Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes

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Roh, Cecilia, Jianrong Han, Alexandros Tzatsos, and Konstantin V. Kandror. Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes. Am J Physiol Endocrinol Metab 284: E322–E330, 2003. First published October 1, 2002; 10.1152/ajpendo.00230.2002.—Leptin biosynthesis in adipose cells in vivo is increased by food intake and decreased by food deprivation. However, the mechanism that couples leptin production to food intake remains unknown. We found that addition of leucine to isolated rat adipocytes significantly increased leptin production by these cells, suggesting that postprandial leptin levels may be directly regulated by dietary leucine. The effect of leucine was inhibited by rapamycin and not by actinomycin D. Besides, leucine administration did not increase the amount of leptin mRNA in adipocytes. Therefore, we concluded that leucine activates leptin expression in adipose cells at the level of translation via a mammalian target of rapamycin (mTOR)-mediated pathway. Because leptin is a secreted protein, its biosynthesis is compartmentalized on the endoplasmic reticulum. To analyze mTOR signaling in this subcellular fraction, we separated adipose cells by centrifugation into a heavy membrane fraction that includes virtually all endoplasmic reticulum and the cytosolic extract. Phosphorylation of the major mTOR targets, the ribosomal protein S6 and the translational inhibitor 4E-binding protein (BP)/phosphorylated heat- and acid-stable protein (PHAS)-1, was stimulated by leucine in the cytosolic extract, whereas, in the heavy fraction, S6 was constitutively phosphorylated and leucine only induced phosphorylation of 4E-BP/PHAS-1. We also found that 60–70% of leptin mRNA was stably associated with the heavy fraction, and leucine administration did not change the ratio between compartmentalized and free cytoplasmic leptin mRNA. We suggest that, in adipose cells, a predominant part of leptin mRNA is compartmentalized on the endoplasmic reticulum, and leucine activates translation of these messages via the mTOR/4E-BP/PHAS-1-mediated signaling pathway.

mammalian target of rapamycin

LEPTIN IS PRODUCED mainly by adipose cells and regulates food intake and whole body energy balance (36). Pursuant to this physiological role, circulating leptin levels rapidly increase after feeding (20) and decrease after food deprivation (9). Because leptin mRNA levels in adipose tissue also follow this pattern (3, 34), it has been generally accepted that leptin expression is controlled at the level of transcription (1). Although this may well be the case, the mechanism that couples food intake, or lack thereof, to leptin production in adipocytes has not yet been elucidated.

Because insulin levels respond to the nutritional status of the body, insulin itself has been suggested as a potential mediator between food intake and leptin production. Indeed, insulin increases leptin production both in vitro and in vivo (summarized in Ref. 1). However, insulin action in isolated rat adipocytes is resistant to actinomycin D, and insulin administration does not change the amount of leptin mRNA in vitro (Ref. 4 and C. Roh and K. V. Kandror, unpublished observation). This suggests that insulin may not regulate or may not exclusively regulate the ob gene expression at the level of transcription.

Alternately, leptin expression may also be regulated at a posttranscriptional level. Mammalian cells possess an important nutrient-sensing pathway that controls protein synthesis at the level of translation. A central player in this pathway is a phosphatidylinositol kinase-related protein kinase called target of rapamycin (mTOR; see Refs. 27, 32, 35). mTOR is activated by free amino acids (13, 32), especially by leucine (23) via a mechanism that is yet unknown. mTOR stimulates translation of stored mRNAs through S6- and/or phosphorylated heat- and acid-stable protein (PHAS/4E-binding protein (BP))-mediated pathways (27, 32, 35). Both pathways are readily activated by leucine in adipocytes (Refs. 7 and 8 and Fig. 3). We proposed that mTOR may be an appropriate nutrient sensor for leptin expression in adipose cells.

