

# Regulation of Interleukin-6-induced Hepatic Insulin Resistance by Mammalian Target of Rapamycin through the STAT3-SOCS3 Pathway\*

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The proinflammatory cytokine interleukin (IL)-6 has been proposed to be one of the mediators that link obesity-derived chronic inflammation with insulin resistance. Signaling through the mammalian target of rapamycin (mTOR) has been found to impact insulin sensitivity under various pathological conditions, through serine phosphorylation and inhibition of insulin receptor substrate by the downstream effector of mTOR, ribosomal S6 kinase 1 (S6K1). However, an involvement of mTOR in IL-6-induced insulin resistance has not yet been reported. Here we show that rapamycin, the inhibitor of mTOR signaling, rescues insulin signaling and glycogen synthesis from IL-6 inhibition in HepG2 hepatocarcinoma cells as well as in mouse primary hepatocytes. IL-6 activates S6K1 in these cells, but unexpectedly, S6K1 is not involved in IL-6 inhibition of insulin signaling, since the effect of IL-6 persists in cells with drastically reduced S6K1 levels induced by RNA interference, suggesting that the function of mTOR signaling is through a mechanism different from the prevailing model of S6K1 phosphorylation of insulin receptor substrate-1. Interestingly, we find that the phosphorylation of STAT3 on Ser<sup>727</sup> and STAT3 transcriptional activity are regulated by mTOR upon IL-6 stimulation and that STAT3 is required for IL-6 inhibition of insulin signaling. Furthermore, IL-6-induced SOCS3 expression is inhibited by rapamycin, and ectopic expression of SOCS3 blocks the ability of rapamycin to enhance insulin sensitivity in the presence of IL-6. Taken together, we propose that mTOR plays a key role in IL-6-induced hepatic insulin resistance by regulating STAT3 activation and subsequent SOCS3 expression.

Insulin resistance, a major risk factor and the principle defect in type II diabetes, is commonly observed with obesity. Chronic production of proinflammatory cytokines is considered a major link between obesity and insulin resistance (1). Elevated local and circulating levels of proinflammatory cytokines, such as

tumor necrosis factor- $\alpha$  and interleukin (IL)-6, are known to be associated with obesity in both human and rodent models (2–5). Although adipose-derived tumor necrosis factor- $\alpha$  may act locally in autocrine and paracrine manners, adipose-derived IL-6 is thought to enter circulation and play a systemic role in modulating insulin actions (6). Depletion of IL-6 improves insulin action in a mouse model of obesity (7), whereas in humans, elevated plasma IL-6 levels positively correlate with obesity and insulin resistance and predict the development of type 2 diabetes (2, 8, 9). Furthermore, IL-6 administration to healthy individuals induces blood glucose increases (10). *In vitro*, IL-6 has been shown to induce insulin resistance in hepatic cells (11). Although the role of IL-6 within peripheral tissues (adipose and skeletal muscle) is still being debated, it is generally accepted that at least in the liver, IL-6 causes insulin resistance (12).

A mechanism of IL-6-induced insulin resistance in the liver has been proposed, which involves the activation of STAT3 (signal transducer and activator of transcription 3) and subsequent induction of suppressor of SOCS3 (cytokine signaling 3) (7, 13, 14), a negative regulator of cytokine signaling (15). Several cytokines and hormones associated with insulin resistance induce the expression of SOCS proteins, which inhibit insulin signaling through several distinct mechanisms, including directly interfering with insulin receptor activation, blocking IRS activation, and inducing IRS degradation (16). The role of SOCS3 in insulin resistance has also been confirmed in animal models. Overexpression of SOCS1 and SOCS3 in liver causes insulin resistance, whereas antisense suppression of SOCS3 expression in obese diabetic mice (*db/db*) ameliorated insulin resistance (17). Furthermore, hepatocyte-specific deletion of the SOCS3 gene improved insulin sensitivity in mice, although unexpected systemic effects of this deletion suggest that hepatic SOCS3 may have dual functions *in vivo* (18).

IL-6 signals through a transmembrane receptor complex containing the common signal transducing receptor glycoprotein gp130, which activates Jak tyrosine kinases, leading to the activation of STAT3 (19). In addition to activation by Tyr<sup>705</sup> phosphorylation, STAT3 also requires phosphorylation on Ser<sup>727</sup> to achieve maximal activity (20, 21). Several reports have suggested that the protein kinases responsible for STAT3 serine phosphorylation vary, probably according to cellular context, and include pro-

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<sup>3</sup> The abbreviations used are: IL, interleukin; mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; shRNA, short hairpin RNA; RT, reverse transcription; IRS, insulin receptor substrate.

tein kinase C, Jun N-terminal kinase, extracellular signal-regulated kinase, the mitogen-activated protein kinase p38, and mammalian target of rapamycin (mTOR) (22).

A member of the phosphatidylinositol kinase-like Ser/Thr kinase family, mTOR is a master regulator of cell growth (23) as well as various types of cellular differentiation (24) in response to nutrient availability and cellular energy levels. Two functionally distinct protein complexes containing mTOR have been characterized, namely mTORC1 and mTORC2, which mediate the rapamycin-sensitive and rapamycin-insensitive signaling of mTOR, respectively (25). The two best characterized downstream targets of mTOR are ribosomal S6 kinase 1 (S6K1) and eIF-4E-binding protein 1, both of which regulate translation initiation (23). Upstream regulators of mTOR signaling include the tuberous sclerosis complex TSC1/2 (26), and phospholipase D, the product of which (the lipid second messenger phosphatidic acid) mediates mitogenic activation of mTOR signaling (27, 28).

