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Regulation of Amino Acid–Sensitive TOR Signaling by Leucine Analogues in Adipocytes

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Abstract In adipocytes, amino acids stimulate the target of rapamycin (TOR) signaling pathway leading to phosphorylation of the translational repressor, eIF-4E binding protein-I (4E-BP1), and ribosomal protein S6. L-leucine is the primary mediator of these effects. The structure-activity relationships of a putative L-leucine recognition site in adipocytes ($LeuR_A$) that regulates TOR activity were analyzed by examining the effects of leucine analogues on the rapamycin-sensitive phosphorylation of the translational repressor, eIF-4E binding protein-I (4E-BP1), an index of TOR activity. Several amino acids that are structurally related to leucine strongly stimulated 4E-BP1 phosphorylation at concentrations greater than the EC_{50} value for leucine. The order of potency was leucine > norleucine > threo-L-β-hydroxyleucine ≃ Ile > Met ≃ Val. Other structural analogues of leucine, such as H-α-methyl-D/L-leucine, S-(−)-2-amino-4-pentenoic acid, and 3-amino-4-methylpentanoic acid, possessed only weak agonist activity. However, other leucine-related compounds that are known agonists, antagonists, or ligands of other leucine binding/recognition sites did not affect 4E-BP1 phosphorylation. We conclude from the data that small lipophilic modifications of the leucine R group and α-hydrogen may be tolerated for agonist activity; however, leucine analogues with a modified amino group, a modified carboxylic group, charged R groups, or bulkier aliphatic R groups do not seem to possess significant agonist activity. Furthermore, the leucine recognition site that regulates TOR signaling in adipocytes appears to be different from the following: (1) a leucine receptor that regulates macroautophagy in liver, (2) a leucine recognition site that regulates TOR signaling in H4IIE hepatocytes, (3) leucyl tRNA or leucyl tRNA synthetase, (4) the gabapentin-sensitive leucine transaminase, or (5) the system L-amino acid transporter. J. Cell. Biochem. 77:234–251, 2000.

Key words: TOR; adipocytes; leucine analogues

Changes in the circulating concentration of certain amino acids that occur in response to various nutritional and disease states regulate protein metabolism associated with growth and development in a number of tissues [May and Buse, 1989; Svanberg et al., 1997; Yoshizawa et al., 1995]. Of these regulatory amino acids, leucine can directly regulate protein metabolism at the levels of both protein synthesis initiation and protein degradation/macroautophagy [for reviews, see May and Buse, 1989; Mortimore et al., 1996; Pain, 1996]. Although the mechanisms involved in the regulation of protein metabolism by amino acids have not been thoroughly delineated, we suspect that they may be similar to the mechanisms by which hormones modulate these processes. For example, both amino acids and insulin can activate cell signaling pathways involving protein kinases (and possibly phosphatases) that regulate protein synthesis [Blommaart et al., 1995; Fox et al., 1998a,b; Iiboshi et al., 1999; Patti et al., 1997; Schott et al., 1985; Wang et al., 1998; Xu et al., 1998a,b].

One of the regulatory protein kinases involved in the cell signaling pathways activated by amino acids is the lipid-related serine-threonine protein kinase target of rapamycin (TOR) [Blommaart et al., 1995; Fox et al., 1998a,b; Iiboshi et al., 1999; Patti et al., 1997; Schott et al., 1985; Wang et al., 1998; Xu et al., 1998a,b]. Downstream targets of the TOR cell signaling pathway are thought to include the ribosomal protein S6 (S6) and translational re-

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pressor protein, 4E-BP1 (also called PHAS-I). This is mainly based on two lines of evidence. First, the antibiotic/immunosuppressant rapamycin [Dumont and Su, 1996] inhibits amino acid-induced phosphorylation of these two proteins. Second, amino acid stimulation of 4E-BP1 and S6 phosphorylation is no longer rapamycin sensitive in cells transfected with a rapamycin-insensitive deletion mutant of TOR [Hara et al., 1998].

S6 and 4E-BP1 regulate protein synthesis through different mechanisms. Phosphorylation of S6 has been associated with preferential translation of mRNAs with a polypyrimidine tract in their 5'-untranslated region (5'UTR) [for review, see Pain, 1996]. A number of mRNA species with a polypyrimidine tract in their 5'UTR encode for proteins involved in protein synthesis. Other mRNAs lacking this polypyrimidine tract can be upregulated secondary to the increases in protein synthetic machinery associated with S6 phosphorylation. By contrast, 4E-BP1 is a translational repressor that works by binding to initiation factor eIF4E, a protein involved in the binding of mRNAs to the ribosome. Initiation factor 4E is thought to be especially important for translation of mRNAs with significant secondary structure in their 5' capped UTR, such as ornithine decarboxylase [for review, see Pain, 1996]. When 4E-BP1 binds to eIF4E, the eIF4E is prevented from interacting with the ribosome, thereby limiting translation. When 4E-BP1 is phosphorylated on multiple residues, for example in cells incubated with insulin or amino acids, binding to eIF4E is reduced and eIF4E is then freed to take part in the initiation of protein synthesis.

TOR appears to be directly responsible for at least some of the phosphorylations on 4E-BP1 [Brunn et al., 1997]. By contrast, TOR does not phosphorylate S6 directly, it acts indirectly by phosphorylating and activating another kinase, 70-kDA ribosomal protein S6 kinase ($p70^{S6k}$). Other unidentified kinase (s) and/or phosphatase (s) also appear to be required to completely explain all the amino acid-stimulated phosphorylations of $p70^{S6k}$ and 4E-BP1 [Hara et al., 1997, 1998; Wang et al., 1998]. While protein kinase B is required for insulin stimulation of TOR signaling, amino acids fail to activate protein kinase B. Therefore, amino acids must use some signaling mechanism distinct from the cascade through protein kinase B.

The ability of amino acids to activate TOR implies the existence of one or more amino acid recognition sites whose occupation is linked, in some way, to TOR activation. Leucine and, in some cases, the other branched-chain amino acids, have been repeatedly implicated in the effects of amino acids on protein metabolism in peripheral tissues. We and others have reported that leucine preferentially stimulates the TOR signaling pathways in adipocytes, hepatocyte cell lines, pancreatic cells and L6 cells in culture [Fox et al., 1998a,b; Patti et al., 1997; Xu et al., 1998a]. As outlined above, activation of TOR would be expected to enhance phosphorylation of 4E-BP1. However, leucine is the only amino acid that can stimulate 4E-BP1 phosphorylation when added by itself to adipocytes [Fox et al., 1998b]. The effects of leucine are stereospecific with an ~6- to 10-fold difference in the EC_{50} values for the L- and D-isomers of leucine. Based on the above findings, we have proposed that adipocytes may possess a stereospecific leucine recognition site linked to TOR activation [Fox et al., 1998b].

In contrast to the situation in freshly isolated adipocytes, a number of amino acids regulate TOR in Jurkat cells [Hara et al., 1998] and Chinese hamster ovary cells overexpressing insulin receptors (CHO-IR cells) [Iiboshi et al., 1999]. For example, deprivation of any one of a number of amino acids caused an ~50–90% reduction in $p70^{S6k}$ activity in Jurkat cells [Iiboshi et al., 1999]. In CHO-IR cells removing Arg, Leu, Lys, Tyr, or Val from a mixture of amino acids inhibited TOR activity [Hara et al., 1998]. Furthermore, in Jurkat cells, amino acid alcohols inhibited amino acid-stimulated TOR signaling, implicating tRNAs or tRNA synthetases as possible recognition sites for amino acid regulation of TOR [Hara et al., 1998].

The present study was designed to evaluate further the role of leucine and other amino acids in the regulation of TOR signaling in adipocytes. To accomplish this goal we have examined the ability of both naturally occurring and synthetic amino acid analogues to regulate 4E-BP1 phosphorylation. Some of the analogues were tested because they have been previously shown to interact with known leucine-binding sites in mammalian cells. The data are consistent with a model in which amino acids activate TOR signaling by interacting with an amino acid recognition site at which leucine is the most potent agonist. The

relative potency of amino acids at this site is related to the structural similarity of each amino acid's R group to the R group of leucine. We conclude from these data that this site may be different from a number of other known leucine binding/recognition sites.

MATERIALS AND METHODS

Materials

The "octopus peptide," Leu_8-α,ε-amino-Lys_4-αε-amino-Lys_2-αε-amino-Lys-β-Ala (MAP-Leu_8), was synthesized as previously described [Miotto et al., 1994]. The synthesized product was determined to contain a single peptide species (measured mass = 1892.94, calculated mass = 1891.78) by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). O-iso-Valeryl-L-carnitine chloride (IVC) was a generous gift of Dr. Giovanni Miotto (Department of Biological Chemistry, Università Degli Studi di Padova, Padua, Italy) [Miotto et al., 1992]. Amino acids and other leucine analogues were purchased from either Acros Organics (Fair Lawn, NJ), Sigma Chemical Co. (St. Louis, MO), or United States Biochemical Corporation (Cleveland, OH). PMSF, disodium EDTA, and benzamidine were all purchased from Sigma. PVDF membrane was purchased from Bio-Rad (Hercules, CA). Horseradish peroxidase (HRP)-linked sheep-anti mouse Ig secondary antibody, HRP-linked goat-anti rabbit Ig secondary antibody, and the ECL Western blotting detection kit were all obtained from Amersham (Arlington Heights, IL). BioMag goat-anti mouse IgG magnetic beads were obtained from PerSeptive Biosystems (Framingham, MA), and the magnetic sample rack from Promega (Madison, WI).

Isolation of Adipocytes

Adipocytes were isolated from 7- to 8-week-old-male Sprague-Dawley rats by collagenase digestion as previously described [Fox et al., 1998a,b]. The cells were washed three times in Krebs-Ringer Hepes (KRH: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM Hepes, 1 mM EDTA, 2 mM glucose, and 200 nM adenosine) buffer containing 2% bovine serum albumin fraction V (BSA) and allowed to incubate at 37°C in albumin-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 in the figure legends.