In agreement with this hypothesis, we found that addition of leucine to isolated rat adipocytes significantly stimulated leptin secretion in a rapamycin-sensitive and an actinomycin D-resistant fashion. Thus dietary leucine may increase leptin production via activation of mTOR and subsequent activation of leptin mRNA translation. This mechanism may provide a long-sought-after connection between food intake and leptin levels in blood.

MATERIALS AND METHODS

Antibodies. Affinity-purified polyclonal antibodies against phosphorylated S6 (Ser235/236), p70 S6 kinase (Thr389), and 4E-BP-1/PHAS-I (Ser45) were from Cell Signaling (Beverly, MA). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MA). Affinity-purified polyclonal antibody against p70 S6 kinase was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody against 4E-BP-1/PHAS-I was characterized earlier (12). Affinity-purified polyclonal anti-calnexin antibody was from StressGen. Affinity-purified polyclonal anti-calreticulin antibody was from Affinity Bioreagents (Golden, CO).

Isolation and fractionation of rat adipocytes. Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats (150–175 g; Taconic) by collagenase digestion (28). All animals had continuous unrestricted access to chow (TAC no. 31) and water. Usually, the fat pads from eight rats were immersed in Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.6 mM Na2HPO4, 0.4 mM Na2PO4, 2.5 mM d-glucose, and 2% BSA, pH 7.4) prepared with diethylpyrocarbonate (DEPC)-treated sterile water, minced, and subjected to collag enase digestion for 35 min at 37°C with constant shaking. Next, the blot was washed three times with PBS and incubated with 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4) or DEPC (0.01% Sigma) and water. Usually, the fat pads from eight rats were immersed in Krebs-Ringer phosphate (KRP) buffer (100 mM KCl, 10 mM MgCl2, 10 mM HEPES, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, and 10 μM leupeptin, pH 7.4, 25°C) prepared with DEPC-treated sterile water. In some experiments, phos- phatase inhibitors (25 mM Na2HPO4, 50 mM NaF, and 5 mM Na3VO4) or DEPC (0.01% final concentration) together with RNAsin (Promega) were included in the buffer. After the second wash, cells in a 80–90% suspension were centrifuged at 14,000 rpm at 4°C for 20 min in a microcentrifuge. The fat layer was removed, and the supernatant and pellet were isolated for further analysis.

Isolation of mRNA and Northern blotting. Total RNA was extracted from adipose cells or from isolated adipocyte supernatants and pellets using Tri-Zol LS and Tri-Zol reagent, respectively. Isolated RNA (10–20 μg) was separated in a 1% agarose/2% formaldehyde gel in 1× MOPS (pH 7, 10 mM sodium acetate and 1 mM EDTA) and transferred to a Hy bond-N nylon membrane (Amersham) in 10× saline-sodium citrate (SSC; American Bioanalytical) and 0.05 M NaOH. After the transfer, the membrane and RNA were auto-cross-linked using an ultraviolet Stratalinker (Stratagene) and subjected to hybridization with the radiolabeled leptin DNA probe using ExpressHyb Hybridization Solution (Clontech) according to the manufacturer’s instructions. Briefly, the blot was prehybridized in hybridization solution for 30 min at 68°C and hybridized with radiolabeled probes (2 × 106 counts·min−1·ml−1) in fresh hybridization solution for 2 h at 68°C with constant shaking. Next, the blot was washed three times with 2× SSC/0.05% SDS at room temperature for 20 min each and then two times with 0.1× SSC/0.1% SDS at 50°C for 20 min each, and then the blot was analyzed in an Instantimagertm (Packard). For generating leptin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes, corresponding cDNA (200 ng) was mixed with 5 μl random primers of 6mers (77 μg/ml) and sterile water in a total volume of 24 μl, incubated at 95°C for 3 min, and then placed on ice for 3 min. dNTP mix (5 μl, 1 mM each, without dCTP), 5 μl of 10× Klenow buffer, 5 μl of 0.5 mg/ml BSA, 5 μl [α-32P]dCTP (NEN), and 1 μl DNA polymerase (Klenow exo-; New England Biolabs) were added to the incubation mixture and incubated at 37°C for at least 2 h. Unincorporated [α-32P]dCTP was removed by a Nuctrap push column (Stratagene).

