IKKß suppression of TSC1 function links the mTOR pathway with insulin resistance

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Abstract. The proinflammatory cytokine TNFα is one of the factors that links obesity-derived chronic inflammation with insulin resistance. Activation of mTOR signaling pathway has been found to suppress insulin sensitivity through serine phosphorylation and the inhibition of IRS1 by mTOR and its downstream effector, S6K1. It remains elusive that whether the mTOR pathway has a role in TNFα-mediated insulin resistance. In the present study, we demonstrated that TNFα-IKKß-mediated inactivation of TSC1 resulted in increasing phosphorylation of IRS1 serine 307 and serine 636/639, impaired insulin-induced glucose uptake, tyrosine phosphorylation of IRS1, and the association between IRS1 and PI3K p85. Furthermore, a higher expression of pIKKß (S181), pTSC1(S511), and pS6(S240/244) was found in livers obtained from both C57BL/6J mice on a high-fat diet and B6.V-Lep$^{ob/J}$ mice. Collectively, dysregulation of the TSC1/TSC2/mTOR signaling pathway by IKKß is a common molecular switch for both cancer pathogenesis and diet- and obesity-induced insulin resistance.

Introduction

Insulin action is essential for growth, development, and metabolism. Upon stimulation with insulin, insulin receptor (IR) stimulates its intrinsic tyrosine kinase activity and phosphorylates IR substrate 1 (IRS1). The tyrosine-phosphorylated IRS1 functions as an adaptor to activate downstream phosphoinositide 3-kinase (PI3K)/AKT signaling, which leads to enhancement of glucose uptake, synthesis of protein and glycogen, and execution of the growth-promoting and metabolic effects of insulin. In contrast with the positive effect of tyrosine phosphorylation of IRS1, serine phosphorylation actually inhibits the function of IRS1 (1). Although serine phosphorylation of IRS1 induced by insulin can function as a feedback control, other factors, such as proinflammatory cytokines, also increase phosphorylation of IRS1 and function as negative regulators. A large number of kinases function as IRS1 serine kinases and play negative regulatory roles in insulin action. These include mammalian target of rapamycin (mTOR)-mediated phosphorylation of IRS1 serine 636 (Ser636) and serine 639 (Ser639) (2), ribosomal S6 kinase 1 (S6K1)-mediated phosphorylation of IRS1 serine 307 [Ser307 (mouse serine 302) (Ser302)] (3) and serine 1101 (4), IκB kinase ß (IKKß)- and c-Jun N-terminal kinase (JNK)-mediated phosphorylation of IRS1 serine 312 [Ser312 (mouse Ser307)] (5,6), and protein kinase ζ-mediated phosphorylation of IRS1 serine 323 (mouse serine 318) (7).

Increased serine phosphorylation of IRS1 leads to insulin resistance, which is commonly associated with obesity. A growing body of evidence suggests that obesity-derived chronic inflammation is a key factor for obesity-mediated insulin resistance. Of the proinflammatory cytokines involved in obesity-mediated insulin resistance, tumor necrosis factor α (TNFα) is considered a major mediator that contributes to this resistance. Elevated plasma TNFα levels are positively correlated with obesity and insulin resistance in obese humans and animals (Zucker fa/fa rats, ob/ob mice, and db/db mice) (8,9) and are significantly related to the development of type 2 diabetes. Researchers demonstrated that the depletion of TNFα by a soluble TNFα receptor improved insulin action in a murine model of obesity (9), indicating the vital role of TNFα signaling in obesity-mediated insulin resistance.

Investigators have proposed that activation of two serine kinases, IKKß and JNK, is involved in TNFα-induced insulin resistance. IKKß- and JNK-mediated phosphorylation of IRS1 Ser312 directly inhibits IR-induced tyrosine phos-

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phorylation of IRS1 and disrupts the association between IRS1 and p85 (a PI3K regulatory subunit), thereby interfering with insulin signaling. Although the involvement of IKKß and JNK in TNFα-mediated insulin resistance is generally approved, recent studies raise interesting possibilities that other mechanisms may be involved in this inhibition. For instance, activation of the mTOR pathway suppresses insulin signaling by modulating the serine phosphorylation of IRS1, which serves as a feedback regulator of the insulin signaling pathway. mTOR and its downstream effector S6K1 suppress IRS1 activity by directly phosphorylating IRS1 at Ser636/Ser639 and Ser307, respectively, which leads to desensitization of insulin signaling (3,10). Remarkably, S6k1-deficient mice are protected against nutritionally and genetically driven insulin resistance (11), and genetic loss of either tuberous sclerosis 1 (TSC1) or tuberous sclerosis 2 (TSC2) results in insulin resistance by increasing the phosphorylation of IRS1 at Ser302 (3), suggesting that hyper-activation of the mTOR signaling cascade attenuates IR signaling.