In recent years, the involvement of mTOR in insulin resistance has drawn a lot of attention. Several conditions known to activate mTOR have been shown to lead to inhibition of insulin signaling (or insulin resistance), including amino acids (29–31), hyperinsulinemia (32), acute and chronic insulin stimulation (33, 34), and deletion of TSC1/2 (35, 36). The current model for the role of mTOR in insulin sensitivity is a negative feedback loop where activated S6K1 phosphorylates IRS-1 on serine residues, which results in degradation of IRS-1, leading to impaired PI3K stimulation (37). In this study, we have examined the role of mTOR signaling in IL-6-induced insulin resistance in liver cells and uncovered a signaling mechanism distinct from the currently established model of mTOR action in insulin resistance.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—HepG2 human hepatocarcinoma cells were obtained from the American Type Tissue Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium with 1 g/liter glucose containing 10% fetal bovine serum at 37 °C with 5% CO<sub>2</sub>. For IL-6 and insulin stimulation, cells were incubated in serum-free Dulbecco's modified Eagle's medium for 24 h prior to various treatments described here. Mouse primary hepatocytes were isolated from 10–12-week-old C57BL/6 mice using the liver perfusion method originally described by Seglen (38) and modified as previously reported (39). All handling of animals was in accordance with IACUC regulations at the University of Illinois. Freshly isolated primary hepatocytes were plated in William's medium with 10% fetal bovine serum. The next day, the cells were serum-starved in plain William's medium overnight and then subjected to glycogen synthesis assays as described below.

**Antibodies and Other Reagents**—Human recombinant insulin and IL-6 were purchased from Sigma and Cell Signaling Technology, respectively. The phospho-STAT3 (Ser<sup>727</sup>) and STAT3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The tubulin antibody was from Abcam. The SOCS3 antibody was from Anaspec. The following antibodies were from Cell Signaling Technology: phospho-Akt (Ser<sup>473</sup>), Akt, phospho-S6K1 (Thr<sup>389</sup>), S6K1, phospho-mTOR (Ser<sup>2481</sup>), mTOR, IRS-1, phospho-IRS-1 (Ser<sup>307</sup>, Ser<sup>312</sup>, and Ser<sup>636/639</sup>), and p85. All secondary antibodies were obtained from Jackson

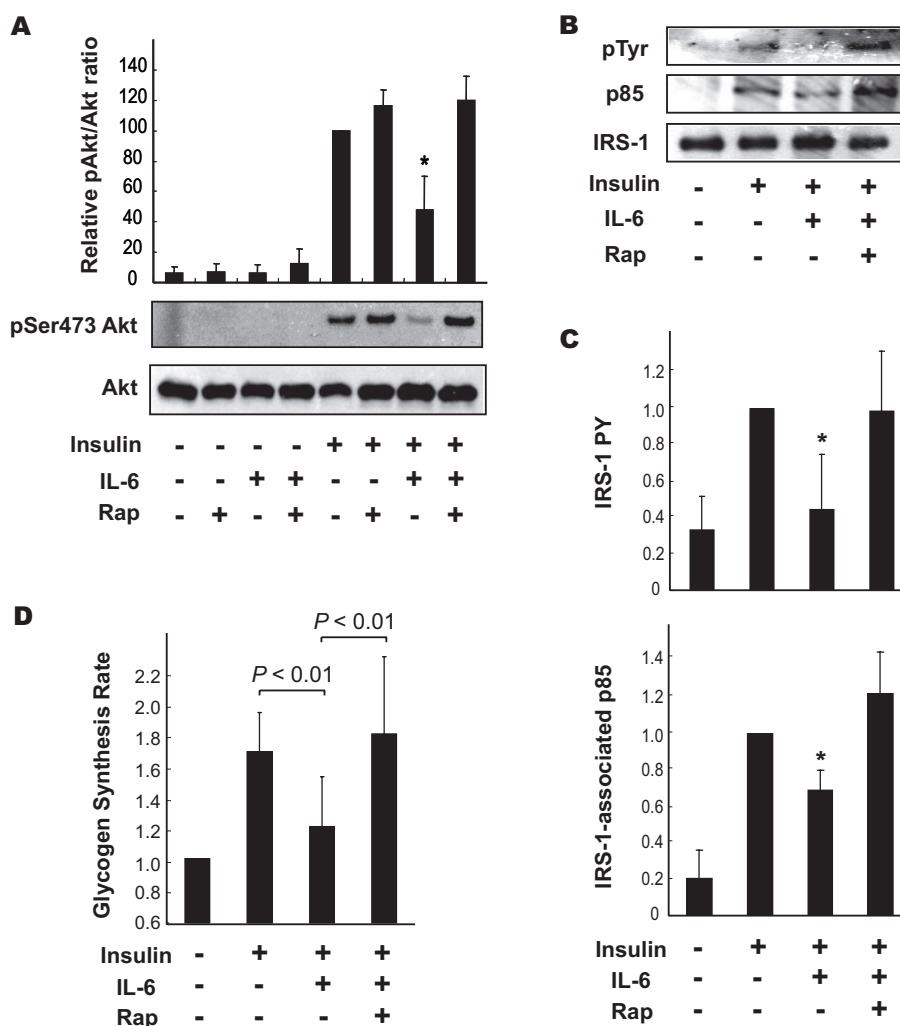
ImmunoResearch Laboratories, Inc. Rapamycin and AG490 were purchased from Calbiochem and Sigma, respectively.

**Plasmids**—The STAT luciferase reporter 3×Ly6E and plasmids expressing wild-type (wt) and S727A STAT3 were generous gifts from the laboratory of James Darnell (20). Human SOCS3 cDNA (in pCMV6-XL6) was obtained from Origene and subcloned into pCDNA3-Myc via NotI.

**Lentivirus-delivered RNA Interference**—All shRNAs used in this study were constructed in pLKO.1-Puro. shRNAs for human S6K1 and STAT3 were purchased from Sigma (Mission shRNA). Each gene set contained five constructs with distinct target sequences, all of which were packaged for viral production and infection and tested for target knockdown. For each gene, two constructs with ≥90% knockdown efficiency were used for further studies. shRNAs for human mTOR and a negative control (scrambled sequence) were obtained from Addgene (40). For viral packaging, pLKO-shRNA, pCMV-dR8.91, and pCMV-VSV-G were co-transfected into 293T cells using Eugene 6 (Roche Applied Science) at 0.5:0.45:0.05 μg (for a 6-well plate). Media containing viruses were collected 48 h after transfection. HepG2 cells were infected with the viruses in the presence of Polybrene (8 μg/ml) for 24 h and then subjected to selection by 2 μg/ml puromycin for 72 h prior to cell lysis or various cell treatments described here. Hairpin sequences in these shRNA constructs are as follows: S6K1-1, 5'-CCGGCCCATGATCTCCAAACGGCC-ACTCGAGTGCCGTTTGGAGATCATGGGTTTTT; S6K1-2, 5'-CGGGCGACATCTTTCTCAACCTTACTCGAGTAAGG-TTGAGAAAGATGTCGCTTTTTT; STAT3-1, 5'-CCGGGCAA-AGAATCACATGCCACTTCTCGAGAAGTGGCATGTGAT-TCTTTGCTTTTTT; STAT3-2, 5'-CCGGGCTGACCAACAAT-CCCAAGAACTCGAGTTCTTGGGATTGTTGGTCAGCTT-TTT; mTOR, 5'-CCGGTTCAGCGTCCCTACCTTCTTCTC-TCGAGAGAAGAAGGTAGGGACGCTGATTTTTTG; negative control, 5'-CCTAAGGTTAAGTCGCCCTCGCTCTAGCGA-GGGCGACTTAACCTTAGG.