RT-PCR. Total RNA (4–5 μg) was reverse transcribed into cDNA using a random hexamer and RT. cDNA was amplified by PCR using the sense primer TGGCTTTGGTCCTCTATCT (87–108 bp) and the anti-sense primer TCTCTACACGGCTGGCTTTCC (310–330 bp; see Ref. 36), and the PCR product was subjected to agarose gel electrophoresis.

Immunosialization of leptin messenger ribonucleaseprotein particle with anti-PHAS-1 antibody. Sheep anti-rabbit IgG Dynabeads m-280 (250 μl; Dynal) were washed with gradient buffer and incubated with either nonspecific rabbit IgG (5 μg; Sigma) or rabbit anti-PHAS-1 antibody (5 μg) overnight at 4°C with constant rotation. Unbound antibodies were removed by washing the beads with gradient buffer containing 0.5% Triton X-100. The supernatant of the broken adipocytes was incubated with Triton X-100 (final concentration of 1%) for 30 min and centrifuged at 4°C for 20 min at 14,000 rpm. The supernatant was incubated with the beads for 2 h at 4°C with constant rotation. The beads were then washed four times with gradient buffer containing 0.5% Triton X-100, and RNA was eluted using Tri-Zol reagent. Isolated RNA was subjected to RT-PCR.

Gel electrophoresis and Western blotting. Proteins were separated by SDS-PAGE without reducing agents and transferred to an Immobilon-P membrane (Millipore) in 25 mM Tris and 192 mM glycine, pH 8.3. After the transfer, the membrane was blocked with 10% nonfat dry milk in PBS-Tween for 1 h at room temperature. Proteins were visualized with specific antibodies, horseradish peroxidase-conjugated secondary antibodies (Sigma), and an enhanced chemilumi- nescent substrate kit (NEN) or a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

RIA. Leptin content was determined with a 125I-labeled leptin RIA kit (Linco) according to the manufacturer’s instructions. Each sample was done in triplicate and counted for the presence of leptin in a γ-counter (Wallac).

Analysis of the secondary structure of mRNA. Secondary structure of leptin mRNA was predicted using “mfold” software by Zucker and Turner (Rensselaer Polytechnic Institute, http://bioinfo.math.rpi.edu/~mfold/rna).

RESULTS

Figure 1, A and C, shows that addition of leucine to isolated rat adipocytes stimulates leptin secretion from these cells. Rapamycin significantly inhibits the leucine-induced increase in leptin secretion, suggesting that this effect is mediated by mTOR. At the same time, the effect of leucine is resistant to 4 μM actino- mycin D (Fig. 1, B and D). Because actinomycin D at this concentration inhibits transcription in isolated adipocytes by 90–95% (results not shown), leucine is likely to stimulate leptin expression at a posttranscriptional level. To confirm this observation, we isolated RNA from leucine-treated and nontreated adipose cells and analyzed the levels of leptin and GAPDH mRNA by Northern blotting. In agreement with previous studies (6), Fig. 2 shows a gradual decrease in leptin mRNA levels in isolated adipocytes. Interestingly, a similar phenomenon was previously reported for GLUT4 mRNA (10). These changes are likely to be caused by
disturbance of as yet unknown regulatory mechanisms upon dissociation of fat tissue into individual cells. In any case, leucine administration does not increase leptin mRNA levels in primary adipocytes in comparison with control (Fig. 2).