Phosphorylation of 4E-BP1

4E-BP1 is phosphorylated at multiple sites and can be resolved into three distinct bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Fadden et al., 1997]. These bands are termed the α (least phosphorylated and fastest migrating), β (intermediate) and γ (most phosphorylated and most slowly migrating) forms. We focused on the formation of the γ-form in the present communication because that form is associated with the release of eIF4E from the eIF4E-4E-BP1 complex, while both the α- and β-forms bind to eIF4E [Lin et al., 1994; Pause et al., 1994]. For these experiments, aliquots of cells (150 μl, 60–80% cytocrit) were generally added to 500 μl of either BSA-free KRH, buffer A, or buffer A containing additional additives as indicated in the figure legends (e.g., leucine or an amino acid analogue) and allowed to incubate at 37°C for 10 min. Buffer A consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, 1 mM EDTA, 2 mM glucose, 200 nM adenosine, 2395μM Ala, 312μM Asn, 176μM Asp, 651μM Arg, 389μM Cys, 2278μM Gln, 1950μM Gly, 385μM His, 649μM Ile, 2335μM Lys, 259μM Met, 326μM Phe, 651μM Pro, 1302μM Ser, 1170μM Thr, 454μM Trp, 371μM Tyr, 1170μM Val. Buffer A does not contain leucine; thus, in the figures, incubation with buffer A is referred to as Am. Acids w/o Leu. When leucine was added, its final concentration was 0.8 mM, unless otherwise indicated. In certain experiments, immediately before the above incubation, cells were preincubated in KRH with potential inhibitors for 15 or 30 min, in order to allow time for these compounds to act on their targets. These potential inhibitors were also present during the 10-min incubations with either buffer A or buffer A with agonist. After the 10-min incubation at 37°C, the buffer was withdrawn from beneath the cells with a syringe, and the cells were frozen in liquid nitrogen.

Subsequently, homogenization buffer (450 μl of 20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1 mM DTT, 0.1 mM

PMSF, 1 mM benzamidine, 0.5 mM Na_3VO_4) was added to 150-μl aliquots of frozen cells. The mixture was processed by one of two methods to measure 4E-BP1 phosphorylation. In method A, the mixture was sonicated on ice, and the samples were centrifuged at 10,000*g* for 10 min at 4°C. 4E-BP1 was immunoprecipitated from aliquots (250 μl) of the resulting fat-free infranatant using a previously described 4E-BP1 monoclonal antibody. The most highly phosphorylated form of 4E-BP1 (the γ-form) was detected by gel shift electrophoresis as previously described in detail [Fox et al., 1998b]. In method B, the mixture was sonicated on ice and centrifuged at 10,000*g* for 10 min at 4°C. The fat-free infranatant was boiled for 10 min and then centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was mixed 1:5 in 5× sample buffer, and 4E-BP1 phosphorylation was detected by gel mobility shift as described as in method A, except that a rabbit anti-recombinant 4E-BP1 antibody was used (1:800 dilution).

The results shown in each figure are from single experiments that are representative of two or more such studies. Statistical analyses were made using the InStat computer program (GraphPad Software, San Diego). Comparisons between different experimental conditions in each experiment for statistical significance were determined by analysis of variance (ANOVA), followed by Dunnett's multiple comparison post-test in most cases in which there was one control to which other data were compared. Alternatively, the Tukey-Kramer post-test was used when multiple comparisons were made.

Analysis of Amino Acid Concentrations in Cell Suspensions

In some experiments, after incubation at 37°C under various conditions and times indicated, the buffer was withdrawn from beneath the cells with a syringe, and ice-cold 10% trichloroacetic acid (TCA) was added to the cells. The amount of leucine, leucine amide or threo-L-β-hydroxyleuycine present in the TCA soluble fraction of cell suspensions was measured by high-performance liquid chromatography (HPLC) after adjusting the TCA-soluble fraction to pH 9.0 and derivatization with dabsyl chloride [Drnevich and Vary, 1993]. The peak heights were measured against a standard curve as previously described [Drnevich and Vary, 1993].

RESULTS

Comparison of Methods and Antibodies for Measuring 4E-BP1 Phosphorylation

We examined 4E-BP1 phosphorylation as a reporter for TOR activity in adipocytes. Figure 1 (top) shows a representative Western blot in

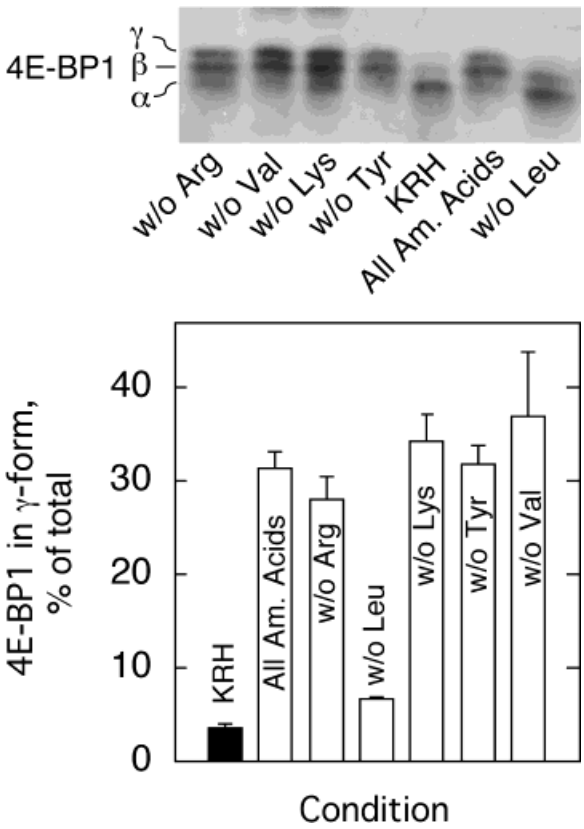

Fig. 1. Leucine is responsible for most of the effects of amino acids on 4E-BP1 phosphorylation in adipocytes. Cells were incubated with KRH (black bar, i.e., no amino acids), a complete mixture of amino acids (white bar labeled All Am. Acids), or a mixture of amino acids in which one amino acid was missing as indicated (e.g., white bars labeled w/o Arg means that Arg was absent). After incubating in these mixtures for 10 min, the buffer was withdrawn from underneath the floating adipocytes and the cells were frozen in liquid nitrogen. Phosphorylation of 4E-BP1 was measured in 4E-BP1 immunoprecipitates and quantitated as described under Materials and Methods. **Top:** Representative Western blot showing the α-, β-, and γ-forms of 4E-BP1 under different conditions. **Bottom:** The results are the mean and standard error from triplicate adipocyte suspensions/immunoprecipitations in a single experiment that is representative of two such experiments. The amount in the γ-form is expressed as a percentage of the total optical density in the α-, β-, and γ-forms. The effects of removing additives to the complete mixture of amino acids (All Am. Acids) were evaluated using ANOVA, followed by Dunnett's post-test. The only two conditions that were significantly different from All Am. Acids at $P < 0.05$ were the KRH and the w/o Leu groups.

which the changes in the distribution of 4E-BP1 among the different isoforms (i.e., the α-, β- and γ-forms) can be seen. In the initial experiments, we tested whether using methods A and B, as described under Materials and Methods, resulted in any significant measurable differences in the amount of 4E-BP1 phosphorylated in the γ-form. No significant differences were detected in the amount of 4E-BP1 in the γ-form detected in the same samples using the two different methods (data not shown). Method B, which is based on the relative heat stability of 4E-BP1, is faster and less expensive than method A. Therefore, we choose method B to analyze 4E-BP1 phosphorylation in all subsequent experiments. We also compared two polyclonal antibodies from our laboratory for their ability to detect the most phosphorylated from of 4E-BP1, the γ-form, in Western blots. No significant differences were observed in the amount of 4E-BP1 in the γ-form when measured by using either a previously described polyclonal antibody [Kimball et al., 1996] or a new rabbit polyclonal antibody we have made to recombinant rat 4E-BP1 (data not shown).

Are the Amino Acids Shown to Be Regulatory for TOR Signaling in CHO-IR and Jurkat Cells Also Regulatory for TOR Signaling in Adipocytes?

We incubated adipocytes in different mixtures of amino acids, having removed one amino acid from each mixture. In contrast to the response seen in CHO-IR and Jurkat cells, the response was not significantly affected when other amino acids, such as Arg, Val, Lys, or Tyr, were removed from an otherwise complete mixture that included leucine (Fig. 1 and Table I). However, removing leucine from the mixture, significantly reduced the extent of 4E-BP1 phosphorylation. Thus, freshly isolated adipocytes appear to be sensitive to removing leucine, but not other amino acids found to be active in the cell lines. This finding is in agreement with our previous finding [Fox et al., 1998b] that at ~4 times (4×) physiological concentrations, only leucine was able to significantly stimulate 4E-BP1 phosphorylation when added by itself (see Table I for rat plasma concentrations of amino acids).

TABLE I. Comparison of Estimated Concentrations of Individual Amino Acids in Incubations of Adipocytes With Buffer A and in Rat Plasma†

Amino acid	Expected amino acid concentrations in our mixtures of buffer A and adipocytes (μM)	Amino acid concentrations measured in rat plasma (μM)
Ala	1842	526
Asn	240	
Asp	135	16
Arg	500	164
Cys	300	60*
Gln	1,752	563
Gly	1,500	293
His	296	66
Iso	500	83
Leu	None added	146
Lys	1,796	390
Met	200	40
Phe	250	50
Pro	500	214
Ser	1,000	200
Thr	900	180
Trp	350	70*
Tyr	370	78
Val	900	177

†The expected concentration of amino acids in mixtures of cells incubated in buffer A assumes a 23% dilution of buffer A based on the addition of 150 μl of cells in KRH to 500 μl of buffer A. The actual concentrations may be higher because (1) the cells may contribute amino acids, and (2) the cytocrit of the cells is ~60–80% and most of their cell volume is lipid. The amino acid concentrations in plasma are values from Sprague-Dawley rats fed ad libitum [Drnevich and Vary, 1993], unless marked with an asterisk. An asterisk (*) indicates values were unavailable from the above report and were obtained from Meijer et al. [1985]. It should be noted that measurements of amino acids in plasma may vary severalfold in different nutritional and pathological states. There are also differences depending on the method used to make the measurements.

