Leucine facilitates insulin signaling through a Gαi protein-dependent signaling pathway in hepatocytes

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Key words: Leucine, insulin sensitivity, insulin resistance, Gαi protein, gluconeogenesis.

Background: The direct effect of leucine on insulin signaling and the associated mechanisms have not been established.

Results: Treatment of hepatocytes with leucine facilitates insulin signaling through Gαi1 protein.

Conclusion: Leucine facilitates insulin signaling via a Gαi protein-dependent pathway.

Significance: Help understand how nutrients modulate insulin signaling directly.

SUMMARY

In this study, we addressed the direct effect of leucine on insulin signaling. In investigating the associated mechanisms, we found that leucine itself does not activate the classical Akt-or ERK1/2 MAP kinase-dependent signaling pathways but can facilitate the insulin-induced phosphorylations of Akt473 and ERK1/2 in a time- and dose-dependent manner in cultured hepatocytes. The leucine-facilitated insulin-induced phosphorylation of Akt at residue 473 was not affected by knocking down the key component of mTORC1 or 2 complexes, but was blocked by inhibition of c-Src (PP2), PI3K (LY294002), Gαi protein (pertussis toxin or siRNA against Gαi1 gene, or β-arrestin 2 (siRNA). Similarly, the leucine-facilitated insulin activation of ERK1/2 was also blunted by pertussis toxin. We further show that leucine facilitated the insulin-mediated suppression of glucose production and expression of key gluconeogenic genes in a Gαi1 protein-dependent manner in cultured primary hepatocytes. Together, these results show that leucine can directly facilitate insulin signaling through a Gαi protein-dependent intracellular
signaling pathway. This is the first evidence showing that macronutrients like amino acid leucine can facilitate insulin signaling through G proteins directly.

Increased plasma levels of branched-chain amino acids such as leucine have been shown to be associated with insulin resistance in humans and animals (1-4). Therefore, leucine has been proposed to be a contributor to the development of insulin resistance. This assertion is supported by many studies in animals and cultured cells. For example, infusion of amino acids or branched-chain amino acid leucine can induce insulin resistance (5-9). Exposure of cultured skeletal muscle cells, adipocytes, or hepatocytes to amino acids or leucine can induce insulin resistance (10-12). In contrast, some studies have shown that leucine supplementation does not have an effect on the development of insulin resistance or can even improve insulin sensitivity and prevent the diet-induced obesity (13, 14). Explanations for these conflicting results are lacking and exact mechanism by which leucine modulates insulin sensitivity has not been established.

It has been shown that infusion of leucine can impair glucose uptake in skeletal muscle and adipose tissue (15) likely through stimulating insulin secretion (16, 17) because it is known that excess exposure to insulin can induce insulin resistance (18-20). Theoretically, leucine is capable of modulating insulin signaling through mTOR and S6K-mediated serine phosphorylations of IRS1/2 (3, 21). It is noteworthy that supplementation of leucine can increase insulin sensitivity in obese animals (13, 14), and the increased level of branched-chain amino acids by knocking out the rate-limiting enzyme that catabolizes branched-chain amino acids is associated with increased instead of decreased insulin sensitivity (22). The increased insulin sensitivity by branched-chain amino acids can be at least partially explained by decreased food intake and elevated energy expenditure (13, 14, 22). Similar to leptin, leucine is capable of activating mTOR that is known to be involved in inhibition of appetite (23). Moreover, leucine and isoleucine can actually stimulate glucose uptake in skeletal muscle (24-26), and leucine can facilitate the insulin-mediated phosphorylation of Akt in a PI3K-independent pathway in adipocytes (27). The direct effect of leucine on hepatic insulin signaling and the associated mechanism are currently unknown. In this study, we addressed the direct effect of leucine on insulin signaling and associated mechanisms.