Leptin contains an NH2-terminal signal sequence (36) that is typical of proteins synthesized in the rough endoplasmic reticulum (ER). To analyze mTOR signaling in this subcellular fraction, primary rat adipocytes were broken open by centrifugation at 14,000 rpm for 20 min in a microfuge (18). Under these conditions, heavy subcellular organelles, including the ER, are recovered in the pellet (Fig. 3A and Ref. 18), whereas free ribosomes and mRNP remain in the supernatant (data not shown). In agreement with previously published results (7, 8), leucine administration to adipocytes causes an increase in phosphorylation of the ribosomal protein S6 and of the translational repressor 4E-BP/PHAS-1 (Fig. 3B). Both effects are likely to be mediated by mTOR, since addition of rapamycin completely blocks phosphorylation of these proteins. Interestingly, a leucine-induced increase in phosphorylation of 4E-BP/PHAS-1 is detectable in both the heavy fraction and cytosol. In contrast, phosphorylation of S6 in response to leucine is observed only in the cytosolic fraction. In the heavy fraction, S6 is constitutively phosphorylated irrespective of leucine treatment. These results indicate that ER-associated ribosomes that continuously synthesize secreted proteins contain phosphorylated S6. Leucine-induced activation of mTOR signaling is readily detectable even after 4 h of incubation with leucine (Fig. 3C). These data are consistent with the dynamics of leptin secretion (Fig. 1) and suggest that stimulation of leptin expression by leucine requires a sustained activation of translation of leptin mRNA.

Activation of leptin expression at the level of translation can be accounted for by recruitment of leptin mRNA from the cytosol on ER membranes or by activation of translation of leptin mRNA already associated with the ER. Thus we isolated RNA separately from the heavy fraction and from cytosol of broken adipocytes and determined the levels of leptin mRNA in both fractions by Northern blot (Fig. 4) and by RT-PCR (data not shown). We found that 60–70% of leptin mRNA in the cell was associated with the heavy fraction and cytosol. In contrast, phosphorylation of S6 in response to leucine is observed only in the cytosolic fraction. In the heavy fraction, S6 is constitutively phosphorylated irrespective of leucine treatment. These results indicate that ER-associated ribosomes that continuously synthesize secreted proteins contain phosphorylated S6. Leucine-induced activation of mTOR signaling is readily detectable even after 4 h of incubation with leucine (Fig. 3C). These data are consistent with the dynamics of leptin secretion (Fig. 1) and suggest that stimulation of leptin expression by leucine requires a sustained activation of translation of leptin mRNA.
ER-containing fraction, whereas 30–40% of leptin message was recovered in the cytosol where it was stored in the form of 80S mRNP particles (data not shown). Leucine administration to adipocytes did not cause a significant redistribution of leptin mRNA between the cytosol and the ER-containing heavy fraction (Fig. 4, A and B).

To confirm this result, we performed immunoprecipitation of leptin mRNA from the cytosolic extract of adipose cells with an anti-PHAS-1 antibody. We found that leptin mRNA was specifically immunoprecipitated with this antibody but not with nonspecific IgG (Fig. 5). This result shows that the translational repressor PHAS-1 is associated with leptin mRNA, probably via an interaction with the cap-binding initiation factor eukaryotic initiation factor (eIF)-4E. In agreement with data shown in Fig. 4, leucine administration did not significantly change the amount of PHAS-1-associated leptin mRNA in the cytosol. Thus correct compartmentalization of leptin mRNA in the cell may be required for its expression and regulation, as previously shown for a variety of other transcripts (17). We were unable to directly determine if translation of compartmentalized leptin mRNA is regulated by PHAS because extraction of this mRNA from the heavy pellet requires high salt treatment that leads to dissociation of mRNA-binding proteins.

