine alone (17 ± 4% in the γ-form, n = 7) and 4× amino acids (43 ± 3% in the γ-form, n = 20) on the phosphorylation of 4E-BP1 were compared, the response of leucine alone was consistently and significantly less (Student’s t-test, P < 0.05). These data indicate either 1) that amino acids other than leucine weakly stimulate the phosphorylation of 4E-BP1 and that their small effects are synergistic when added together with leucine or 2) that one or more other amino acids are required to observe maximal effects of leucine on this parameter.

**Figure 1.** Effect of amino acids on amount of eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1) associated with eIF-4E. Adipocytes were incubated in absence (Control) or presence of 4× concentrations of amino acids as indicated for 10 min before buffer was withdrawn and cells were frozen in liquid nitrogen. Amount of 4E-BP1 associated with eIF-4E was measured in 500-µl aliquots of postmitochondrial supernatant and was corrected for amount of immunoprecipitated eIF-4E. Results are means ± SE of separate immunoprecipitations from 3 different aliquots of adipocyte suspension in a single experiment that are representative of 2 such experiments.

**Figure 2.** Effects of insulin or amino acids on 4E-BP1 phosphorylation. Aliquots (150 µl) of cell suspension were placed in BSA-free Krebs-Ringer-HEPES (KRH) buffer alone (Control) or in presence of 100 nM insulin, 4× concentrations of amino acids, or a 4× mixture of amino acids in which leucine was not added. Adipocytes were incubated under these conditions for 10 min. Buffer was then withdrawn, and cells were frozen in liquid nitrogen. Phosphorylation of 4E-BP1 in 4E-BP1 immunoprecipitates from cell lysates was assessed by conversion of 4E-BP1 to γ-form, which has decreased mobility during SDS-PAGE. A: representative Western blot showing relative mobility of α-, β-, and γ-forms of 4E-BP1 under different conditions as indicated. B: percent of total 4E-BP1 in γ-form was determined under different conditions and from different experiments. Results are means ± SE from 3 separate cell preparations. *Response to 4× amino acids was significantly different from response to 100 nM insulin.
It is possible that a metabolite of leucine, such as α-ketoisocaproate (α-KIC), is responsible for the effects of leucine. To compare the effects of leucine and α-KIC, we examined the effects of amino acid mixtures in which α-KIC was substituted for leucine. Figure 3 shows that when α-KIC was substituted for leucine in the amino acid mixtures, 4E-BP1 phosphorylation was stimulated to the same level as with the leucine-containing mixtures. Thus the efficacy of leucine and α-KIC in stimulating 4E-BP1 phosphorylation appears to be equivalent in isolated adipocytes.

Reversible interconversion of leucine and α-KIC is catalyzed by branched chain amino transferase activity. (Aminooxy)acetic acid (AOAA) has been used to block the activity of transaminases (10, 32), although the branched chain amino acid transferases are more resistant to this compound than other transaminases.

**Fig. 3.** Effects of leucine, α-ketoisocaproic acid (α-KIC), and (aminooxy)acetic acid (AOAA) on 4E-BP1 phosphorylation. Cell suspensions to be treated with AOAA were incubated in BSA-free KRH containing 0.2 mM AOAA for 20 min before beginning of experiment, while remainder of adipocytes were incubated in BSA-free KRH. All buffer was removed from cells, and 150-µl aliquots of adipocytes were added to BSA-free KRH buffer (with or without AOAA), a complete mixture of 4× amino acids (with or without AOAA), or a 4× mixture of amino acids in which α-KIC replaced leucine (with or without AOAA). Cells were incubated for 10 min. All buffer was then removed from beneath cells, and cells were frozen in liquid nitrogen for subsequent analysis of 4E-BP1 phosphorylation. A: representative experiment. B: means ± SE from quadruplicate determinations from a representative experiment. *Significant difference between L- and D-leucine as determined using Student’s t-test (P < 0.05).

**Fig. 4.** Stereospecificity of leucine stimulated 4E-BP1 phosphorylation. Aliquots of cell suspension (150 µl) were incubated for 10 min in either BSA-free KRH or BSA-free KRH containing either 1 or 10 mM L- or D-leucine. Buffer was withdrawn from beneath adipocytes, and cells were then frozen in liquid nitrogen for subsequent analysis of 4E-BP1 phosphorylation. A: representative experiment. B: means ± SE from quadruplicate determinations from a representative experiment. *Significant difference between L- and D-leucine as determined using Student’s t-test (P < 0.05).