EXPERIMENTAL PROCEDURES

Reagents: Leucine, human insulin, N\(^6\),2'-\(O\)-dibutyryladenosine 3'-5'-cyclic monophosphate sodium (cAMP), dexamethasome (Dex) and G\(\alpha\)i protein inhibitor pertussis toxin (PT) were from Sigma (St.Louis, MO). The c-Src tyrosine kinases inhibitor PP2 and the PI3 K inhibitor LY294002 were purchased from Calbiochem (San Diego, CA). The siRNA against rictor and related scrambled siRNA were from Sigma. The siRNAs against G\(\alpha\)i1, G\(\alpha\)i2, G\(\alpha\)i3, raptor, \(\beta\)-arrestin 2 were from IDT (Coralville, Iowa). Lipofectamine\(^\text{TM}\) RNAi-MAX transfection reagents were from Invitrogen. Antibodies against total/phospho- Akt (serine 473), and Rictor were purchased from Cell Signaling Technology (Danvers, MA). Antibody against G\(\alpha\)i1/2/3 was from Santa Cruz. Antibodies against G\(\alpha\)i2 and G\(\alpha\)i3 were from Novus (Littleton, CO) and GeneTex (Irvine, CA), respectively. Other materials were all obtained commercially and are of analytical quality.

Cell culture: Hepa1c1c7 and C2C12 myocyte cell lines were obtained from The American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 \(\mu\)g/ml) (Invitrogen, Paisley, UK) at 37°C in a 5% \(CO_2\) humidified incubator at 2x10\(^5\) cells/ml density. Cells were split every two days.

Isolation and culture of primary hepatocytes: Primary hepatocytes were isolated from un-fasted C57BL/6 male mice (8–10 weeks old, Charles River, NC) as previously described (28-37). Mice were fed ND ad libitum. In brief,
under anesthesia with pentobarbital (intraperitoneal, 50 mg/kg body weight), isolated liver was perfused with Hanks’ balanced solution containing 0.5 mM EGTA, 0.004 N NaOH, 10 mM HEPES (Invitrogen) at 5 ml/min for 8 min, followed by continuous perfusion with the serum-free Williams’ Medium E containing collagenase (Worthington, type II, 50 units/ml) and 10 mM HEPES for 12 min. Hepatocytes were harvested and purified with Percoll (Sigma). The viability of hepatocytes was examined with trypan blue exclusion. Only cells with viability over 95% were used. Hepatocytes were inoculated into collagen coated-plates (5×10^5 cells/well in 6-well plates and 1.25×10^5 cell per well in 24-well plates) in Williams’ Medium E with 10% fetal bovine serum, and were incubated for 24 h before experimentation.

**Measurement of glucose production:** Glucose production was assayed as described before (37). Briefly, hepatocytes were pre-treated with 1ug/ml pertussis toxin (PT) for 30 min or Gαi1 for 36 h, then treated with 0.6 mM leucine and/or 10 nM insulin for 30 min in the presence or absence of PT, followed by stimulation with cAMP/dexamethasone (10 µM/50 nM) in glucose-free DMEM containing 2 mM sodium lactate for 2.5 hour in the continuous presence or absence of insulin, leucine and PT. The culture media were subsequently collected for measuring glucose and cells were lysed with the lysis buffer for protein determination using the Pierce BCA protein assay kit (Thermo Scientific). Glucose concentrations were determined with an YSI glucose analyzer (YSI, Yellow Springs, OH) and normalized to protein concentrations.

**Quantitative real-time polymerase chain reaction (PCR):** Total RNA was extracted from liver samples with Trizol (Invitrogen), and reverse transcribed into cDNA by using a high capacity cDNA archive kit (Applied Biosciences). The cDNAs were quantified by using a SYBR green-based qRT-PCR kit (Applied Biosciences) and specific oligo primers. Following gene-specific primers were used for mRNA quantification in this study: G-6-Pase (mouse), 5’-GTGGCTGGAGTCTTGTCAAGG-3’ (forward), 5’-ATTGTAGATGCCCCGATG-3’ (reverse); PEPCK (mouse), 5’-ATGGTTGGCGATGACA TT-3’ (forward), 5’-AACCCGTTTTCTGGGTTG AT -3’ (reverse); GAPDH (mouse), 5’-AGCTT GTCATCAACGGGAAG-3’ (forward), 5’-TTTG ATGTTAGTGGGTCCTCG-3’.

**Immunoblotting:** Hepatocytes were lysed in lysis buffer (20mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 2 µg/ml leupeptin, and 10µg/ml aprotinin), supplemented with 1 mM phenylmethylsulfonyl fluoride before use. Lysates (50µg/lane) were resolved in 4-12% Tris glycerine gels (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). The presence of proteins was detected by immunoblotting with primary antibodies as indicated and alkaline phosphatase-conjugated secondary antisera. Fluorescent bands were visualized with a Molecular Imager VersaDoc MP 4000 System (Bio-Rad), and then quantified by densitometry analysis using QuantityOne (Bio-Rad).