**Immunoprecipitation and Western Blotting**—For immunoprecipitation studies, HepG2 cells were lysed in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% protease inhibitor mixture (Sigma). The cell lysates were cleared by centrifugation at 12,000 × g at 4 °C for 5 min and incubated overnight with anti-IRS-1 antibody followed by protein G-agarose (Upstate Biotechnology, Inc.) at 4 °C for 3 h. The beads were washed with the lysis buffer three times and boiled in 2× SDS sample buffer. Whenever only Western analysis was necessary, the cells were lysed in 1× SDS sample buffer, sonicated to shear the DNA, and boiled. Samples were run on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and incubated with various antibodies following the manufacturer's recommendations. Quantification of Western band intensities was performed with images on x-ray film using the NIH Image software.

**Glycogen Synthesis Assay**—HepG2 cells or mouse primary hepatocytes were serum-starved overnight and then treated for 2.5 h with IL-6 (20 ng/ml) in the absence or presence of rapamycin, followed by incubation in 1.5 μCi of <sup>14</sup>C-D-glucose and 100 nM insulin for 3 h. The cells were washed three times with ice-cold PBS and scraped in 0.3 ml of 10 N KOH. After adding



**FIGURE 1. Rapamycin ameliorates IL-6-induced insulin resistance in HepG2 cells.** A, human hepatocarcinoma HepG2 cells were serum-starved overnight and then stimulated with 100 nM insulin for 10 min, with or without pretreatment by 20 ng/ml IL-6 for 2.5 h. When indicated, 100 nM rapamycin was added 30 min prior to any stimulation. Cell lysates were subjected to Western analyses for phospho-Akt (Ser<sup>473</sup>) and total Akt. Three independent experiments were quantified to yield the ratio between phospho-Akt and total Akt. Representative blots are also shown. \*,  $p < 0.05$  compared with insulin treatment alone (Student's *t* test). B, lysates of HepG2 cells treated as in A were immunoprecipitated with IRS-1 antibody and then immunoblotted with various antibodies as indicated. C, four independent experiments in B were quantified to yield the relative ratio of phospho-Tyr-IRS-1 or IRS-1-associated p85 versus total IRS-1. \*,  $p < 0.05$  compared with insulin treatment alone (Student's *t* test). D, the cells were incubated in <sup>14</sup>C-D-glucose and 100 nM insulin for 3 h, followed by cell lysis and glycogen precipitation. Relative glycogen synthesis rates are shown as the average of four independent experiments. The results of Student's *t* test (*P*) are shown above the graph. pTyr, phospho-Tyr; Rap, rapamycin.

glycogen (4 mg) as a carrier, the samples were boiled for 30 min and precipitated with two volumes of 95% ethanol overnight at 4 °C, followed by centrifugation at  $10,000 \times g$  for 10 min. The pellets were washed once with 66% ethanol, resuspended in 0.5 ml of water, and subjected to scintillation counting.

**Transient Transfection and Luciferase Assays**—HepG2 cells grown on 12-well plates at 60–70% confluence were transfected with 1  $\mu$ g of 3  $\times$  Ly6E reporter using Eugene 6. When indicated, 1  $\mu$ g of STAT3 wild type or S727A plasmid was cotransfected. After various treatments, the cells were lysed in 200  $\mu$ l/well of passive lysis buffer (Promega). Luciferase activity was measured using a luciferase assay system (Promega) following the manufacturer's protocol.

**Quantitative RT-PCR**—Total RNA was extracted from HepG2 cells by using the RNeasy Mini Kit (Qiagen), according

to the manufacturer's instructions. cDNA was synthesized from 2  $\mu$ g of total RNA with Superscript II reverse transcriptase (Invitrogen) using oligo(dT) primer (Invitrogen). Quantitative RT-PCR was performed on a Bio-Rad iCycler system using SYBR green chemistry in a MicroAmp 96-well reaction plate following the manufacturer's protocols.  $\beta$ -Actin was used as a reference to obtain the relative -fold change for target samples using the comparative  $C_T$  method. The primers used are as follows:  $\beta$ -actin forward, 5'-GCACTCTTCCAGCCTTCCT;  $\beta$ -actin reverse, 5'-AGGTCTTTGCGGATGTCCAC; SOCS3 forward, 5'-CTTCAGCTCCAAGAGCGA-GTA; SOCS3 reverse, 5'-GAGCTGTCGCGGATCAGAAAG.