Effects of Leucine Analogues Containing Carboxyl-Group Modifications

Mortimore and others have characterized a plasma membrane leucine recognition site ($LeuR_m$) thought to regulate macroautophagy in liver [for review, see Mortimore et al., 1996]. $LeuR_m$ appears to be activated not only by leucine, but also by leucine analogues lacking the carboxyl (C) group of leucine. Two such analogues that strongly inhibit macroautophagy are MAP-Leu_8 [Miotto et al., 1994] and isovaleryl-L-carnitine [Miotto et al., 1992]. Recently, Blommaart et al. [1995, 1997] postulated that $LeuR_m$ also mediates the effects of leucine on the mTOR signaling pathway in hepatocytes. Therefore, we examined the effects of MAP-Leu_8 and isovaleryl-L-carnitine on 4E-BP1 phosphoryla-

tion in order to determine whether or not $LeuR_m$ might regulate TOR signaling in adipocytes. We found that while 0.8 mM leucine significantly stimulated 4E-BP1 phosphorylation, neither 0.8 mM MAP-Leu_8 nor up to 8 mM isovaleryl-L-carnitine had any significant effect (Fig. 2). We conclude that $LeuR_m$ may be different from the leucine recognition site in adipocytes ($LeuR_A$), which mediates the effects of leucine on the mTOR signaling pathway.

We then investigated the effects of leucinol on amino acid stimulation of 4E-BP1 phosphorylation in adipocytes. Leucinol is a leucine analogue which can be formed from the reduction of the carboxylic acid group (structure shown in Fig. 3). It is of interest for several reasons. First, leucinol and other amino acid alcohols are competitive inhibitors of tRNA synthetases [e.g., Vaughan and Hansen, 1973] and thereby affect the initiation of protein synthesis. Second, the modified carboxy-group of leucinol al-

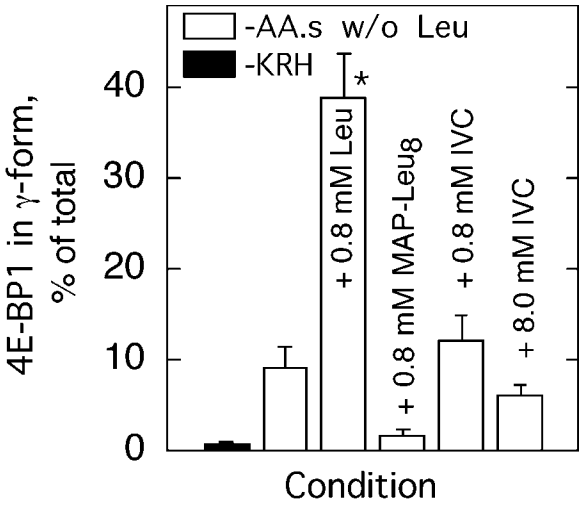

Fig. 2. Effect of leucine analogues that regulate macroautophagy in hepatocytes on 4E-BP1 phosphorylation state in adipocytes. Cells were incubated with KRH (black bar, i.e., no amino acids) or buffer A (white bars) plus or minus either Leu (0.8 mM), Leu_8-αε-amino-Lys_4-α,ε-amino-Lys_2-α,ε-amino-Lys-βAla (MAP-Leu_8, 0.8 mM) or isovaleryl-L-carnitine (IVC, 0.8 or 8.0 mM) as indicated. After 10 min, the buffer was withdrawn and the cells were frozen in liquid nitrogen. Phosphorylation of 4E-BP1 was measured in 4E-BP1 immunoprecipitates and quantitated as described under Materials and Methods. The results are the mean and standard error of separate immunoprecipitations from three different adipocyte suspensions in a single experiment that is representative of two such experiments. The effects of additives to the buffer A were evaluated using ANOVA, followed by Dunnett's post-test, in which the effects of the additives were compared with the second bar from the left (AA.s w/o Leu, i.e., buffer A alone). An asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

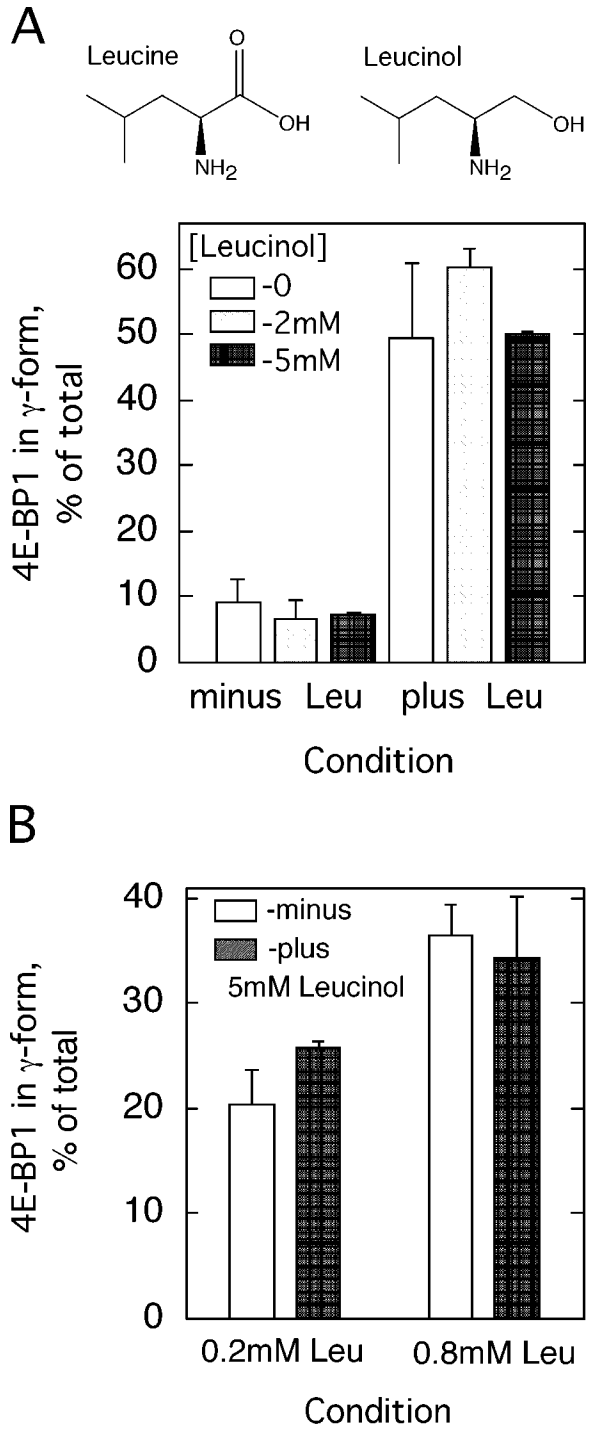

Fig. 3. Effects of leucinol on 4E-BP1 phosphorylation in adipocytes. The cells were preincubated with either 0, 2, or 5 mM leucinol dissolved in KRH. **A:** After 15 min, the buffer beneath the cells was removed and replaced with the same concentration of leucinol dissolved in either buffer A (minus Leu) or buffer A containing 0.8 mM leucine (plus Leu). After a 10-min incubation under these conditions, the cells were frozen and processed for quantitation of 4E-BP1 phosphorylation. **B:** Lack of effect of 5 mM leucinol on amino acid stimulation of 4E-BP1 phosphorylation. Buffer A contained either 0.2 or 0.8 mM leucine as indicated.

lows us to evaluate the role of this group in the activation of TOR signaling. Lastly, L-leucinol and other L-amino acid alcohols block amino acid stimulation of TOR signaling in Jurkat cells [Iiboshi et al., 1999]. Iiboshi et al. [1999] have therefore hypothesized that the extent of tRNA charging state may be a signal regulating the TOR signaling pathway in mammalian cells.

Figure 3A shows the effects of 2 mM and 5 mM leucinol on 4E-BP1 phosphorylation in adipocytes. Surprisingly, leucinol neither stimulated 4E-BP1 phosphorylation nor prevented the subsequent phosphorylation in response to 0.8 mM leucine. We explored possible reasons for this discrepancy between our results (Fig. 3A) and those of Iiboshi et al. [1999]. In our experiments, adipocytes were incubated for 15 min in leucinol-containing KRH (during which leucinol did not have to compete with added amino acids for transport or intracellular binding sites) and then for an additional 10 min in either buffer A (minus leu) or buffer A plus leucine (plus leu). Thus, leucinol-exposed adipocytes received a total of 25 min of leucinol exposure. This should have been sufficient time for blocking the response. In both our study (Fig. 3A) and that of Iiboshi et al. [1999], 2 mM leucinol was used. Furthermore, one concentration of leucinol in our experiments, 5 mM, was greater than the concentration that ablated the effects of amino acids in Jurkat cells, 2 mM. Increasing the leucinol concentration to 10 mM proved toxic to adipocytes: we observed cell clumping, leaking of cell 4E-BP1 immunoreactivity into the medium, and leaking of cytoplasmic contents, as detected by microscopic inspection with trypan blue dye in several of our experiments (data not shown). The concentration of leucine used in Figure 3A (0.8 mM) was both comparable to Iiboshi et al. [1999] and submaximal in adipocytes [Fox et al., 1998b]. While leucinol should have been able to compete readily with the added leucine under these conditions, we subsequently examined the effects of a lower concentration of leucine to allow an even better situation for competitive inhibition by leucinol. We measured the effect of 5 mM leucinol on the stimulation of 4E-BP1 phosphorylation by 0.2 mM leucine (Fig. 3, bottom). Again, 5 mM leucinol (more than twice the concentration used by Iiboshi et al), did not significantly affect 4E-BP1 phosphorylation in adipocytes, even at the lower concentration of leucine. It seems likely, therefore, that the reason why leucinol does not inhibit amino acid stimulation of TOR in adipocytes is that, unlike in Jurkat cells, the mechanism by which amino acids activate TOR is not leucinol-sensitive in adipocytes.