**Gene Silencing with siRNA:** The siRNA against Rictor or scrambled siRNA was introduced into hepa1c1c7 hepatocytes by reverse transfection with Lipofectamine™ RNAiMAX transfection reagents with the standard approach. In brief, the siRNA transfection mixture was applied to 6 well-plates right before plating hepa1c1c7 hepatocytes in complete DMEM without antibiotics. The siRNAs against Gαi1, Gαi2, Gαi3 or scrambled siRNA was introduced into primary mouse hepatocytes by forward transfection with Lipofectamine™ RNAiMAX transfection reagents with the standard approach. In brief, the siRNA transfection mixture was added to 6 well-plates the day after plating primary hepatocytes in complete Williams’ Medium E without antibiotics. After 36 h, cells were treated with leucine or/and insulin as noted.
Statistical Analysis: Data are presented as mean ± SE of at least three independent experiments. Data were analyzed with one-way ANOVA, followed by LSD or Dunnett’s. The SPSS 15.0 software package was used for all statistical analyses. Differences at values of p<0.05 were considered significant.

RESULTS

Leucine facilitates the insulin-induced phosphorylation of Akt in a dose- and time-dependent manner. To investigate the direct effect of amino acid leucine on insulin signaling, hepatic hepatocytes were treated with increasing amount of leucine in the presence or absence of insulin for 30 min, followed by evaluating phosphorylation of Akt at residue 473. As shown in Fig. 1A, leucine alone did not stimulate phosphorylation of Akt at residue 473 while insulin did. Addition of leucine enhanced the insulin-mediated phosphorylation of Akt at residue 473 in a dose-dependent manner and 0.3 mM of leucine showed an obvious effect while 0.6 mM of leucine induced a maximal level of effect. (Note: The concentration of leucine in the culture media used in this study was 157 µM; and the plasma level of leucine in normal subjects ranges between 132.5-176.6 µM but between 150.2-234.5 µM in obese subjects) (4, 38). To determine the optimized time point of leucine effect, hepatic cells were treated with 0.6 mM of leucine with or without insulin for different amount of time (10-60 min), followed by evaluating phosphorylation of Akt. As shown in Fig. 1B, leucine facilitated the insulin-mediated phosphorylation of Akt at residue 473 at 30 min clearly, but not at 10 min, 20 min, or 60 min. Similarly, leucine facilitated the insulin-induced phosphorylation of Akt at residue 308 (Fig. 1C). The facilitating effect of leucine in insulin-mediated Akt phosphorylation was also observed in differentiated C2C12 myocytes (Fig. 2D). Together, these results show that leucine can facilitate the insulin-mediated phosphorylation of Akt in a dose- and time-dependent manner.

Leucine facilitates the insulin-induced phosphorylation of ERK1/2 in a dose- and time-dependent manner. To investigate the direct effect of amino acid leucine on the other main branch of insulin signaling, hepatic hepatocytes were treated with increasing amount of leucine in the presence or absence of insulin for 10 min, followed by evaluating phosphorylation of ERK1/2. As shown in Fig. 2A, leucine alone did not stimulate phosphorylation of ERK1/2 while insulin did clearly. Addition of 0.3 mM leucine appeared to enhance the insulin-mediated phosphorylation of ERK1/2 but did not reach a statistical significance until 0.6 mM. To determine the optimized time point of leucine effect, hepatic cells were treated with 0.6 mM of leucine with or without insulin for different amount of time (10-60 min), followed by evaluating phosphorylation of ERK1/2. As shown in Fig. 2B, leucine facilitated the insulin-mediated phosphorylation of ERK1/2 at 10 min clearly and faded gradually. Together, these results show that leucine can facilitate the insulin-mediated phosphorylation of ERK1/2 in a dose- and time-dependent manner.

The leucine-facilitated insulin-induced phosphorylation of Akt473 is mTORC-independent. It has been shown that phosphorylation of Akt at residue 473 requires mTORC2 (39). So we went to determine whether mTORC2 activation was required for the leucine-facilitated and insulin-induced phosphorylation of Akt at residue 473 by knocking down the core component of mTORC2, Rictor, with specific siRNA. As shown in Fig. 3A, leucine alone, insulin alone, or leucine plus insulin all stimulated phosphorylation of Akt. The siRNA knocked down Rictor efficiently, but had no effect on the leucine- and insulin-induced phosphorylation of Akt at residue 473 (Fig. 3B). Similarly, knockdown of the core component of mTORC1, Raptor, had no effect on leucine- and insulin-induced phosphorylation of Akt (Fig. 3C). Together, these results demonstrate that leucine facilitates the insulin-mediated phosphorylation of
Akt at residue 473 through a pathway independent of mTORC complexes.