**Fig. 5.** Concentration-related effects of leucine on 4E-BP1 phosphorylation. Aliquots of cells were incubated for 10 min either in BSA-free KRH containing 4× concentrations of all amino acids except leucine (0 mM) or in BSA-free KRH containing 4× concentrations of all amino acids and various concentrations of L-leucine as indicated. Buffer was withdrawn from beneath adipocytes, and cells were then frozen in liquid nitrogen for subsequent analysis of 4E-BP1 phosphorylation. Relative amount of 4E-BP1 phosphorylation was quantitated from densitometric scans using National Institutes of Health Image computer program. EC50 values were determined by nonlinearizing curve fitting using Sigma-Plot software and the equation f(x) = (x × Emax)/(EC50 + x), where Emax is maximal amount of 4E-BP1γ that would be formed at a supra-maximal concentration of leucine as determined from curve fitting. Results are representative of 2 such studies. In first experiment, EC50 for leucine was 0.43 ± 0.08 mM. In second experiment, it was 0.85 ± 0.34 mM. Difference in EC50 between these 2 experiments was not significant.
The concentrations used for in situ inhibition of branched chain transaminase isozymes in different tissues range from 0.2 to 5 mM. However, the higher concentrations can be expected to lower ATP concentrations; therefore, we used a lower concentration (0.2 mM). Adding 0.2 mM AOA to adipocytes had no significant effect on basal 4E-BP1 phosphorylation or 4E-BP1 phosphorylation in response to 4× amino acids (Fig. 3B). However, AOA significantly attenuated the response to a mixture of amino acids in which α-KIC replaced leucine (Fig. 3). These results suggest that the effect of α-KIC is dependent on its conversion to leucine.

Figure 4 shows that the effect of leucine displayed stereospecificity for the L-stereoisomer. When added with 4× concentrations of the other amino acids, 1 mM d-leucine was only 36% as effective as L-leucine at stimulating the phosphorylation of 4E-BP1 (as determined by conversion of 4E-BP1 to the γ-form). This difference increased to 52% at 10 mM leucine (Fig. 4).

Finally, the concentration dependence of the leucine response was evaluated. The EC50 of leucine in generating the γ-phosphorylated form of 4E-BP1 in the presence of other amino acids was 0.43 ± 0.08 mM in one experiment (Fig. 5). This was not significantly different from 0.85 ± 0.34 mM, measured in a repetition of that experiment.

DISCUSSION

In this communication, we show that amino acids, like insulin (e.g., Ref. 23), stimulate a functional phosphorylation of the translational repressor 4E-BP1 (Figs. 1 and 2). Leucine was the only amino acid that individually produced reproducible changes in 4E-BP1 phosphorylation. However, our data indicate that other amino acids must be present to observe a full effect of leucine. The effect of leucine is probably not mediated by its metabolite α-KIC, since the transaminase inhibitor AOA attenuated the α-KIC response (Fig. 3).

Several findings reported in this communication suggest that the mechanism used by amino acids to stimulate the functional phosphorylation of 4E-BP1 involves the interaction of leucine with a specific binding site. The supporting evidence is as follows: 1) the effects of leucine are concentration dependent (Fig. 5), 2) the dose-response curve fits a model involving binding of a ligand to a single site (Fig. 5), 3) other amino acids closely related to leucine, i.e., isoleucine and valine, do not elicit the same effects (data not shown), 4) the effects of leucine are stereospecific (Fig. 4), and 5) the effects of α-KIC on the phosphorylation of 4E-BP1 may require its conversion to leucine (Fig. 3). Leucine has previously been identified as an amino acid that is able to mimic the effects of insulin on peripheral protein metabolism in both liver (2, 20, 36, 38, 46) and skeletal muscle (7–9, 28).

Changes in peripheral protein metabolism occur after a protein-rich meal (30, 47). In the past, these effects have been attributed to postprandial changes in circulating insulin concentrations. However, recent evidence suggests that some responses to a protein-rich meal might actually be due to postprandial increases in the concentrations of circulating amino acids (44, 50). Leucine and certain other amino acids approximately double in concentration after a protein-rich meal and probably reach much higher concentrations within the portal system (1). Because the EC50 for leucine stimulation of 4E-BP1 phosphorylation was above the 1× concentration (Fig. 5), the linear portion of the response to leucine in adipocytes seems to occur within the range over which the circulating concentration of leucine can change after a meal. Thus it is conceivable that transient changes in leucine after a meal may be detected by adipocytes through the increased occupancy of a leucine binding site on or in adipocytes that at least regulates the FRAP/TOR signaling pathway.

The effect of nutrients such as amino acids on adipocyte biology and metabolism are of interest because overnutrition can lead to obesity, the relative excess of adipose tissue. In vitro, amino acids augment the effects of insulin both on protein synthesis (29), required for hypertrophic growth (19), and on multilayered clusters of adipocytes, an in vitro behavior that may reflect adipose tissue morphogenesis in vivo (4, 15). The growth-promoting actions of nutrients may act on adipose tissue, to some extent indirectly, through postprandial excursions in insulin. However, it is tempting to speculate from the present studies that some nutrients such as amino acids may also exert direct regulatory effects on adipocytes that act together with insulin to regulate adipose tissue growth and metabolism.

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REFERENCES