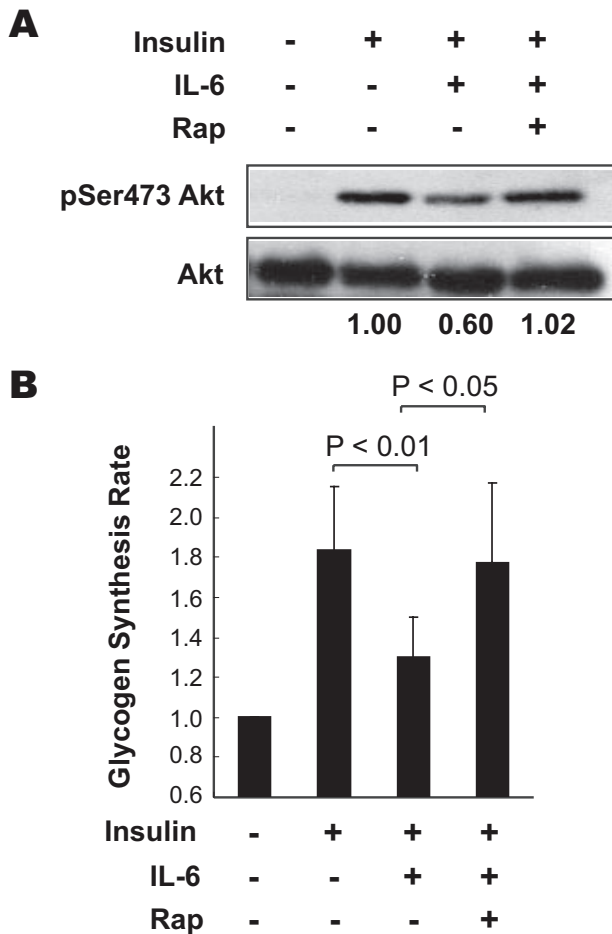
## RESULTS

**Rapamycin Ameliorates IL-6-induced Insulin Resistance in Liver Cells**—Previously, IL-6 was reported to induce insulin resistance in human hepatocarcinoma HepG2 cells (11), a frequently utilized *in vitro* system for studying insulin's effects on hepatic cells. Indeed, we also found that pretreatment of serum-starved HepG2 cells by IL-6 significantly dampened their response to acute insulin stimulation, as measured by Akt phosphorylation at Ser<sup>473</sup> (Fig. 1A). To determine the involvement of mTOR in IL-6-induced insulin resistance, rapamycin was added to the cells during IL-6 treatment. As shown in Fig. 1A, rapamycin treatment restored

insulin-stimulated Akt phosphorylation in the presence of IL-6. Phosphorylation of Akt at Thr<sup>308</sup> displayed a similar pattern, although phosphorylation at that site was less easily detectable by Western blotting than Ser<sup>473</sup> phosphorylation (data not shown). As additional readouts for insulin signaling, IRS-1 tyrosine phosphorylation and the association of the p85 subunit of phosphatidylinositol-3 kinase with IRS-1 were also examined. As shown in Fig. 1, B and C, both events were inhibited by IL-6 pretreatment, and rapamycin relieved this inhibition. However, from here on we would use phospho-Ser<sup>473</sup> as a positive indicator of downstream insulin signaling because its detection was more sensitive and reliable than that of phospho-IRS-1 or p85 association with IRS-1.

As a metabolic end point of insulin action in the liver, glycogen synthesis was examined in HepG2 cells with <sup>14</sup>C-D-glucose incorporated. A 1.7-fold increase of glycogen production was

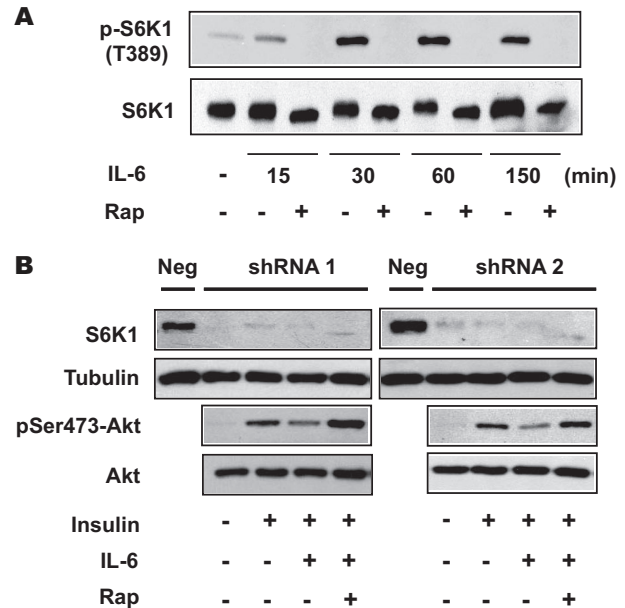




**FIGURE 2. Rapamycin ameliorates IL-6-induced insulin resistance in mouse primary hepatocytes.** A, mouse primary hepatocytes were serum-starved overnight and then stimulated with 100 nM insulin for 10 min, with or without pretreatment by 20 ng/ml IL-6 for 2.5 h. When indicated, 100 nM rapamycin was added 30 min prior to any stimulation. Cell lysates were subjected to Western analyses. Two independent experiments yielded similar results, one of which is shown. The relative ratios of phospho-Akt (Ser<sup>473</sup>) versus total Akt are indicated at the bottom of the blots. B, the cells were incubated in <sup>14</sup>C-D-glucose and 100 nM insulin for 3 h, followed by cell lysis and glycogen precipitation. Relative glycogen synthesis rates are shown as the average of three independent experiments. The results of Student's *t* test (*P*) are shown above the graph. Rap, rapamycin.

observed upon insulin stimulation (Fig. 1C), consistent with the degree of insulin activation reported in the literature (11). IL-6 treatment blunted this insulin response as expected, and, importantly, rapamycin treatment fully rescued insulin activation of glycogen synthesis in the presence of IL-6 (Fig. 1C).

To further validate the physiological relevance of the observations above, we examined the effect of rapamycin on IL-6 modulation of insulin signaling in mouse primary hepatocytes. As shown in Fig. 2A, insulin-induced Akt phosphorylation was dampened by IL-6 pretreatment in the primary cells, and rapamycin abrogated the IL-6 effect. Furthermore, insulin-stimulated glycogen synthesis in these cells was also suppressed by IL-6, and importantly, rapamycin ameliorated the IL-6-induced insulin resistance (Fig. 2B). Thus, a rapamycin-sensitive pathway probably plays a key role in IL-6-induced insulin resistance in liver cells. These observations suggest that in addition to mediating insulin resistance resulting from perturbation of the