**Leucine facilitates insulin signaling through a Gαi1-dependent pathway.** Since phosphorylation of Akt at residue 473 induced by leucine and insulin was not mediated by mTORC as shown in Fig. 3, we examined the signaling pathways upstream of Akt by using inhibition of c-Src, PI3K, or Gα proteins. As shown in Fig. 4A, phosphorylation of Akt at residue 473 induced by insulin alone was prevented by PI3K inhibitor LY294002 (LY) but not by c-Src inhibitor PP2 or Gα protein inhibitor pertussis toxin (PT). However, phosphorylation of Akt at residue 473 facilitated by leucine in the presence of insulin was prevented by either PI3K inhibitor LY or pertussis toxin (Fig. 4B). Leucine or insulin alone stimulated phosphorylation of ERK1/2 and leucine plus insulin together further enhanced phosphorylation of ERK1/2 (Fig. 4B). Inhibitors against c-Src (PP2), PI3K (LY), or Gα1 (PT) all appeared to decrease phosphorylation of ERK1/2 induced by leucine plus insulin (Fig. 4B). To determine the isoform of Gαi proteins involved in the leucine effect, we used siRNA to knock down each of the 3 known Gαi proteins. As shown in Fig. 4C, the siRNA against each isoform decreased the level of the target protein significantly, but only the knockdown of Gαi1 prevented the facilitating effect of leucine in phosphorylation of Akt. Together, these results show that leucine facilitates insulin signaling through Gαi1 protein.

**Leucine facilitation of insulin-induced phosphorylation of Akt requires β-arrestin 2.** To further investigate the mechanism by which leucine facilitates insulin signaling, the involvement of a Gαi protein scaffold protein, β-arrestin 2, was examined. It has previously been shown that β-arrestin 2 as a scaffold of Akt and Src plays an important role in insulin-induced Akt activation (40), but it is unclear whether β-arrestin is involved in the leucine facilitation of insulin signaling. As shown in Fig. 5, leucine facilitated the insulin-mediated phosphorylation of Akt. The effects of leucine and insulin on phosphorylation of Akt were all gone when the β-arrestin 2 gene was knocked down with siRNA. The control siRNA had no effect. Thus, these results show that leucine facilitation of insulin signaling in phosphorylation of Akt involves G protein scaffold protein β-arrestin.

**Leucine facilitates insulin inhibition of glucose production through a Gαi1 protein-dependent signaling pathway.** To further define the mechanism by which leucine facilitated insulin signaling, the role for Gαi protein in leucine facilitation of insulin-mediated inhibition of glucose production was examined in cultured primary mouse hepatocytes. As shown in Fig. 6A, glucose production was stimulated by cAMP and dexamethasone, but the stimulation was significantly inhibited by insulin alone, negligibly reduced by leucine alone, and completely prevented by the combination of insulin and leucine. The combined inhibitory effect of insulin and leucine was also partly blocked by using either pertussis toxin (Fig. 6A) or Gαi1 gene silencing but not by siRNA against Gαi2 or 3 (Fig. 6B). Similarly, the leucine- facilitated inhibition of gluconeogenic gene (G-6-pase and PEPCK) expression was also partially reversed by inhibition of Gαi with PT or siRNA against Gαi1 (Fig. 7). Together, these results further support that leucine facilitates insulin signaling via a Gαi1 protein-dependent signaling pathway.

**DISCUSSION**

Reports about the regulatory effect of leucine on insulin sensitivity are currently conflicting and the associated mechanisms have not been fully established. In this study, we investigated the direct effect of leucine on insulin signaling and associated mechanisms in cultured hepatocytes. We made some important findings.