Another leucine analogue with a carboxy-group modification is L-leucine amide. This is one of two leucine analogues reported by Shigematzu et al. [1999] to stimulate TOR activity in H4IIE hepatocytes. Shigematzu et al. [1999] reported L-leucine amide was more active than leucine at stimulating TOR signaling in H4IIE hepatocytes using a $p70^{S6k}$ assay as a reporter for TOR activity. The structure of L-leucine amide is shown in Figure 4A (to form this analogue, the carboxy-group of leucine is converted to an amide linkage; in this respect, L-leucine amide is similar in structure to MAP-Leu_8). In adipocytes, both 0.8 and 8.0 mM L-leucine amide robustly stimulated 4E-BP1 phosphorylation to the same extent as leucine (Fig. 4A). Two lower concentrations of L-leucine amide (0.2 and 0.4 mM) stimulated 4E-BP1 phosphorylation approximately 50% more than the corresponding concentrations of leucine (data not shown).

Since L-leucine amide, but not MAP-Leu_8, stimulated 4E-BP1 phosphorylation, we considered the possibility that the effects of L-leucine amide might be the result of its conversion to leucine by intracellular leucine amino peptidase or other amidase activities. To evaluate this possibility, adipocytes were incubated for 5, 10, or 30 min in either KRH media or KRH media containing either no additions, 0.8 mM (800 nmol/ml) leucine or 0.8 mM L-leucine amide. At the indicated times, most of the buffer ($\sim$50% cytocrit) was then removed from below the cells and the amount of leucine or leucine amide present in 10% TCA-soluble portion of these cell suspensions was measured by HPLC and quantitated against a standard curve as previously described [Drnevich and Vary, 1993]. Figure 4B shows that adipocytes incubated in the absence of leucine did not release appreciable amounts of leucine. When adipocytes were incubated with 0.8 mM leucine, close to half of the leucine was lost within 30 min (Fig. 4B). This is probably a result of metabolic conversion beginning with branched-chain amino acid transaminase activity. By contrast, appreciable amounts of leucine were produced from adipocytes incubated with L-leucine amide. The increase in leucine corresponded with a decrease in

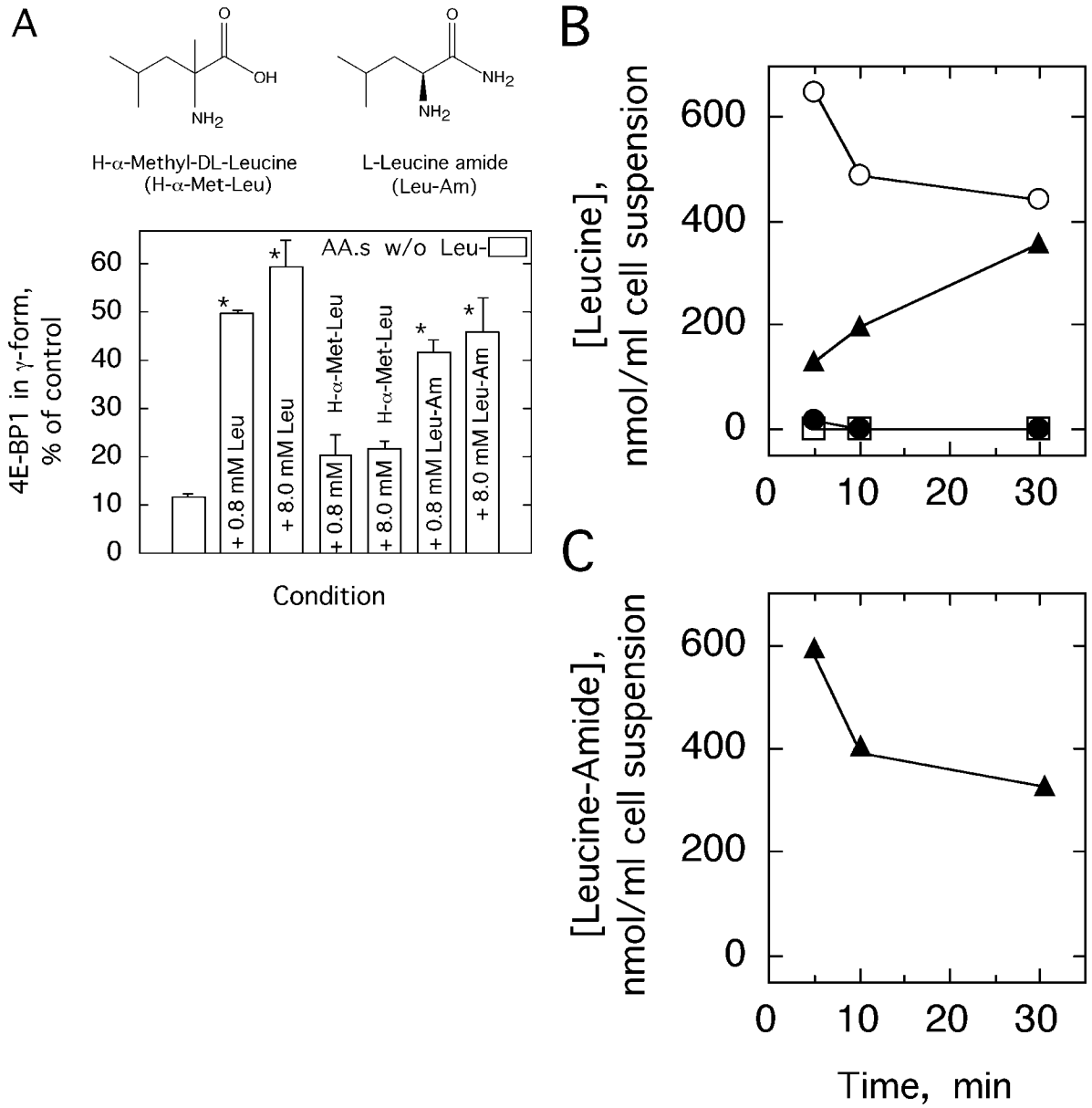

Fig. 4. Leucine, H-α-methyl-D/L-leucine and L-leucine amide effects on 4E-BP1 phosphorylation. **A:** Adipocytes were incubated with buffer A, or buffer A plus the indicated concentration of either leucine, H-α-methyl-D/L-leucine or L-leucine amide. Phosphorylation of 4E-BP1 in 4E-BP1 immunoprecipitates was quantitated as described in Fig. 1. Bars show the mean and standard error of separate immunoprecipitations from three different aliquots of adipocyte suspension. The effects of additives to the buffer A were evaluated using ANOVA, followed by Dunnett's post-test, in which the effects of the additives were compared with the second bar from the left (AA.s w/o Leu, i.e., buffer A alone). Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$. **B:** Suspensions of cells were incubated with either 0.8 mM leucine (○), 0.8 mM leucine amide (▲), 0.8 mM H-α-methyl-D/L-leucine (●) or KRH (□) as described under Materials and Methods for the indicated times. Most of the buffer was then removed from underneath the floating adipocytes and leucine concentrations were measured in TCA acid-soluble fractions of these cell suspensions as described under Materials and Methods. **C:** Leucine amide concentrations (▲) were measured in the suspensions of cells incubated with 0.8 mM leucine amide from **B**.

L-leucine amide concentrations (Fig. 4C). Thus, the possibility exists that the efficacy of L-leucine amide on 4E-BP1 phosphorylation is a result of its conversion to leucine. The amounts of leucine produced would be expected to be at a higher concentration around the microenvironment of the floating adipocytes or in the cytosol and should therefore be sufficient to stimulate $LeuR_A$. Thus, it cannot be proved that the parent compound, L-leucine amide, is

actually the stimulatory ligand at the amino acid recognition site in adipocytes that is responsible for leucine stimulation of 4E-BP1 phosphorylation.

Effect of a Leucine Analogue With an α-Hydrogen Substitution

Figure 4A shows the structure of H-α-methyl-D/L-leucine. Shigematzu et al. [1999] reported that H-α-methyl-D/L-leucine stimulated TOR signaling in the H4IIE cell line. The efficacy was similar to leucine. While 0.8 mM H-α-methyl-D/L-leucine appears to stimulate 4E-BP1 phosphorylation, the effects were not statistically significant in this experiment. Increasing the concentration of H-α-methyl-D/L-leucine to 8 mM did not result in any further stimulation (Fig. 4A). Concentration-response analysis beginning at 0.2 mM H-α-methyl-D/L-leucine was performed; however, at each of the concentrations the same level of 4E-BP1 phosphorylation was observed. Some of these did achieve statistical significance (data not shown). Thus, H-α-methyl-D/L-leucine may be a weak or partial agonist in adipocytes. Figure 4B shows that by contrast to L-leucine amide, H-α-methyl-D/L-leucine was not converted to leucine. The weak agonist activity of H-α-methyl-D/L-leucine in adipocytes compared with its strong activity H4IIE cells may suggest that leucine activation of TOR in H4IIE and adipocytes is mediated by different leucine recognition sites. Alternatively, the weak effect of H-α-methyl-D/L-leucine in adipocytes may indicate that two leucine recognition sites are present, but that only one interacts with H-α-methyl-D/L-leucine. Cycloleucine and 2-amino-2-norbornane carboxylic acid (BCH) also lack the α-hydrogen. Their effects and interpretation are considered later, as they are also R-group modified leucine analogues.

Effects of Leucine Analogues Containing Amino-Group Modifications

Figures 3 and 5 compare the chemical structures of leucine, α-ketoisocaproic acid, α-hydroxyisocaproic acid, and N-methyl-L-leucine and their effects on 4E-BP1 phosphorylation. While neither α-hydroxyisocaproic acid nor N-methyl-L-leucine significantly stimulated 4E-BP1 phosphorylation, α-ketoisocaproic acid displayed a robust stimulation (Fig. 5).

Perhaps the reason that α-ketoisocaproic acid so robustly stimulated 4E-BP1 phosphorylation is that the amino group on leucine was not necessary. If this is true, it seems surprising that the hydroxyl analogue and the analogue with a secondary amine were incapable of stimulating this response. Another explanation is that α-ketoisocaproic acid is rapidly transaminated to leucine which, in turn, activates TOR signaling. Indeed, we have previously shown that the effects of α-ketoisocaproic acid are diminished by the transaminase inhibitor aminooxyacetic acid while those of leucine are not [Fox et al., 1998b].