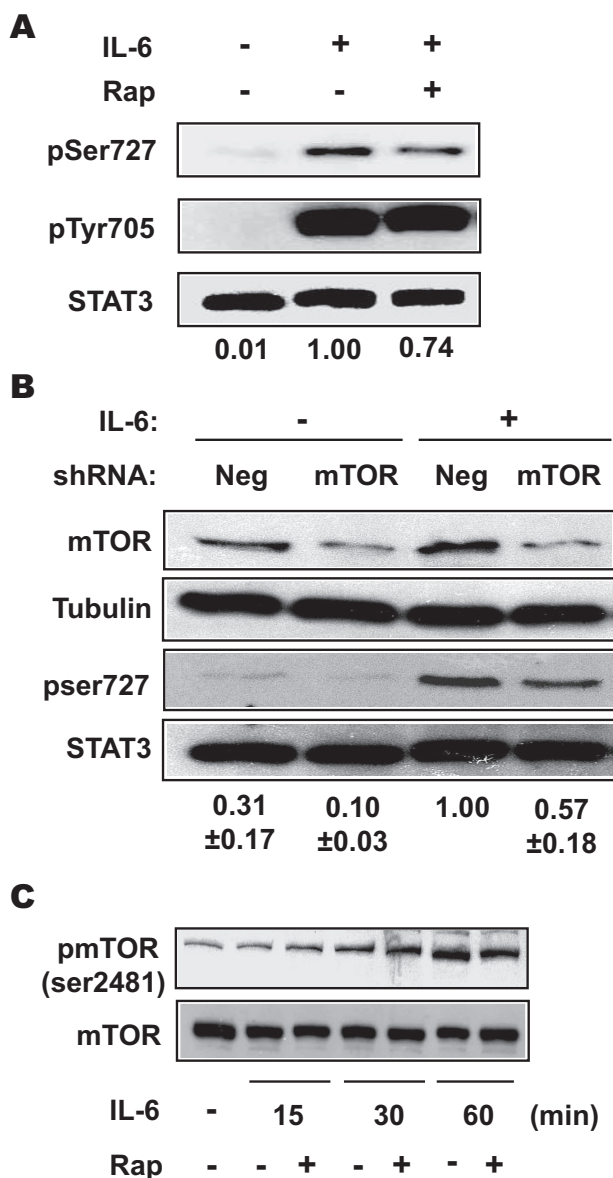


**FIGURE 3. S6K1 is not involved in IL-6 inhibition of insulin signaling.** A, serum-starved HepG2 cells were stimulated with 20 ng/ml IL-6 for various periods of time as indicated, in the presence or absence of 100 nM rapamycin. Cell lysates were analyzed by Western blot for phospho-Thr-389 or total S6K1. B, HepG2 cells were infected with lentiviruses expressing two independent S6K1 shRNA as described under "Experimental Procedures." Cells infected by viruses expressing a scrambled hairpin sequence served as a negative control (Neg) for knockdown. After puromycin selection for 3 days, the cells were serum-starved overnight and treated as described in Fig. 1A. The cell lysates were analyzed by Western blotting with various antibodies. Rap, rapamycin.

nutrient-sensing pathway, mTOR signaling is potentially involved in the modulation of insulin sensitivity by proinflammatory cytokines.

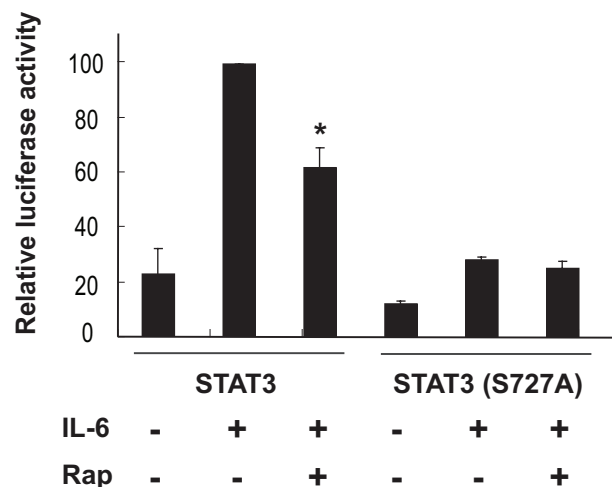
**S6K1 Is Not Responsible for the Involvement of mTOR in IL-6 Inhibition of Insulin Signaling**—A well accepted mechanism of rapamycin-sensitive insulin resistance is through the serine phosphorylation of IRS-1 by S6K1 (37). To consider whether S6K1 may also be involved in IL-6-induced insulin resistance, we first examined S6K1 activity in IL-6-stimulated HepG2 cells. As shown in Fig. 3A, S6K1 activation, as measured by the phosphorylation of Thr<sup>389</sup>, was detectable after 15 min of IL-6 stimulation of the quiescent cells and then reached a plateau at 30 min; the activity was maintained throughout the 2.5 h of IL-6 treatment. As expected, this activation was abolished by rapamycin treatment. These results seemed consistent with a role for S6K1 downstream of IL-6 stimulation in these cells.

To more definitively assess the role of S6K1 in the effect of IL-6, we depleted the S6K1 protein from HepG2 cells by RNA interference. As shown in Fig. 3B, two lentiviral delivered shRNAs each knocked down S6K1 by ~90% in HepG2 cells, but IL-6 was still able to dampen insulin signaling, as measured by Akt Ser<sup>473</sup> phosphorylation. Also, rapamycin restored Akt phosphorylation from IL-6 inhibition in these S6K1-deficient cells. These results clearly indicated that S6K1 is not essential for the effect of IL-6 on insulin signaling in liver cells. We also examined serine phosphorylation sites on IRS-1 that have been reported to be sensitive to rapamycin and negatively regulate IRS-1 activity or stability, including Ser<sup>307</sup>, Ser<sup>312</sup>, and Ser<sup>636/639</sup> (corresponding to Ser<sup>302</sup>, Ser<sup>307</sup>, and Ser<sup>632/635</sup> in



**FIGURE 4. mTOR regulates IL-6-induced STAT3 Ser<sup>727</sup> phosphorylation.** A, serum-starved HepG2 cells were stimulated with 20 ng/ml IL-6 in the presence or absence of 100 nM rapamycin, lysed, and then subjected to Western analysis for Ser<sup>727</sup> phosphorylation of STAT3. The relative ratio of phospho-Ser<sup>727</sup> versus total STAT3 is indicated at the bottom of the blots. B, cells infected by viruses expressing an mTOR shRNA or a scrambled sequence (Neg) were stimulated with IL-6 for 15 min and analyzed by Western blot. The relative ratios of phospho-Ser<sup>727</sup> versus total STAT3 are indicated at the bottom of the blots as the average results of three independent experiments. C, mTOR catalytic activity was examined by autophosphorylation at Ser<sup>2481</sup> upon IL-6 stimulation for various periods of time. pmTOR, phospho-mTOR; Rap, rapamycin.

mouse IRS-1) (35, 41–43). None of these sites was affected by prolonged IL-6 treatment (data not shown). Furthermore, S6K1 knockdown significantly blocked the phosphorylation of all three sites (data not shown), yet IL-6 inhibition of insulin signaling persisted (Fig. 3B). Taken together, our data strongly argue against S6K1 phosphorylation of IRS-1 as a key mediator of IL-6-induced insulin resistance in HepG2 cells. Thus, we proceeded to consider alternative possibilities for a mechanistic explanation of the involvement of mTOR signaling in the cross-talk between IL-6 and insulin signaling.