Leucine facilitates insulin signaling through a Gαi1 protein-dependent signaling pathways. Leucine has been shown by many to impair insulin signaling (10-12). The presumed mechanism is associated with activations of
mTOR/S6K and consequent serine phosphorylations of IRS1/2 (10, 21). Nevertheless, elevated plasma levels of branched-chain amino acids including leucine by knocking out the rate-limiting enzyme of the catabolic pathway of branched-chain amino acids is linked to increased insulin sensitivity (22). Others have shown that branched-chain amino acids including leucine and isoleucine promote glucose uptake in skeletal muscle cells (24-26). Leucine has also been shown to facilitate insulin-induced phosphorylation of Akt via a PI3K-independent manner in adipocytes (27, 39, 41). It is noteworthy that activation of mTOR may be able to promote phosphorylation of Akt at residue 473 and consequently facilitate phosphorylation of Akt at residue 308 (39). The full activation of Akt requires phosphorylations of both 308 and 473 residues (39). The mTOR-promoted activation of Akt473 has been shown to be mediated by mTORC2 (39). In this study, we found that the leucine-facilitated insulin-induced phosphorylation of Akt at residue 473 was not affected by knocking down the core components of mTORC1 or 2, Rictor or Raptor, suggesting that the leucine facilitated insulin signaling is not mediated by mTOR. Moreover, our results clearly show that the leucine-facilitated insulin-induced phosphorylation of Akt at residue 473 was c-Src and PI3K-dependent. These results together implicate that the effect of leucine on insulin signaling comes from somewhere upstream of mTOR and Akt. Interestingly, we observed that leucine facilitation of insulin-mediated phosphorylation of Akt was prevented by the Gαi protein inhibitor, pertussis toxin or siRNA against Gαi protein 1 or β-arrestin, but not by siRNA against Gαi2 or 3. It is noteworthy that it has previously been shown that amino acids such as leucine may be able to trigger intracellular signaling through amino acid transporters, small G proteins, or currently unidentified membrane receptor(s) (42-44). However, the involvement of G protein in the leucine-mediated intracellular signaling has not been reported. Furthermore, our results clearly demonstrate that treatment of cultured hepatocytes with leucine further increases insulin suppression of glucose production and expression of key gluconeogenic genes. The leucine-facilitated insulin suppression of glucose production and gluconeogenesis was blunted by inhibition of Gαi protein. Finally, our results show that leucine can also facilitate insulin-induced phosphorylation of ERK1/2. Therefore, we have found a brand new signaling pathway by which leucine facilitates insulin signaling.

In summary, the findings in this study will not only help explain how macronutrients like leucine directly regulate insulin signaling and metabolism but also help explain why results are sometimes conflicting among different studies.
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FIGURE LEGENDS

Figure 1. Leucine facilitates insulin in stimulating phosphorylation of Akt. (A) Hepa1c1c7 cells were incubated with increasing amount of leucine in the presence or absence of insulin (10 nM) for 30 min, followed by evaluations and quantifications of total and phosphorylated Akt by immunoblotting. (B) Hepa1c1c7 cells were treated with insulin (10 nM), leucine (0.6 mM), or both together as noted for a different amount of time, followed by evaluations of total and phosphorylated Akt and quantification by immunoblotting. (C) Hepa1c1c7 cells were similarly treated as described in (B), phosphorylation of Akt at residue 308 was detected by using immunoblotting. (D) C2C12 myocytes were differentiated and then similarly treated as described in (B), phosphorylated and total Akt were detected by using immunoblotting. Results were presented as the mean ± SE of 4 independent experiments, each in duplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p < 0.05). **: P < 0.01 vs. control.

Figure 2. Leucine facilitates insulin in stimulating phosphorylation of ERK1/2 MAP kinase. (A) Hepa1c1c7 cells were incubated with increasing amount of leucine in the presence or absence of insulin (10 nM) for 10 min, followed by evaluations and quantifications of total and phosphorylated ERK1/2 by immunoblotting. (B) Hepa1c1c7 cells were treated with insulin (10 nM), leucine (0.6 mM), or both together as noted for a different amount of time, followed by evaluations of total and phosphorylated ERK1/2 and quantification by immunoblotting. Results were presented as the mean ± SE of 4 independent experiments, each in duplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p < 0.05).

Figure 3. Leucine facilitates insulin signaling through a pathway independent of mTORC2. Hepa1c1c7 hepatocytes were treated with leucine, insulin, or insulin plus leucine for 30 min, followed by evaluations of phosphorylated mTOR, total mTOR, and β-actin by immunoblotting and then quantified. (B) Hepa1c1c7 cell were transfected with siRNAs against Rictor or scrambled siRNA for 36 h, and were then treated with leucine or insulin alone or insulin plus leucine for 30 min, followed by evaluations of phosphorylated Akt (at serine 473), total Akt, total Rictor, and β-actin by using immunoblottings with specific antibodies. (C) Hepa1c1c7 cell were transfected with siRNAs against Raptor or scrambled siRNA for 36 h, and were then treated with leucine (0.6 mM) or insulin (10 nM) alone or insulin plus leucine for 30min, followed by evaluations of phosphorylated Akt (at serine 473), total Akt, total Raptor, and β-actin by using immunoblottings. Results were presented as the mean ± SE of 4 independent experiments, each in duplicate. Bars not sharing a letter are statistically different (p <0.05).

