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 Ser727 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-α and interleukin (IL)3–6, are known to be associated with obesity in both human and rodent models (2–5). Although adipose-derived tumor necrosis factor-α 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 cytokine signaling 3 (SOCS3) (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 Tyr705 phosphorylation, STAT3 also requires phosphorylation on Ser727 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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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% CO2. 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 glycosylation 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 antibody was from Cell Signaling Technology, 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 (Ser473), Akt, phospho-S6K1 (Thr389), S6K1, phospho-mTOR (Ser2481), mTOR, IRS-1, phospho-IRS-1 (Ser307, Ser312, and Ser326/329), 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 3×1.6E6 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 Fugene 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′-CCGGGCCCATGATCTCACAACGCCTCATTGGTGGAGGATGTCGAGTTGTGATCTTTTTGTTTTGACATCTTTCTCAACCTTACACTGAGTTGTTGCTTCTTTGCTTTTT; negative control (scrambled sequence) were obtained from Addgene.

**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 14C-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.
to the manufacturer’s instructions. cDNA was synthesized from 2 μg 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. β-Actin was used as a reference to obtain the relative -fold change for target samples using the comparative Ct method. The primers used are as follows: β-actin forward, 5’-GAGCCATCATCCATGTCGCGGATCAGAAAGG; SOCS3 reverse, 5’-CTTCAGCTCCAAGACGAGCTA; SOCS3 forward, 5’-CTTCAGCTCCAAGACGAGCTA; SOCS3 reverse, 5’-GAGCCTTGGCCTGATCAGAAG.

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 Ser473 (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 Thr308 displayed a similar pattern, although phosphorylation at that site was less easily detectable by Western blotting than Ser473 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. 1B and C, both events were inhibited by IL-6 pretreatment, and rapamycin relieved this inhibition. However, from here on we would use phospho-Ser473 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 14C-D-glucose incorporated. A 1.7-fold increase of glycogen production was...
S6K1 is not involved 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 Thr389, 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 Ser473 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 Ser307, Ser312, and Ser536/639 (corresponding to Ser302, Ser307, and Ser532/635 in
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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 Ser727 (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 Ser727 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 Ser727 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 Ser2481) 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 Ser727 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-γ 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 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.

FIGURE 4. mTOR regulates IL-6-induced STAT3 Ser727 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 Ser727 phosphorylation of STAT3. The relative ratio of phospho-Ser727 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-Ser727 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 Ser2481 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 crosstalk between IL-6 and insulin signaling.
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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 with 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 with 20 ng/ml IL-6 for 30 or 45 min with or without 10 μM 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 μM AG490 was added 30 min prior to stimulation. Cell lysates were subjected to Western analyses for phospho-Akt (Ser473) and total Akt. Rap, rapamycin.

reporter was observed in the presence or absence of rapamycin. These observations confirmed the requirement of Ser727 phosphorylation for STAT3 activation through Ser727 phosphorylation. The partial inhibition of STAT3 activation by rapamycin (Fig. 5) was mirrored by the incomplete removal of Ser727 phosphorylation (Fig. 4A), implying that another kinase(s) may also contribute to the phosphorylation of Ser727 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 Ser727 phosphorylation and activation (Figs. 4A and 4C).

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 Ser727 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
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 Ser307, Ser312, and Ser636/639, 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 Ser727 on STAT3 has long been proposed to be required for maximal activation of STAT3 (20, 21). Our observation that inhibition of Ser727 phosphorylation by rapamycin abolishes the capacity of IL-6 to impact insulin signaling underscores the notion that Ser727 phosphorylation is essential for STAT3 function. Many kinases have been found to phosphorylate Ser727, and the specificity appears to be dependent on the cell type or context (22). mTOR has been reported to be a kinase for Ser727, 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 Ser727 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 Ser727 phosphorylation in HepG2 cells (data not shown). It is possible that Ser727 phosphorylation is dependent on Tyr705 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 PPARγ 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−/− 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 adipocyte 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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