DISCUSSION

We show here that leptin production in isolated rat adipocytes is increased two- to threefold by leucine within 2–4 h (Fig. 1). In live rats, plasma leptin levels also rise 2.5- to 3-fold within 3 h after food intake (20). Therefore, the leucine-induced increase in leptin secretion may account for the effect observed in vivo and thus may provide a direct connection between food intake and plasma leptin levels. Leucine does not increase the amount of leptin mRNA and, also, demonstrates its effect in the presence of the transcriptional inhibitor actinomycin D (Figs. 1B and 2). For these reasons, we believe that leucine stimulates leptin ex-
pression at a posttranscriptional level. It has been shown previously that actinomycin D alone may increase leptin secretion from isolated rat adipocytes (4, 6). This unusual effect of actinomycin D may, for example, be attributed to a putative short-lived inhibitor of leptin expression that can be downregulated by actinomycin D. We have not detected a stimulatory effect of actinomycin D on leptin production (Fig. 1). This may be explained by different experimental conditions, such as a higher concentration of actinomycin D, used in our experiments. Also, we measured leptin production in actinomycin D-treated adipocytes after 4 h of incubation, whereas the stimulatory effect of the inhibitor may become readily detectable only after 24 h (6).

In any case, we found that the effect of leucine on leptin expression is sensitive to rapamycin and, therefore, is likely to be mediated by the mTOR pathway. This observation is consistent with several previously described effects. In particular, it has been known for several years that leptin expression in adipocytes depends on energy metabolism (25) and correlates very well with the level of intracellular ATP (19). Because mTOR may represent an ATP sensor in the cell (5), it may mediate the effect of ATP on leptin expression. In addition, there is a strong correlation between adipo-

![Fig. 3. Mammalian target of rapamycin (mTOR) signaling in different subcellular fractions of rat adipocytes. A: isolated rat adipocytes were centrifuged at 14,000 rpm for 20 min in a microcentrifuge. The lipid cake was removed, and the pellet (heavy fraction) and supernatant (cytosol) were analyzed by PAGE (50 μg total protein/lane) and Western blotting with antibodies against endoplasmic reticulum markers, calnexin and calreticulin. B: isolated rat adipocytes were treated with leucine (5 mM) and/or rapamycin (100 nM) as indicated for 30 min and separated in the heavy fraction and cytosol, as described in A. Both fractions (50 μg total protein/lane) were analyzed by Western blotting. C: isolated rat adipocytes were treated with leucine (5 mM) for the indicated periods of time and separated in the heavy fraction and cytosol, as described in A. Both fractions (50 μg total protein/lane) were analyzed by Western blotting. A-C show representative results of at least 3 independent experiments. 4E-BP, 4E-binding protein; PHAS-1, phosphorylated heat- and acid-stable protein.](http://ajpendo.physiology.org/)

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cyte size and the amount of leptin produced by the cell (reviewed in Ref. 1). Because mTOR regulates cell growth (32, 35), this correlation suggests that mTOR may be involved in both adipocyte growth and leptin expression.

mTOR exerts its biological effects via phosphorylation of ribosomal protein S6 and of the repressor 4E-BP/PHAS, which represent two independent pathways of translational control in the cell (27). The first mechanism is thought to involve expression of messages with a 5' terminal oligopyrimidine tract, and the second mechanism is thought to be required for initiation of translation of mRNAs with double-stranded regions in the 5'-untranslated region (UTR). Translation of such mRNAs depends on the recruitment of the RNA helicase eIF-4A that, in conjunction with its protein cofactor eIF-4B, unwinds inhibitory secondary structures in the 5'-UTR, allowing the 40S ribosomal subunit to slide toward the 3' terminus of mRNA until the initiation codon is located (11). RNA helicase eIF-4A is recruited to the initiation complex by the scaffolding protein eIF-4G, which, in turn, interacts with the cap-binding protein eIF-4E. Binding of eIF-4G and the translational repressor 4E-BP/PHAS to eIF-4E is mutually exclusive. Phosphorylation of 4E-BP/PHAS by mTOR causes its dissociation from eIF-4E, thus allowing for the interaction with eIF-4G and initiation of translation (11).