Figure 4. Leucine facilitates insulin signaling through Gαi1 protein. (A) Hepa1c1c7 hepatocytes were treated with insulin (10 nM) in the presence of PP2 (100 nM), LY294002 (LY, 10 µM), or pertussis toxin (PT, 1 µg/ml) for 30 min as noted, followed by evaluations of phosphorylated and total Akt using immunoblotting and then quantification. (B) Hepa1c1c7 hepatocytes were treated with leucine (0.6 mM) or insulin (10 nM) alone or leucine plus insulin together in the presence of PP2 (100 nM), LY294002 (LY, 10 µM), or pertussis toxin (PT, 1 µg/ml) for 30 min as noted, followed by evaluations of phosphorylated and total Akt or ERK1/2 using immunoblotting and then quantification. (C) Hepa1c1c7 hepatocytes were first transfected with siRNA against Gαi1, 2, or 3, or scrambled siRNA for 36 h, and then treated with leucine (0.6 mM) or insulin (10 nM) alone or leucine plus
insulin together for 30 min as noted, followed by evaluations of phosphorylated and total Akt, each Gαi isoform protein, and β-actin using immunoblotting and then quantification. Results were presented as the mean ± SE of 4 independent experiments, each in duplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p <0.05).

Figure 5. Leucine facilitation of insulin-mediated phosphorylation of Akt requires β-arrestin 2. Hepa1c1c7 hepatocytes were first transfected with siRNA against β-arrestin 2 or scrambled siRNA for 36 h, and then treated with leucine (0.6 mM) or insulin (10 nM) alone or leucine plus insulin together for 30 min as noted, followed by evaluations of phosphorylated and total Akt, β-arrestin 2, and β-actin using immunoblotting and then quantification. Results were presented as the mean ± SE of 3 independent experiments, each in duplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p <0.05).

Figure 6. Leucine facilitates insulin suppression of hepatic glucose production through a Gαi1 protein-dependent signaling pathway. Primary hepatocytes were isolated from mouse and cultured as detailed in “Experimental Procedures”. (A) Cells were pretreated with PT (1 μg/ml) for 30 min, then with leucine or insulin alone or leucine plus insulin as noted for another 30 min before the stimulation with cAMP (10 μM) and dexamethasone (Dex, 50 nM) in the continuous presence of leucine (0.6 mM), insulin (10 nM) and PT (1 μg/ml) as noted in the serum- and glucose-free DMEM supplemented with gluconeogenic substrate sodium lactate (2 mM) for 2.5 h. Glucose production was determined, calculated, and normalized to protein concentrations as detailed in “Materials and Methods.” (B) Primary hepatocytes were transfected with siRNA against Gαi1, Gαi2, Gαi3 or a scrambled siRNA for 36 h, and treated with leucine, insulin, or leucine plus insulin as indicated for 30 min before the stimulation with cAMP (10 μM) and dexamethasone (Dex, 50 nM) in the continuous presence of leucine (0.6 mM) and insulin (10 nM) as noted in the serum- and glucose-free DMEM supplemented with gluconeogenic substrate sodium lactate (2 mM) for 2.5 h. Glucose production was determined, calculated, and normalized to protein concentrations as detailed in “Materials and Methods.” Results were presented the mean ± SE of 3 independent experiments, each in triplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p <0.05).