Another transaminase inhibitor that has been used to probe the regulatory actions of leucine and α-ketoisocaproic acid is L-cyclo-

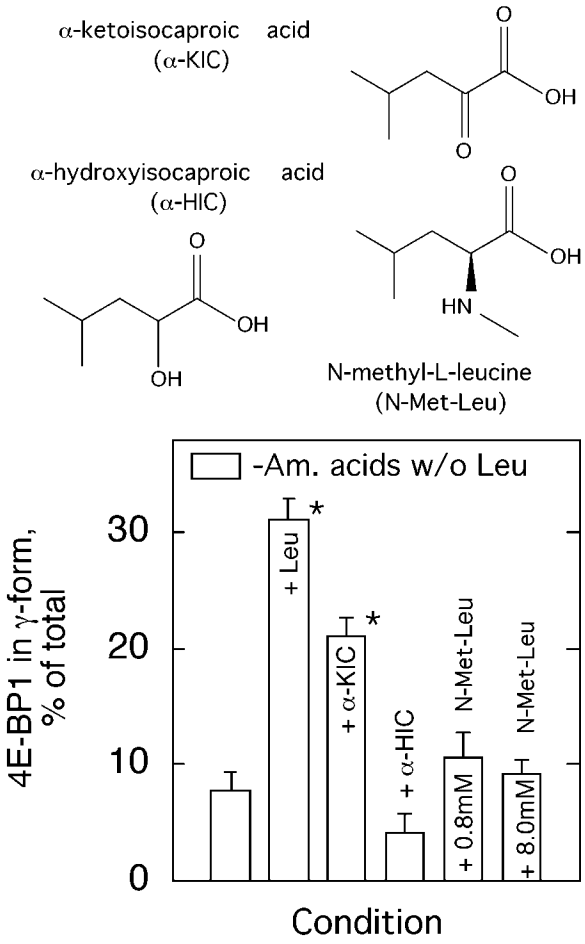

Fig. 5. Effects of amino residue modifications on stimulation of 4E-BP1 phosphorylation. Adipocytes were incubated with either buffer A, buffer A plus leucine, or buffer A plus the indicated leucine analogue. Phosphorylation of 4E-BP1 in 4E-BP1 immunoprecipitates was quantitated as described in Fig. 1. Bars show the mean and standard error of separate immunoprecipitations from three different aliquots of adipocyte suspension. Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

TABLE II. Transaminase Inhibitor, L-Cycloserine, Attenuates Effects of α-KIC, But Not Leucine, on 4E-BP1 Phosphorylation†

Condition	Minus L-cycloserine 4E-BP1 in γ-form % of total 4E-BP1	Plus L-cycloserine 4E-BP1 in γ-form % of total 4E-BP
Control (buffer A)	7.6 + 1.4	10.1 + 0.7
Plus leucine	31.1 ± 3.5	32.6 ± 5.7
Plus α-KIC	23.4 ± 0.2	13.0 ± 2.5*

†Adipocytes were incubated for 30 min ±5 mM L-cycloserine in KRH buffer (no amino acids). The buffer was removed from beneath the floating cells. They were subsequently incubated for 10 min in the absence (control) or presence of either 0.8 mM leucine or α-ketoisocaproate in buffer A or buffer A with 5 mM cycloserine (i.e., in order to continue exposure to this inhibitor). Phosphorylation of 4E-BP1 was examined by method B in triplicate. The mean and standard error are shown. An asterisk (*) indicates the response in the presence and absence of cycloserine is significantly different as determined using ANOVA, followed by the Tukey-Kramer post-test for multiple comparisons ($P < 0.05$).

serine [Tischler et al., 1982]. Table II shows that preincubation of adipocytes with L-cycloserine inhibited the activity of α-ketoisocaproic acid, but not that of leucine. These findings are consistent with the apparent activity of α-ketoisocaproic acid being related to enzymatic conversion of added α-ketoisocaproic acid to leucine.

Shigematzu et al. [1999] reported that two N-acetyl leucine analogues blocked leucine stimulation of TOR signaling in adipocytes. Figure 6 shows the structures of these two analogues, N-acetyl-leucine amide and N-acetyl-leucine methylamide. In H4IIE cells, these compounds caused a 50%–60% inhibition when ten times the concentration of leucine was used [Shigemitsu et al., 1999]. In the present study, neither of these compounds stimulated 4E-BP1 phosphorylation at a concentration of 8 mM in adipocytes. Furthermore, preincubation with either N-acetyl-leucine amide or N-acetyl-leucine methylamide at a concentration of 8 mM did not significantly inhibit the stimulation of 4E-BP1 phosphorylation by 0.8 mM leucine (Fig. 6).

We conclude from the results of the experiments shown in Figures 5 and 6 that if there is a leucine recognition site ($LeuR_A$) responsible for the rapamycin-sensitive phosphorylation of

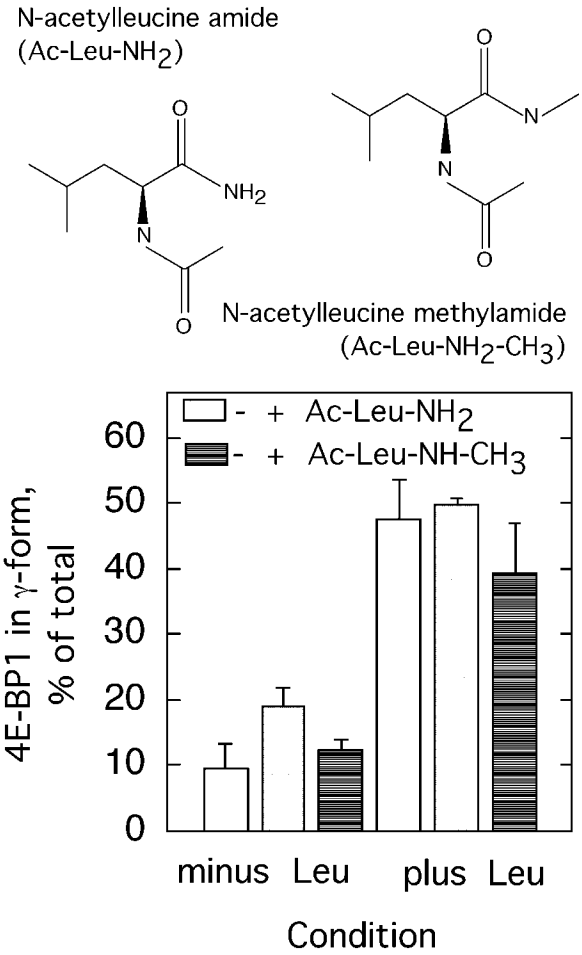

Fig. 6. Effects of N-acetyl-leucine amide and N-acetyl-leucine methylamide on 4E-BP1 phosphorylation. The cells were preincubated in the absence or presence of either 8 mM N-acetyl-leucine amide or N-acetyl-leucine methylamide dissolved in KRH. After 15 min, the buffer beneath the cells was removed and replaced with the same concentration of N-acetyl-leucine amide or N-acetyl-leucine methylamide dissolved in either buffer A (minus Leu) or buffer A containing 0.8 mM leucine (plus Leu). After a 10-min incubation under these conditions, the cells were frozen and processed for quantitation of 4E-BP1 phosphorylation. Bars show the mean and standard error of separate immunoprecipitations from three different aliquots of adipocyte suspension. All of the "plus Leu" conditions were significantly different from the response to buffer A alone at $P < 0.05$.

4E-BP1 and $p70^{S6k}$ in adipocytes, (1) it may require the availability of an intact amino group, and (2) it is pharmacologically distinct from the leucine recognition site that regulates TOR signaling in the H4IIE cell line. Alternatively, since we cannot rule out the possibility that the recognition is intracellular, it is possible that some of the compounds tested have no effect because they are not transported in adipocytes but are in other cells such as H4IIE cells.

Effects of Leucine Analogues Containing R-Group Modifications

We investigated the effects of three leucine analogues with bulky ring structures on 4E-BP1 phosphorylation: gabapentin, cycloleucine, and 2-amino-2-norbornane carboxylic acid (BCH). None had any significant effect at concentrations of up to 8 mM (Fig. 7). Pretreatment with 8 mM BCH did not block the effects of subsequent addition of 0.8 mM leucine.

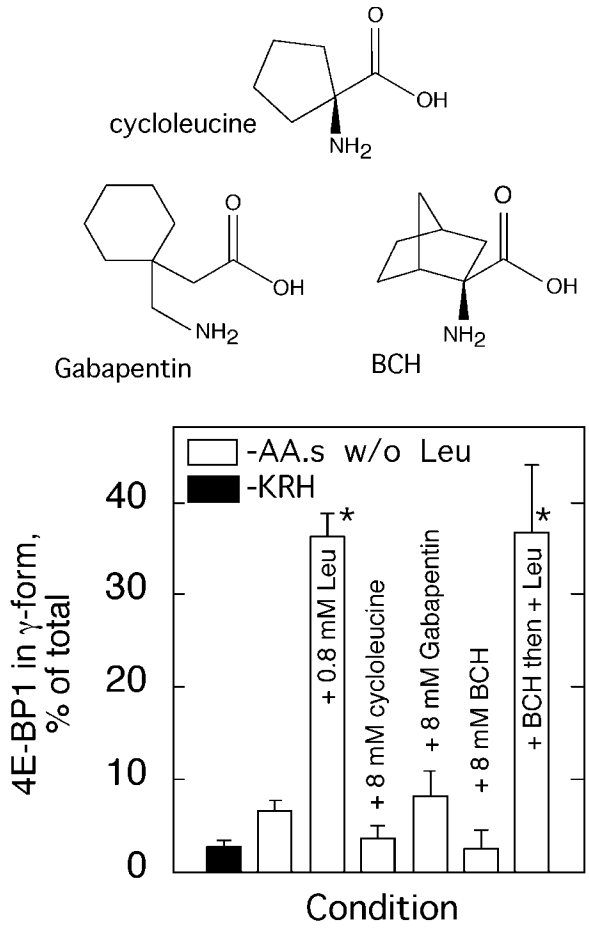

Fig. 7. Effects of leucine analogues in which the branched-chain R-group is replaced with a ring structure. Adipocytes were preincubated for 15 min with KRH containing 8 mM BCH (bar labeled BCH then Leu) or KRH alone (all other bars). The buffer in the BCH preincubated cells was withdrawn and replaced for 10 min with 8 mM BCH and 0.8 mM Leu in buffer A (bar labeled BCH then Leu). The buffer in the cells which had been preincubated in KRH was withdrawn and replaced for 10 min with either KRH (black bar) or buffer A (unlabeled white bar) or buffer A containing one of the indicated additives: 0.8 mM Leu, 8 mM cycloleucine, 8 mM Gabapentin, or 8 mM BCH. Phosphorylation of 4E-BP1 was quantitated in 4E-BP1 immunoprecipitates as described in the legend to Fig. 1. Bars show the mean and standard error of separate immunoprecipitations from three different aliquots of adipocyte suspension. Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