**FIGURE 5. Rapamycin inhibits IL-6 activation of STAT3.** HepG2 cells were co-transfected with the 3×Ly6E luciferase reporter and wild type or S727A STAT3, serum-starved overnight, and then stimulated by IL-6 with or without rapamycin. Cell lysates were subjected to luciferase assays. Relative luciferase activities are shown as the average results of three independent experiments. \*,  $p < 0.05$  compared with IL-6 stimulation in the absence of rapamycin (Student's  $t$  test). Rap, rapamycin.

**mTOR Regulates IL-6-induced STAT3 Activation**—The activation of STAT3 and the subsequent induction of SOCS3 are thought to mediate IL-6-induced inhibition of insulin signaling (7, 13, 14). Previously, in neuronal cells mTOR has been reported to be a kinase for STAT3 on Ser<sup>727</sup> (44, 45), phosphorylation of which is required for maximal activation of STAT3 (20, 21). To test whether mTOR participates in IL-6 signaling in liver cells by regulating STAT3 activity, we first examined the effect of rapamycin on Ser<sup>727</sup> phosphorylation. As shown in Fig. 4A, IL-6 enhanced phosphorylation of STAT3, which was diminished by rapamycin treatment. On the other hand, IL-6-induced STAT3 tyrosine phosphorylation on residue 705 was not affected by rapamycin (Fig. 4A). Knockdown of mTOR by a previously characterized shRNA (40) also impaired IL-6-induced Ser<sup>727</sup> phosphorylation (Fig. 4B). These observations support the possibility that mTOR has kinase activity toward STAT3. Furthermore, consistent with the notion that mTOR may be a direct kinase for STAT3 in IL-6-treated HepG2 cells, the catalytic activity of mTOR (as measured by autophosphorylation at Ser<sup>2481</sup>) was stimulated by IL-6 (Fig. 4C). As expected (46), this phosphorylation was not sensitive to rapamycin. Although it is a well established fact that insulin also activates mTOR, we did not observe obvious STAT3 phosphorylation on Ser<sup>727</sup> when the cells were stimulated by insulin alone (data not shown).

To probe a functional link between mTOR and STAT3, we studied STAT3 transcriptional activity using a STAT reporter containing a luciferase gene and three copies of the interferon- $\gamma$  activation sites from the Ly6E gene (3×Ly6E) (20). The reporter was co-expressed with the wild-type or nonphosphorylatable mutant (S727A) of recombinant STAT3 in HepG2 cells. As shown in Fig. 5, in the presence of wild-type STAT3, a 5-fold induction of the reporter was observed with IL-6 stimulation, and rapamycin treatment diminished this activation. When the S727A STAT3 mutant was expressed, no activation of the

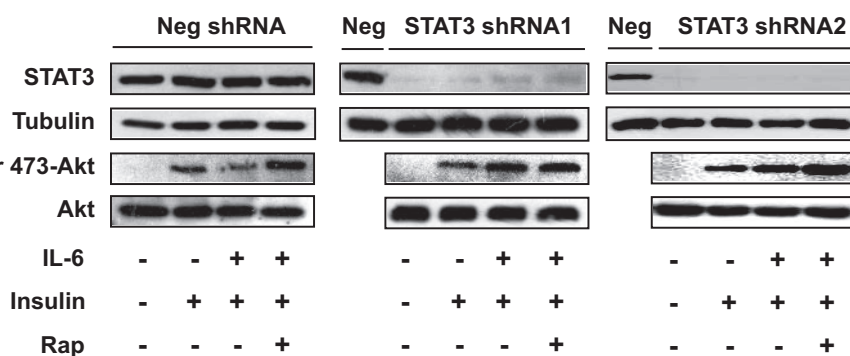


FIGURE 6. **Knockdown of STAT3 eliminates the negative effect of IL-6 on insulin signaling.** HepG2 cells infected with lentiviruses expressing two independent STAT3 shRNAs or a scrambled sequence (Neg) were selected with puromycin for 3 days and then treated as described in the legend to Fig. 1A. The cell lysates were analyzed by Western blotting with various antibodies. Rap, rapamycin.

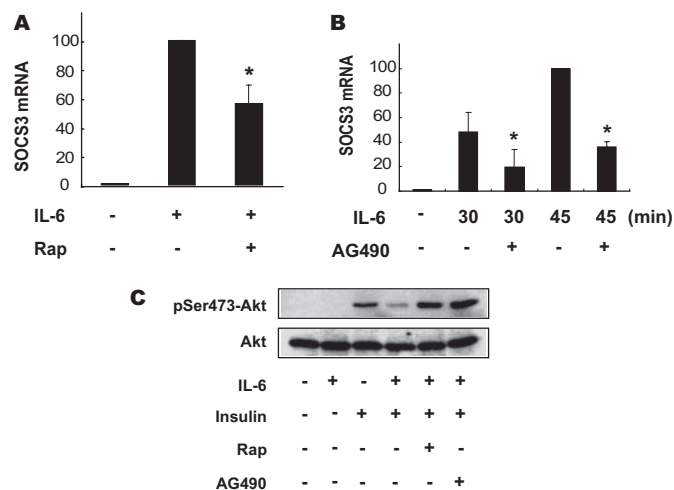


FIGURE 7. **A Jak inhibitor rescues insulin signaling from IL-6 inhibition.** A, serum-starved HepG2 cells were stimulated by 20 ng/ml IL-6 with or without 100 nM rapamycin for 1 h. Relative levels of SOCS3 mRNA were determined by quantitative RT-PCR. The average results of three independent experiments are shown with error bars representing S.D. \*,  $p < 0.01$  compared with IL-6 stimulation in the absence of rapamycin (Student's *t* test). B, serum-starved HepG2 cells were stimulated by 20 ng/ml IL-6 for 30 or 45 min with or without 10  $\mu$ M AG490. Relative levels of SOCS3 mRNA were determined by quantitative RT-PCR. The average results of three independent experiments are shown with error bars representing S.D. \*,  $p < 0.05$  when comparing data with and without AG490 treatment at each IL-6 stimulation time point (Student's *t* test). C, serum-starved cells were stimulated with 100 nM insulin for 10 min, with or without pretreatment by 20 ng/ml IL-6 for 2.5 h. When indicated, 100 nM rapamycin or 10  $\mu$ M AG490 was added 30 min prior to stimulation. Cell lysates were subjected to Western analyses for phospho-Akt (Ser<sup>473</sup>) and total Akt. Rap, rapamycin.

reporter was observed in the presence or absence of rapamycin. These observations confirmed the requirement of Ser<sup>727</sup> phosphorylation for STAT3 activity and supported a role for mTOR as a regulator of STAT3 activation through Ser<sup>727</sup> phosphorylation. The partial inhibition of STAT3 activation by rapamycin (Fig. 5) was mirrored by the incomplete removal of Ser<sup>727</sup> phosphorylation (Fig. 4A), implying that another kinase(s) may also contribute to the phosphorylation of Ser<sup>727</sup> in these cells.