Figure 7. Leucine facilitates insulin suppression of gluconeogenic gene expression through a Gαi protein-dependent signaling pathway. Primary hepatocytes were isolated from mouse and cultured as detailed in “Experimental Procedures”. Cells were pretreated with PT (1 μg/ml) for 30 min (A-B) or siRNA against Gαi1, Gαi2, Gαi3 or a scrambled siRNA for 36 h (C-D), then with leucine or insulin alone or leucine plus insulin as noted for another 30 min before the stimulation with cAMP (10 μM) and dexamethasone (Dex, 50 nM) in the continuous presence of leucine (0.6 mM) and insulin (10 nM) and PT (1 μg/ml) as noted for 2.5 h, followed by measurements of mRNA levels of glucose-6-phosphatase (G-6-P) and phosphoenolpyruvate carboxykinase (PEPCK) genes using real time RT-PCR. Results were presented the mean ± SE of 4 independent experiments, each in triplicate. Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different (p <0.05).
Fig. 1

A. P-Akt⁴⁷³/T-Akt

B. 10 min 20 min 30 min 60 min

P-Akt⁴⁷³/T-Akt

C. p-Akt³⁰⁸/T-Akt

D. P-Akt⁴⁷³/T-Akt

Leucine (mM) 0 0.3 0.6 1.2 2.4

Insulin - - - - + + + + + +

Leucine (0.6 mM) - - + + - - + + - + +

Leucine   0    0.3   0.6   1.2   2.4    0    0.3    0.6  1.2   2.4

Insulin   -      -       -      -       -      +      +     +      +      +

Leucine   -     +     -     +     +     -     +    +     -     +     +     -     +

Insulin   -     -      +    +     -      +    +     -     +    +     -      +    +

P-Akt⁴⁷³/T-Akt

0.0

0.5

1.0

0.0

0.5

1.0

Leucine (mM)

Insulin

β-actin

P<0.05

**
### Table 1

| Leucine (mM) | 0  | 0.3 | 0.6 | 1.2 | 2.4 |
|--------------|----|-----|-----|-----|-----|
| Insulin (10 nM) | -  | -   | -   | -   | +   |
|               | -  | +   | +   | +   | +   |

### Bar Graph 1

- **x-axis**: Leucine (mM)
- **y-axis**: P-ERY1/2/T-ERY1/2
- **Legend**: Different conditions are indicated with different symbols and letters.

### Bar Graph 2

- **x-axis**: Leucine (0.6 mM)
- **y-axis**: P-ERY1/2/T-ERY1/2
- **Legend**: Different conditions are indicated with different symbols and letters.

### Figure 2

- **A.** Western blot analysis of P-ERK1/2 and T-ERK1/2.
- **B.** Graph showing the percentage of P-ERK1/2/T-ERK1/2 activity over time (10 min, 20 min, 30 min, 60 min).

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**Note:** All data points are accompanied by error bars indicating standard deviation.
A. $\text{P-mTOR}^{2448}/\text{T-mTOR}$

B. $\text{P-Akt}^{473}/\text{Akt}$

C. $\text{p-Akt}^{473}/\text{T-Akt}$

Leucine

Insulin

Scr siRNA

Rictor siRNA

Fig. 3
### A.

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
|              | c               | a    |
| Insulin -    |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| PP2 -        |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| LY -         |                 | -    |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| PT -         |                 | -    |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |

### B.

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Leucine -    |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Insulin -    |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| PP2 -        |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| LY -         |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| PT -         |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

### C.

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Leucine -    |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Insulin -    |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Scrambled siRNA |                 | -    |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |
| +            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Gai1 siRNA    |                 | -    |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Gai2 siRNA    |                 | -    |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |

| Treatment     | P-Akt\(^{473}/\text{Akt}\) | T-Akt |
|---------------|-----------------|------|
| Gai3 siRNA    |                 | -    |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |
| -            |                 |      |

### Fig. 4

A. Bar graph showing the effect of different treatments on P-Akt\(^{473}/\text{Akt}\) and T-Akt. Significant differences are indicated by different letters.

B. Bar graph showing the effect of different treatments on P-Akt\(^{473}/\text{Akt}\) and T-Akt. Significant differences are indicated by different letters.

C. Western blots showing the expression levels of P-Akt\(^{473}\), T-Akt, Gai1, Gai2, Gai3, and \(\beta\)-actin. Significant differences are indicated by different letters.
Fig. 5

- Insulin
- Leucine
- Scr siRNA
- β-arrestin siRNA

β-arrestin 2
β-actin

p-Akt^{473}
T-Akt

P-Akt/T-Akt

Leucine
- + - + + +

Insulin
- - + + + +

Scr siRNA
- - - - + -

β-arrestin siRNA
- - - - - +

Bar graph showing the relative expression levels of p-Akt and T-Akt.

Significance markers: a, b, c, bc
A. Glucose production (mg/g protein)

B. Glucose production (mg/g protein)
Leucine facilitates insulin signaling through a Gαi protein-dependent signaling pathway in hepatocytes

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