These analogues are of interest because they can be transported via system L, the transport system for carrier-mediated leucine uptake into cells. For example, gabapentin, a centrally acting drug, is transported into the brain on system L [Su et al., 1995]. Its activity as an antiseizure medication may be related to its ability to inhibit a newly discovered leucine transaminase rarely found outside of central neurons [Hutson et al., 1998] or possibly other leucine binding sites [for review see Su et al., 1995]. On the other hand, 2-amino-2-norbornane carboxylic acid (BCH) is widely used to study system L amino acid transporters because it competitively inhibits L-leucine transport [e.g., Su et al., 1995]. We conclude from our findings that the targets of these analogues are probably not involved in the regulation of 4E-BP1 phosphorylation by leucine. The lack of agonist activity may either be due to the presence of a bulky cyclic R-groups, or possibly, due to the absence of the α-hydrogen on these two compounds. Of these two options the former would seem more likely.

In contrast with leucine analogues with a bulky cyclic R-groups, the aliphatic analogue of leucine, norleucine, produced a robust response when added with the leucine-deficient buffer A (Fig. 8). Despite its name, norleucine is often considered more of an analogue of methionine than leucine (cf. structures in Fig. 8). In this regard, norleucine can substitute for methionine in the charging of bacterial initiator tRNA and met tRNA during bacterial protein synthesis [Barker and Bruton, 1979; Kerwar and Weissbach, 1970]. Because of this structural similarity, the effects of norleucine on 4E-BP1 phosphorylation were also compared with the responses to methionine (Fig. 8). Adipocytes were incubated with buffer A containing a much higher concentration of methionine than we have used before (8 mM). At this concentration, 4E-BP1 phosphorylation was significantly stimulated by leucine, norleucine, and methionine (Fig. 8). However, the stimulation in response to methionine was significantly less than that observed with leucine or norleucine. Thus, with respect to 4E-BP1 phosphorylation, norleucine seemed to mimic the response to leucine rather than the response to methionine.

While leucine seems to be responsible for most amino acid effects on 4E-BP1 phosphory-

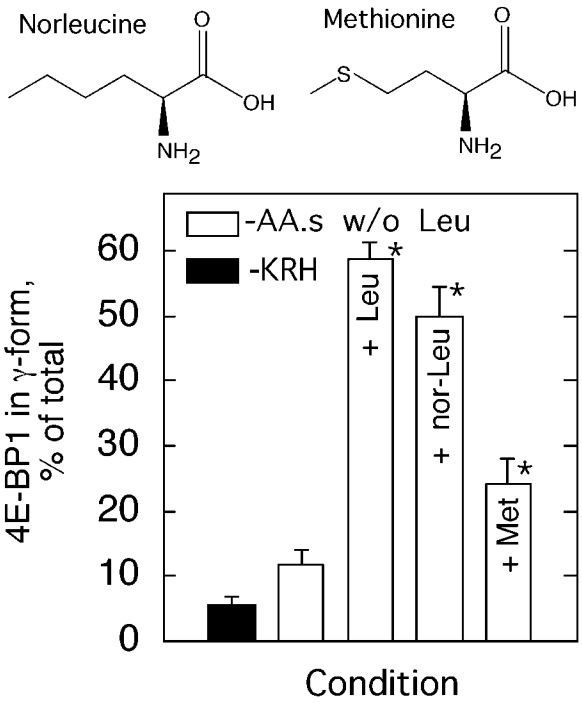

Fig. 8. Effects of norleucine and methionine on 4E-BP1 phosphorylation. The conditions were either buffer A alone (Am. Acids w/o Leu) or buffer A with one of the following additives at a final concentration of 8 mM: Leu (+Leu), norleucine (+nor-Leu) or methionine (+Met). The final concentration of Met and other amino acids in a mixture of plain buffer A and adipocyte suspension is shown in Table I. The effects of additives to the buffer A were evaluated using ANOVA, followed by Dunnett's post-test, in which the effects of the additives were compared with the second bar from the left (AA.s w/o Leu, i.e., buffer A alone). Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

lation, there is a significant difference between the 4E-BP1 phosphorylation state of cells incubated with KRH and those incubated with buffer A (e.g., Figs. 1, 2, 7). We considered the possibility that methionine was responsible for this effect because Shigematzu et al. [1999] reported that methionine was about a third to half as active as leucine at stimulating $p70^{S6k}$ activity in H4IIE cells. While buffer A contains methionine at a concentration that is many times higher than the circulating plasma concentration in rats (Table I), it is not as high as the concentration of methionine used in Figure 8. To address this question, we examined 4E-BP1 phosphorylation in response to buffer A or A lacking any one of the following amino acids: methionine, threonine, isoleucine, or valine (data not shown). No reproducible difference in 4E-BP1 phosphorylation was observed under these different incubation conditions. Thus, none of these amino acids alone seems to be responsible for the small difference in phosphorylation we observed between cells incubated with KRH and the leucine-free KRH. However, it is possible that additive weak effects of several amino acids working at $LeuR_A$ or working through another mechanism that also impacts TOR signaling are responsible for this small effect.

Figure 9A compares the effects of leucine and threo-L-β-hydroxyleuycine on 4E-BP1 phosphorylation. Like leucine, threo-L-β-hydroxyleucine stimulated 4E-BP1 phosphorylation. The response to 8.0 mM threo-L-β-hydroxyleucine ranged from 50% to 66% of the maximal leucine response in different experiments. Another amino acid, threonine, which is structurally related to threo-L-β-hydroxyleucine, had no significant effect on 4E-BP1 phosphorylation at the same concentration (Fig. 9B). The apparent activity of threo-L-β-hydroxyleucine might be secondary to biological dehydroxylation and conversion to leucine. The possible conversion of this compound to leucine in adipocytes was examined (Fig. 9B). Cells were incubated with either threo-L-β-hydroxyleucine or leucine for 10 min and then acid extracts were prepared from the floating cells. Amino acid analysis of these cell extracts indicated that there was no significant conversion of the threo-L-β-hydroxyleucine to the leucine (Fig. 9B). Thus, the effects of threo-L-β-hydroxyleucine on 4E-BP1 phosphorylation are most likely due to activity of the parent compound.

Other leucine analogues with R-group modifications were examined (Fig. 10). Both S- (−)-2-amino-4-pentenoic and 3-amino-4-methylpentanoic acids stimulated 4E-BP1 phosphorylation. While these effects were statistically significant, they were rather small when compared with 0.8 mM leucine. L-tert-leucine, which has the added bulk of an a methyl group on the R group branch, had no significant effect on phosphorylation of 4E-BP1 (e.g., Fig. 10).

It appeared that, while the amino and carboxylic group may be necessary for activation of TOR signaling in adipocytes, there might be some flexibility in the recognition of the R-group. We were surprised because our previous studies found that isoleucine and valine were not active at all [Fox et al., 1998b]. However, only 4–5× concentrations were employed in our previous study. Therefore, we re-examined the ability of these amino acids to alter

phosphorylation of 4E-BP1. In these experiments, we raised the concentration of isoleucine and valine to 8 mM. At the higher concentration, isoleucine and valine did significantly stimulate 4E-BP1 phosphorylation, while glycine had no effect (Fig. 11). Thus, isoleucine and valine can partially substitute for leucine if their concentrations are sufficiently elevated. Isoleucine was consistently more efficacious in this regard than valine.