**STAT3 Is Required for IL-6 Inhibition of Insulin Signaling—**To directly assess the role of STAT3 in IL-6 modulation of insulin signaling, we knocked down STAT3 by RNAi. Two lentiviral delivered shRNAs each knocked down STAT3 by ~90% in HepG2 cells (Fig. 6). In the STAT3-depleted cells, IL-6 did

not inhibit insulin stimulation of Akt phosphorylation, and rapamycin treatment no longer had a significant effect, suggesting that both STAT3 and mTOR are critical components of the cross-talk between IL-6 and insulin signaling. Interestingly, a slight increase of phospho-Akt was repeatedly observed upon IL-6 treatment in STAT3 knockdown cells. The mechanism underlying this phenomenon is not clear, but one might speculate that the removal of STAT3 eliminated the negative feedback control by SOCS3, leading to enhanced Jak activation

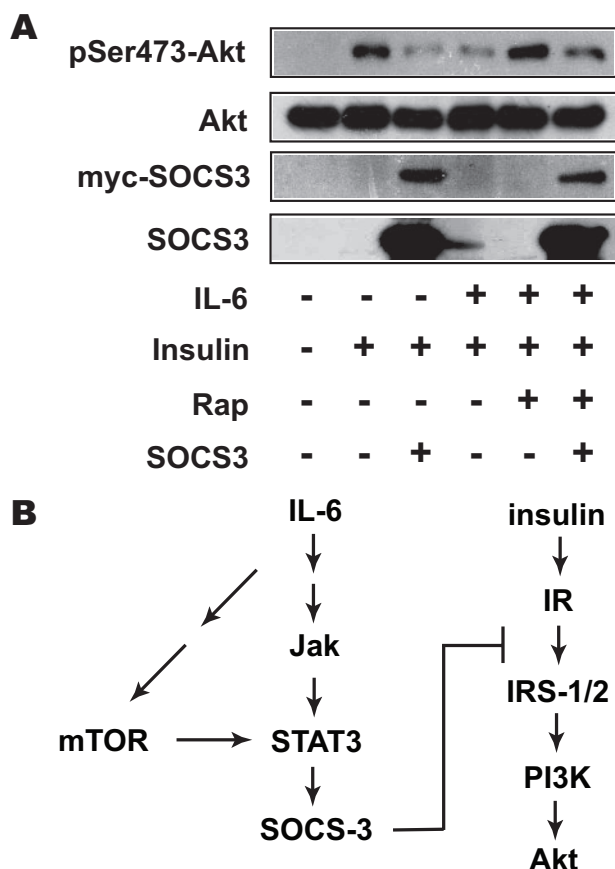
by IL-6, and hyperactive Jak might contribute to Akt activation by directly phosphorylating IRS on tyrosine residues, a cross-talk frequently observed with Jak signaling (47).

**SOCS3 Mediates the Role of mTOR in IL-6 Inhibition of Insulin Signaling—**Induction of SOCS3 by STAT3 activation has been reported to mediate the negative effect of IL-6 on insulin signaling. If mTOR indeed acts through STAT3 activation to modulate IL-6-induced insulin resistance, one would expect SOCS3 to play a key role. The mRNA level of SOCS3, measured by quantitative RT-PCR, was acutely induced by IL-6 treatment in HepG2 cells, and this induction was reduced by ~50% upon the addition of rapamycin (Fig. 7A). This partial inhibition by rapamycin corresponds with the partial effect of rapamycin on STAT3 Ser<sup>727</sup> phosphorylation and activation (Figs. 4A and 5). It is noteworthy that although rapamycin only partially blocked SOCS3 mRNA expression, the SOCS3 protein was undetectable in rapamycin-treated cells (Fig. 8A, bottom panel, lane 5), implying that rapamycin might act at an additional level to inhibit SOCS3 protein production. However, detection of the endogenous SOCS3 protein had been difficult with available antibodies. As a result, we were not certain that the lack of a Western signal in rapamycin-treated cells could be interpreted as ablation of the SOCS3 protein.

Since the physiological relevance of only a partial inhibition of the STAT3-SOCS3 pathway remained to be established, we wished to investigate whether incomplete inhibition of SOCS3 expression would be sufficient to lead to the drastic rescue of insulin signaling by rapamycin. Although the issue could not be addressed directly without certain difficulty, further observations of Jak inhibition could provide important insight. AG490 is a Jak inhibitor that blocks STAT3 tyrosine phosphorylation and subsequent activation. As shown in Fig. 7B, AG490 inhibition of SOCS3 mRNA production was also partial. Nevertheless, cells administered an identical AG490 treatment and then assessed for Ser<sup>473</sup> phosphorylation of Akt were found to have fully rescued insulin signaling from IL-6 inhibition (Fig. 7C). Thus, it is likely that a certain threshold of SOCS3 levels may be required for its action on insulin signaling, so that a reduction of SOCS3 below the threshold is sufficient to block its inhibitory effect on insulin signaling.