Although leucine administration to rat adipocytes activates both S6- and 4E-BP/PHAS-mediated pathways (Fig. 3B; see also Refs. 7 and 8), translation of leptin mRNA is more likely controlled by phosphoryla-
tion of 4E-BP/PHAS. First, leptin mRNA lacks a 5'-terminus oligopyrimidine tract but contains predicted hairpins in the 5'-UTR (Fig. 6). Second, repressed leptin mRNA in the cytosol is associated with PHAS-1 (Fig. 5). Third, S6 is constitutively highly phosphorylated in the ER-associated ribosomes that translate leptin mRNA, and leucine does not seem to have any additional effect on phosphorylation of S6 in this fraction (Fig. 3B).

Approximately two-thirds of leptin mRNA is associated with the heavy membrane fraction, and only one-third is found in the cytosol in a form of 80S mRNP. We have shown that the cytosolic pool of leptin mRNA is not regulated by leucine (Figs. 4 and 5). Thus compartmentalization of leptin mRNA may be required for its expression and translational regulation. Based on our preliminary fractionation experiments, we suggest

![Fig. 6. Predicted secondary structure of 5'-untranslated region of mouse and human leptin mRNA. Initiation codon is underlined.](image)

![Fig. 7. Proposed link between insulin-regulated glucose transport machinery and leptin secretion in adipose cells. Food intake causes activation of insulin secretion by β-cells in the pancreas. Insulin stimulates translocation of intracellular GLUT4-containing vesicles to the plasma membrane in fat and skeletal muscle cells. The two major protein constituents of these vesicles in both cell types are GLUT4 and an insulin-responsive (leucine) aminopeptidase, or IRAP (14). The latter represents an ectoenzyme with the extracellular catalytical domain. Upon translocation to the cell surface, IRAP locally generates free leucine from as yet unidentified substrates. Leucine activates mTOR that stimulates translation of leptin mRNA and leptin secretion. An increase in circulating leptin may cause satiety and inhibit food intake. In parallel, GLUT4 clears blood glucose, insulin levels decrease, and GLUT4 and IRAP are internalized from the cell surface in an intracellular vesicular compartment.](image)
that, in adipocytes, the major pool of leptin mRNA is associated with the ER. Usually, intracellular localization of mRNA is defined by the 3′-UTR (17). Leptin mRNA has a long 3′-UTR with multiple structural elements (36) that may include a localization signal. Studies are in progress to further explore specific compartmentalization of leptin mRNA in the cell.

Because insulin is known to activate the mTOR pathway in adipocytes (21, 22, 26), we propose that the previously described effects of insulin on leptin expression (reviewed in Ref. 1) may be mediated, at least in part, by mTOR (also see Ref. 4). Although our results indicate that mTOR regulates leptin expression primarily at a posttranscriptional level, we do not exclude the possibility that, in vivo, this pathway may also be involved in the regulation of transcription of the ob gene. Experiments are in progress to test this hypothesis.

In any case, regulation of leptin production in adipose cells is controlled at several levels. In addition to transcriptional (reviewed in Ref. 1) and translational (current study) control, leptin release from adipose cells is regulated at the level of secretion. We and others have previously shown that adipose cells possess a regulatable pool of presynthesized leptin (2, 4, 30, 31, 33) that is compartmentalized in specialized insulin- and serum-regulated secretory vesicles (30, 31). We believe that a multilevel structure of leptin regulation allows for accurate adjustment of leptin secretion to match the nutritional needs of the body and for maintenance of energy homeostasis in the mammalian organism.

In particular, translational regulation of leptin mRNA by leucine via mTOR may provide a direct connection between food intake and postprandial leptin levels and thus may be essential for the formation of the satiety signal. Moreover, the involvement of mTOR in the regulation of leptin expression may help to establish a link between insulin resistance and obesity. First, normal insulin-stimulated glucose transport machinery may have other regulatory functions. In particular, in fat, it may provide a feedback device that controls metabolic homeostasis in the body via mTOR-mediated production of leptin and, possibly, other secreted products (Fig. 7).

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