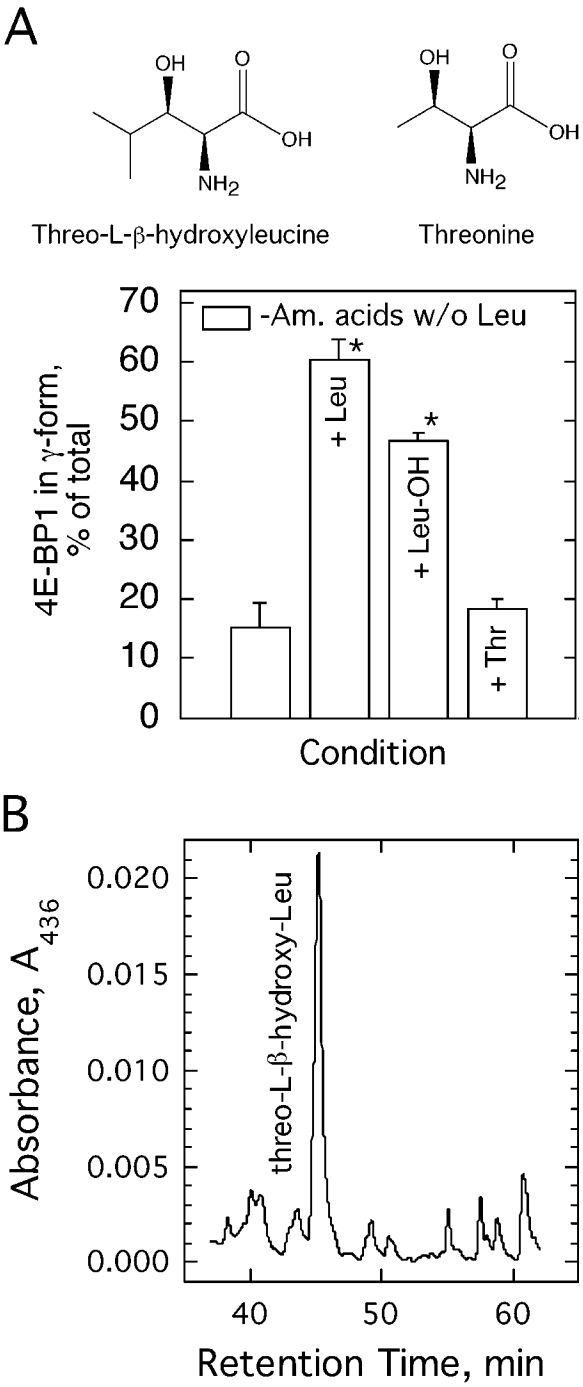

Fig. 9. Effects of threo-L-β-hydroxyleucine (Leu-OH) and threonine on 4E-BP1 phosphorylation. **A:** 4E-BP1 phosphorylation was measured in cells incubated for 10 min in buffer A alone (Am. acids w/o Leu) or with buffer A containing either 8 mM leucine (+Leu), threo-L-β-hydroxyleucine (+Leu-OH) or threonine (+Thr). Bars show the mean and standard error from triplicate determinations from different cell suspensions in a single experiment that is representative of three. Asterisk indicates a response was significantly different from control ($P < 0.05$). **B:** Adipocytes were incubated with either threo-L-β-hydroxyleucine or the same concentration of leucine at 37°C in KRH. After 10 min, the buffer was withdrawn and the cells were TCA precipitated to release free amino acids. The amino acids in the soluble fractions were dabsylated and separated by high-performance liquid chromatography (HPLC), with peak detection at 436 nm. Peaks were identified based on the mobility of standards in separate HPLC runs (not shown). The gray tracing shows the peak tracing from cells incubated with leucine. The one large peak co-migrates with leucine standard and the other smaller peaks represent other background levels of amino acids present in the cells incubated with KRH. The black colored tracing is from extracts of cells incubated with threo-L-β-hydroxyleucine and the one large peak co-migrates with threo-L-β-hydroxyleucine standard. The results are representative of data from two separate cell preparations.

Concentration Dependency of Leucine and Active Leucine Analogues on 4E-BP1 Phosphorylation

We next established the concentration dependency of leucine and active leucine analogues on 4E-BP1 phosphorylation (Fig. 12). Leucine was the most potent with half-maximal concentrations in different experiments typically around 0.4 mM or slightly lower. The half-maximal concentration for norleucine was some six-fold higher (2.3 ± 0.2 mM) while that for isoleucine and threo-L-β-hydroxyleucine was approximately 8 mM. The EC_{50} values for Met and Val were greater than 10 mM. Thus, the order of potency of leucine analogues for this response was as follows: leucine > norleucine > Ile ≃ threo-L-β-hydroxyleucine > Met ≃ Val. Once again, we observed differences between adipocytes and H4IIE cell leucine recognition sites in the regulation of TOR activity.

DISCUSSION

The present study was designed to elucidate some of the structural requirements for agonist activity at a putative leucine recognition site ($LeuR_A$) that regulates TOR activity in adipocytes. Leucine was found to be responsible for most of the TOR activating effects of a mixture of amino acids in adipocytes in agreement with

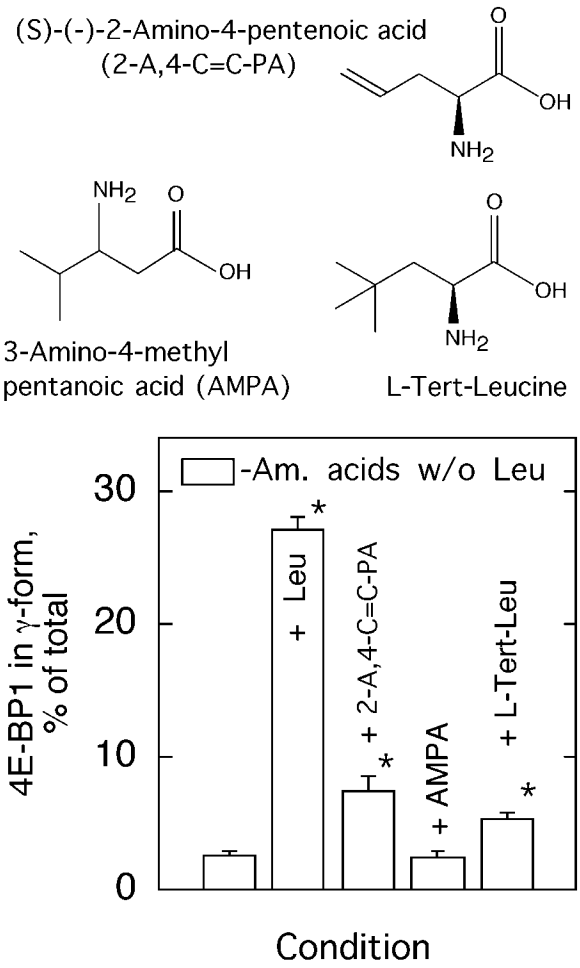

Fig. 10. Activity of leucine analogues containing other R-group modifications. Aliquots of cells were incubated for 10 min in buffer A alone (Am. Acids w/o Leu) or buffer A containing either 0.8 mM Leucine, 8 mM (S)- (−)-2-amino-4-pentenoic acid (2-A, 4-C = C-PA), 8 mM 3-amino-4-methyl pentanoic acid (AMPA), or 8 mM L-tert-leucine. 4E-BP1 phosphorylation was quantitated after 10 min as described in Fig. 1. The results are representative of three separate experiments. Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

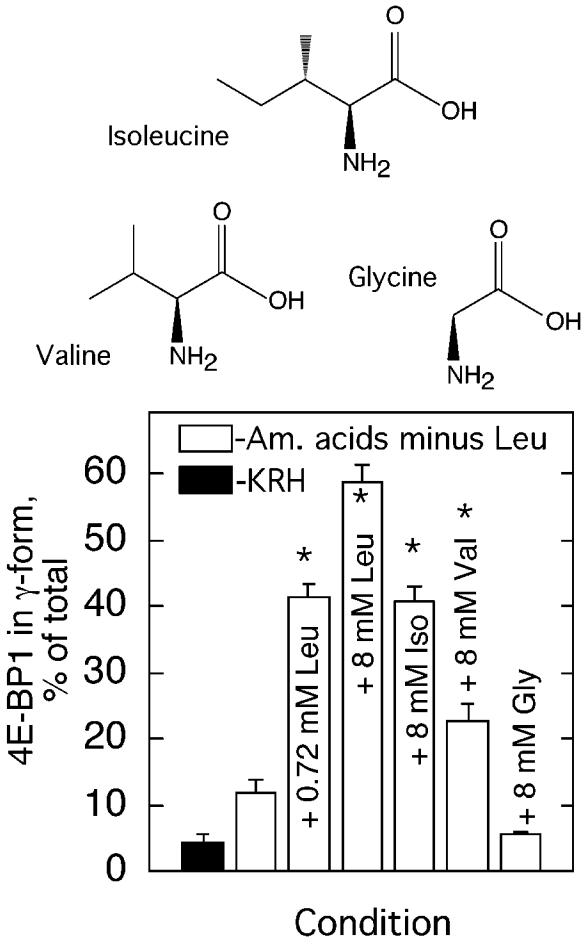

Fig. 11. Effects of other branched-chain amino acids and glycine on 4E-BP1 phosphorylation. Aliquots of cells were incubated for 10 min in buffer A (Am. Acids w/o Leu), with buffer A containing leucine at the indicated concentrations, or with buffer A with either additional isoleucine, valine, or glycine added to the final concentration indicated. The 8-mM final concentration of either isoleucine, valine, and glycine is much higher than the concentration of these amino acids in buffer A (cf. Table I; buffer A is the buffer that has a mixture of all the amino acids except leucine). 4E-BP1 phosphorylation was quantitated after 10 min as described in Fig. 1. The results are representative of three separate experiments. Asterisk indicates that the response was significantly different from the response to buffer A alone at $P < 0.05$.

our previous studies [Fox et al., 1998a,b]. Similar preferences for leucine have been reported for rapamycin-sensitive $p70^{S6k}$ activation in FAO hepatocytes and, more recently, in H4IIE hepatocytes [Shigemitsu et al., 1999]. However, in addition to leucine, non-leucine amino acids such as Arg, Lys, Tyr and others have been reported to fully or partially activate TOR in other cells [Hara et al., 1998; Iiboshi et al., 1999].

These non-leucine amino acids may contribute to the TOR signaling by two possible mechanisms. In mechanism 1, they may interact with their own highly specific receptors. In mechanism 2, they may interact with only one receptor that is somewhat less specific. The less specific receptor may recognize several amino acids, possibly with different K_d values for different amino acids (e.g., leucine may be the most potent amino acid at such a site). Mechanism 1 may exist in CHO-IR and Jurkat cells in which a number of structurally unrelated amino acids appear to be stimulators of $p70^{S6k}$ and 4E-BP1 phosphorylation [Hara et al., 1998; Iiboshi et al., 1999]. In adipocytes, we

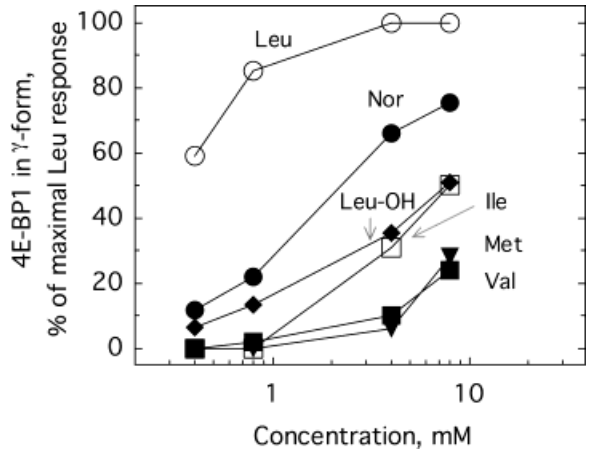

Fig. 12. Concentration dependence of leucine and leucine analogues stimulating 4E-BP1 phosphorylation. 4E-BP1 phosphorylation state was determined as in Fig. 1 after cells were incubated for 10 min in buffer A or in buffer A containing the indicated concentrations of either leucine (○), norleucine (●), threo-L-β-hydroxyleucine (◇), isoleucine (□), methionine (▼), or valine (■) as indicated. The results are computed as the percentage of the maximal leucine response. Symbols show the mean from triplicate determinations from different cell suspensions in a single experiment that is representative of three experiments.

hypothesize that mechanism 2 is present; i.e., only a single amino acid recognition site activates TOR signaling. We have termed this putative recognition site $LeuR_A$. Within the context of this putative mechanism, we speculate that leucine is the primary agonist. At physiologically relevant concentrations other amino acids individually provide a weak stimulation of $LeuR_A$, the exact strength of which is presumably related to each amino acid's structural-similarity to leucine or affinity for $LeuR_A$. For example, structurally, isoleucine, methionine, and valine (which were active at concentrations greater than the EC_{50} for leucine) are more closely related to leucine than, for example, threonine (which was inactive at up to 8 mM). While the action of each of the non-leucine amino acids may have been difficult to observe when they were removed from buffer A, when the action of all of these amino acids was summed together, their combined stimulation of $LeuR_A$ may have significantly added to the actions of leucine. Thus, this putative mechanism could explain why the additive presence of other amino acids in buffer A provided the slight but statistically significant increase in 4E-BP1 phosphorylation observed when comparing the effects of KRH (no amino acids) and buffer A (amino acids without leucine) on 4E-BP1 phosphorylation.