We reasoned that if SOCS3 indeed acted downstream of mTOR to exert a negative impact on insulin signaling, ectopic





**FIGURE 8. SOCS3 mediates the role of mTOR in IL-6 inhibition of insulin signaling.** A, HepG2 cells transfected with Myc-SOCS3 or empty vector were serum-starved and then stimulated with 100 nM insulin for 10 min, with or without pretreatment by 20 ng/ml IL-6 for 2.5 h. When indicated, 100 nM rapamycin was added 30 min prior to stimulation. Cell lysates were subjected to Western analyses with various antibodies as indicated. B, a proposed model for mTOR regulation of IL-6-induced insulin resistance (for details, see "Results"). Rap, rapamycin.

expression of SOCS3 would override the ability of rapamycin to reverse IL-6 induced insulin resistance. To probe this theory, we transiently expressed a Myc-tagged SOCS3 in HepG2 cells and enriched the transfected cells by brief drug selection. As shown in Fig. 8A, the recombinant SOCS3 was expressed at a level far exceeding that of IL-6-induced endogenous SOCS3 (*bottom panel*), and it effectively reduced insulin activation of Akt (*top panel, lane 3*), consistent with a previous report (14). Importantly, the positive effect of rapamycin on insulin-activated Akt in the presence of IL-6 was significantly blocked by the expression of the recombinant SOCS3 (Fig. 8A, compare the *last two lanes*). Taken together, these observations are consistent with a model in which SOCS3 plays a key role in mediating mTOR involvement in IL-6-induced insulin resistance.

## DISCUSSION

From a recent collective body of work, mTOR signaling has emerged as a key player in insulin resistance resulting from a variety of conditions, including excessive amino acid concentrations, hyperinsulinemia, chronic insulin stimulation, and deletion of TSC1/2. Our observations described here unravel for the first time a critical role for mTOR signaling in cellular insulin resistance induced by IL-6, a proinflammatory cytokine

known to mediate obesity-derived chronic inflammation. More importantly, our results suggest a mechanism distinct from the currently established model for mTOR modulation of insulin sensitivity. All previously reported cases of mTOR inhibition of insulin signaling involve serine phosphorylation of IRS and subsequent inactivation or degradation of IRS. The downstream target of mTOR signaling, S6K1, is responsible for the phosphorylation of several relevant serine sites on IRS-1 (37), although mTOR itself has also been proposed to be a candidate kinase for at least one of the sites on IRS-1 (31). Deletion of the S6K1 gene in mice ablated this negative feedback loop, resulting in protection of insulin sensitivity on a high fat diet in these mice (43). In contrast, we have found that despite its activation by IL-6, S6K1 does not play a unique, if any, role in IL-6-induced insulin resistance, as evidenced by the persistent IL-6 effect in cells with significantly reduced S6K1 levels as a result of RNA interference (Fig. 3B). Furthermore, none of the previously characterized rapamycin-sensitive serine phosphorylation sites on IRS-1, including Ser<sup>307</sup>, Ser<sup>312</sup>, and Ser<sup>636/639</sup>, contributes to IL-6 action on insulin signaling (data not shown). Instead, our data strongly support a novel model (Fig. 8B), in which mTOR impinges on IL-6 signaling by regulating STAT3 phosphorylation and activation, and the subsequent expression of SOCS3 inhibits insulin signaling, presumably by one or several mechanisms reported in the literature (16).

Phosphorylation of Ser<sup>727</sup> on STAT3 has long been proposed to be required for maximal activation of STAT3 (20, 21). Our observation that inhibition of Ser<sup>727</sup> phosphorylation by rapamycin abolishes the capacity of IL-6 to impact insulin signaling underscores the notion that Ser<sup>727</sup> phosphorylation is essential for STAT3 function. Many kinases have been found to phosphorylate Ser<sup>727</sup>, and the specificity appears to be dependent on the cell type or context (22). mTOR has been reported to be a kinase for Ser<sup>727</sup>, but this connection has been limited to cells of neuronal lineage thus far (44, 45). Our studies reported here implicate mTOR as a kinase for STAT3 outside of neurons, suggesting that the mTOR-STAT3 relationship may be a prevalent one and should be examined in other biological contexts in the future.

Interestingly, the catalytic activity of mTOR, as measured by its autophosphorylation, is drastically stimulated by IL-6 treatment in HepG2 cells, which correlates well with the increase of STAT3 Ser<sup>727</sup> phosphorylation (Fig. 4). Previously, another cytokine, CNTF, has also been reported to activate mTOR kinase activity (44). It is not clear whether the canonical Jak pathway initiated by cytokines regulates mTOR catalytic activity; although a Jak inhibitor blocked IL-6-induced S6K1 activation, it did not affect mTOR autophosphorylation (data not shown). The mechanism underlying the activation of mTOR catalytic activity by cytokines presents an intriguing question for future investigation. A potentially related issue is the specificity of cytokine-activated mTOR toward STAT3. Although insulin also stimulates mTOR, it does not detectably induce STAT3 Ser<sup>727</sup> phosphorylation in HepG2 cells (data not shown). It is possible that Ser<sup>727</sup> phosphorylation is dependent on Tyr<sup>705</sup> phosphorylation, which is stimulated in these cells by IL-6 and not insulin. Alternatively, mTOR activity toward STAT3 may require additional modifications or regulators of

mTOR that are specifically conferred by cytokine receptor activation. It will certainly be interesting to ask whether the known upstream regulators of mTOR, such as TSC, Rheb, and raptor, are involved in the activation of mTOR by IL-6.

mTOR signaling is intimately involved in adipogenesis and fat metabolism. Required for preadipocyte differentiation (48–52), mTOR mediates nutrient-sensing regulation of the adipogenic transcriptional network by controlling PPAR $\gamma$  activity via a yet-to-be-identified mechanism (51). Deletion of the S6K1 gene in mice leads to increased basal lipolysis and reduced adipose mass, and the S6K1<sup>-/-</sup> mice are protected against obesity due to altered fat metabolism (43). On the other hand, double knock-out of 4E-BP1 and 4E-BP2 in mice manifests increased sensitivity to diet-induced obesity, accelerated adipogenesis, and reduced lipolysis and energy expenditure (53). Our finding that mTOR mediates the action of an adipose-derived proinflammatory cytokine on hepatic insulin sensitivity in an S6K1-independent (and presumably 4E-BP-independent) manner potentially adds a new mechanistic link to the intricate relationship between adipose function, obesity, and mTOR signaling, further cementing the crucial role of mTOR in insulin sensitivity. Future *in vivo* studies are needed to validate these *in vitro* observations, which may potentially provide a new therapeutic approach to diabetes, a disease of epidemic proportions in the Western world.

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**Regulation of Interleukin-6-induced Hepatic Insulin Resistance by Mammalian  
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