Pharmacological Distinctions Between $LeuR_A$ and Other Leucine Recognition Sites

$LeuR_A$ is pharmacologically distinct from a number of other known leucine binding sites. Blommaart et al. [1995, 1997] proposed that $LeuR_M$ and a leucine recognition site that mediates TOR signaling in hepatocytes may be the same. However, we have shown that two agonists of $LeuR_M$—MAP-Leu8, and O-isovaleryl-L-carnitine chloride—had no significant effect on 4E-BP1 phosphorylation, even at concentrations at which they regulated macroautophagy in liver (Fig. 2). On the basis of this evidence, it seems unlikely that $LeuR_M$ is responsible for leucine effects on 4E-BP1 phosphorylation in adipocytes.

Several lines of pharmacological evidence from this study also clearly distinguish $LeuR_A$ from the site that regulates leucine-stimulated TOR activity in H4IIE hepatocytes. First, H-α-methyl-D/L-leucine was a weak agonist in adipocytes (Fig. 4A) and a full agonist in H4IIE cells [Shigemitsu et al., 1999]. Second, two compounds that were leucine signaling antagonists in H4IIE cells, N-acetyl-leucine amide and N-acetyl-leucine methylamide, failed to block leucine stimulation of 4E-BP1 phosphorylation in adipocytes. Lastly, leucine, isoleucine, valine, and methionine effected different relative responses. In H4IIE cells, 0.24 mM methionine stimulated TOR signaling; however, neither 0.46 mM isoleucine nor 1 mM valine affected TOR signaling [Shigemitsu et al., 1999]. In adipocytes, we showed that isoleucine was more potent than methionine, whereas valine appeared to be equipotent with methionine. This would seem to suggest a different order of potency of leucine-related amino acids in H4IIE cells than in adipocytes.

Another leucine recognition site that was considered as a possible candidate for $LeuR_A$ was leucyl-tRNA synthetase. Ibioshi et al. [1999] have provided evidence that the tRNA charging state or activity of tRNA synthetases may represent a signal for activating TOR in Jurkat cells. However, several lines of pharmacological evidence suggest that $LeuR_A$ is neither leucyl-tRNA nor leucyl-tRNA synthetase. First, the stereospecificity of $LeuR_A$ does not match the stereospecificity expected for leucyl-tRNA or leucyl-tRNA synthetase. For example, the difference in the EC_{50} values for $LeuR_A$

stimulation of 4E-BP1 phosphorylation in response to L- and D-leucine is about 6- to 10-fold [Fox et al., 1998b]. On the other hand, D-amino acids do not appear to be substrates for the tRNA synthetase activity. In fact, they inhibit RNA synthetase-catalyzed ATP-PP_i exchange reaction like amino acid alcohols do [Santi and Webster, 1976]. Thus, if tRNA charging was the signal for L-leucine stimulation of 4E-BP1 phosphorylation in adipocytes, D-leucine would not be expected to possess the agonist activity we observed [Fox et al., 1998b]. Indeed the difference in the Km for L-amino acids and the K_i value for D-amino acid inhibition of the rat tRNA synthetase ATP-PP_i exchange reactions is typically greater than 100-fold [e.g., Santi and Webster, 1976]. A second line of evidence that $LeuR_A$ is not leucyl-tRNA synthetase is the difference between the K_m value for leucyl tRNA synthetase, $\sim$10 μM [Tischler et al., 1982], and the EC_{50} for leucine stimulation of 4E-BP1 phosphorylation in adipocytes, which has ranged from $\sim$350–800 μM in different experiments (Fig. 12, data not shown) [Fox et al., 1998b]. The third line of evidence that $LeuR_A$ is not leucyl-tRNA synthetase comes from experiments with leucinol. Leucinol is a competitive inhibitor of leucyl tRNA synthetase; however, $LeuR_A$ appears to be insensitive to inhibition by L-leucinol (Fig. 3). This is important because in Jurkat cells, 2 mM L-leucinol ablated amino acid stimulation of $p70^{S6k}$ in the presence of amino acid concentrations that were similar to those employed here. In adipocytes, however, we observed no effect of L-leucinol, even when using higher leucinol concentrations and lower leucine concentrations. A fourth line of evidence that $LeuR_A$ is not leucyl-tRNA synthetase comes from the consideration of the relative activity of leucine analogues at stimulating 4E-BP1 phosphorylation. Norleucine, isoleucine, valine, methionine, and threo-L-β-hydroxyleucine stimulated 4E-BP1 phosphorylation when added to the leucine-deficient buffer A. Threo-L-β-hydroxyleucine is capable of charging leucyl tRNA and can be misincorporated into mammalian proteins when replaced for leucine [McAndrew et al., 1991; Schwartz, 1988]. By contrast, while norleucine stimulates protein synthesis in rat skeletal muscle, it has not been reported to misincorporate into leucyl sites in proteins of either prokaryotic or eukaryotic organisms [Schott et al., 1985]. Furthermore isoleucine, valine, or methionine should not charge leucyl tRNA. Thus, by contrast to studies utilizing Jurkat cells, we conclude from the above findings that it is unlikely the either leucyl tRNA or leucyl tRNA synthetase are involved in the mechanism by which amino acids regulate TOR signaling in adipocytes.

Structural Requirements for Activation of the Putative $LeuR_A$

Based on the analysis of a limited number of analogues on 4E-BP1 phosphorylation, we predicted that $LeuR_A$ agonist activity may require leucine analogues with intact carboxyl and amino residues. The requirement of the carboxy residue is suggested from experiments with leucinol, isovaleryl-L-carnitine and MAP-Leu_8. No increase in 4E-BP1 phosphorylation was observed when the carboxyl residue of leucine was altered while preserving the four-carbon branched chain of leucine (i.e., as is the case for leucinol, isovaleryl-L-carnitine and MAP-Leu_8). While it is true that leucine amide was active at stimulating 4E-BP1 phosphorylation, its apparent activity could be secondary to metabolic conversion to leucine. On the other hand, the importance of the amino group of leucine is indicated by the lack of effect of α-hydroxy-isocaproic acid, N-methyl-L-leucine, isovaleryl-L-carnitine, N-acetyl-leucine amide and N-acetyl-leucine methylamide on 4E-BP1 phosphorylation. The apparent agonist activity of α-ketoisocaproic acid, which lacks the amino group of leucine, appears to be the result of metabolic conversion to leucine. Using incubation conditions previously shown to inhibit the leucine transaminase in muscle [Tischler et al., 1982], preincubation of adipocytes with L-cycloserine inhibited the activity of α-ketoisocaproic acid, but not of leucine 4E-BP1 phosphorylation. These findings are consistent with our previous data showing that aminooxyacetic acid, another inhibitor of branched-chained amino acid transaminases, attenuated α-ketoisocaproic acid-stimulated, but not leucine-stimulated, 4E-BP1 phosphorylation [Fox et al., 1998b].

Leucine analogues with small lipophilic modifications of the leucine R group were capable of stimulating 4E-BP1 phosphorylation at concentrations above the EC_{50} value for leucine. The order of potency of such analogues for stimulating phosphorylation of 4E-BP1 in adipocytes was: leucine $<$ norleucine $<$ threo-L-β-hydroxyleucine $\simeq$ Ile $<$ Met $\simeq$ Val. Leucine analogues with bulky ring structures replacing

the R group were inactive, as was the addition of an amino group on the branched chain. Thus, Ile and Val may indeed activate this signaling pathway in adipocytes, but only at higher concentrations. Norleucine and methionine possessed agonist activity. Since these amino acids lack the branched-chain R-group found in leucine, we conclude that the branched chain is not an absolute requirement for agonist activity.

Physiological Relevance of the In Vitro Data

The activity of isoleucine and valine on 4E-BP1 phosphorylation in adipocytes at concentrations greater than 1 mM may be comparable to pancreatic β-cells where higher concentrations were examined by Xu et al. [Xu et al., 1998a]. Although these higher concentrations were not physiological, they nevertheless provided information on the structural basis for amino acid stimulation of TOR. The EC_{50} values we observed, in vitro, may be slightly lower in vivo, because more of the 4E-BP1 was initially in the β-form in vivo (unpublished data). This may be due to the presence, in vivo, of circulating insulin and other growth hormones known to activate TOR. We would therefore predict that the concentration responses curves would be shifted to the left in vivo relative to in vitro. Thus, in vivo changes in circulating Leu and possibly Ile, but probably not Val, may regulate $LeuR_A$ over the range of concentrations found in various nutritional states (0.5–2 times their physiological concentrations). Even if the EC_{50} values in vivo are not left-shifted, the circulating range of concentrations that have been reported for leucine are, nevertheless, within the linear range of the in vitro leucine concentration response curve.

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