Furthermore, we recently found that TNFα activates the mTOR pathway through IKKß-mediated phosphorylation and inactivation of TSC1, which contributes to inflammation-mediated tumorigenesis via increased vascular endothelial growth factor production (12,13). Collectively, this raises the question of whether IKKß-induced mTOR activation contributes to TNFα-mediated insulin resistance. In the present study, we examined the role of phosphorylation of TSC1 by IKKß in TNFα-induced insulin resistance, and uncovered a previously unrecognized signaling mechanism distinct from the established model by which TNFα induces insulin resistance through IKKß- and JNK-mediated phosphorylation of IRS1 Ser312. Based on our present and previous findings (12), we concluded that dysregulation of the TSC1/TSC2/mTOR signaling pathway by IKKß is a common molecular switch for both cancer pathogenesis and diet- and obesity-induced insulin resistance. Our finding may provide a target for clinical intervention for treatment of diet- and obesity-induced insulin resistance.

Materials and methods

Antibodies and chemicals. We used antibodies against Myc (11667203001; Roche), S6K1 (SC-230); IRS1 (SC-559); IκBα (SC-371) (the above 3 from Santa Cruz Biotechnology), phosphorylated S6K1(T389) [pS6K1(T389), 9205]; phosphorylated 4EBP1(S65) [p4EBP1(S65), 9451]; 4EBP1 (9452); phosphorylated S6(S240/244) [pS6(S240/244)], 2215; phosphorylated IRS1(S307) {phosphorylated IRS1(S302) [pIRS1(S302) in mice], 2384}; phosphorylated IRS1(S636/639) [pIRS1(S636/639), 2388]; phosphorylated IKKß(S181) [pIKKß(S181), 2681]; phosphorylated AKT(S473) [pAKT(S473), 9271 and 9277] (the above 8 from Cell Signaling Technology), PI3K p85 (06-195); phosphorylated Tyr 4G10 (05-321) (the above 2 from Upstate Biotechnology), and actin (A2066; Sigma). Antibodies against TSC1 Ser511 phosphorylation were described previously (12). Recombinant human TNFα was purchased from Roche.

Stable transfectants. MDA-MB-453 TSC1 stable transfectants were selected and maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum and 500 μg/ml G418.

Immunoprecipitation and immunoblotting. Cells were lysed in RIPA-B buffer (20 mM Na_2HPO_4, pH 7.4, 150 mM NaCl, 1% Triton X-100) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 2 mM sodium orthovanadate, 3 μg/ml aprotinin, and 750 μg/ml benzamidine) and processed as described previously (14). For immunoprecipitation, samples were precleaned with protein A-agarose for 1 h at 4˚C. Precleaned cell lysates were immunoprecipitated with an anti-IRS1 antibody overnight at 4˚C, incubated with protein A-agarose for 3 h at 4˚C, washed with ice-cold RIPA buffer containing protease inhibitors four times, resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membranes. For immunoblotting, membranes were blocked with TBST buffer (10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.05% Tween 20) with either 5% bovine serum albumin or 5% skim milk, incubated with indicated primary antibodies, subsequently incubated with horseradish peroxidase-conjugated secondary antibodies, and detected using enhanced chemiluminescence (Amersham Biosciences).

Glucose uptake assay. An insulin-stimulated glucose uptake assay was performed for detecting insulin response, as described previously (15). Briefly, MDA-MB-453 TSC1 stable transfectants plated in a six-well plate with complete medium (Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal calf serum) were washed with phosphate-buffered saline (PBS) and serum-starved overnight. Cells were either left untreated or treated with 10 μM insulin for 30 min followed by the addition of 2-deoxy-D-[2,6-^{3}H]glucose (1 μCi/ml) and 2-deoxy-D-glucose (0.1 mM) for an additional 5 min at 37˚C. After washing three times with ice-cold PBS, cells were solubilized in 0.4 ml of 1% SDS, and the level of tritium radioactivity was determined using liquid scintillation counting. Nonspecific uptake was measured in the presence of cytochalasin B (20 μM) and subtracted from the total uptake to determine the specific level of glucose uptake.

Animal model of insulin resistance. Male 6-week-old C57BL/6J and B6.V-Lep$^{ob/J}$ (ob/ob) mice were purchased from The Jackson Laboratory and maintained in the animal care facility of the Department of Veterinary Medicine at The University of Texas M.D. Anderson Cancer Center. The C57BL/6J mice were fed either a high-fat diet (HFD) (D12331; Research Diets) or a normal chow diet (NCD), whereas the ob/ob mice were fed an NCD only. Diet-induced insulin resistance was assessed in the mice as described previously (16). After 16 weeks of feeding on these diets, the mice were sacrificed, and frozen sections of their livers were prepared for immunofluorescent staining. All animal studies were performed following an animal protocol approved by the Institutional Animal Care and Use Committee at M.D. Anderson Cancer Center.

Immunofluorescent staining. Immunofluorescent staining of the frozen liver sections was performed as described previously (17). Briefly, 4-μm frozen sections were fixed; washed with

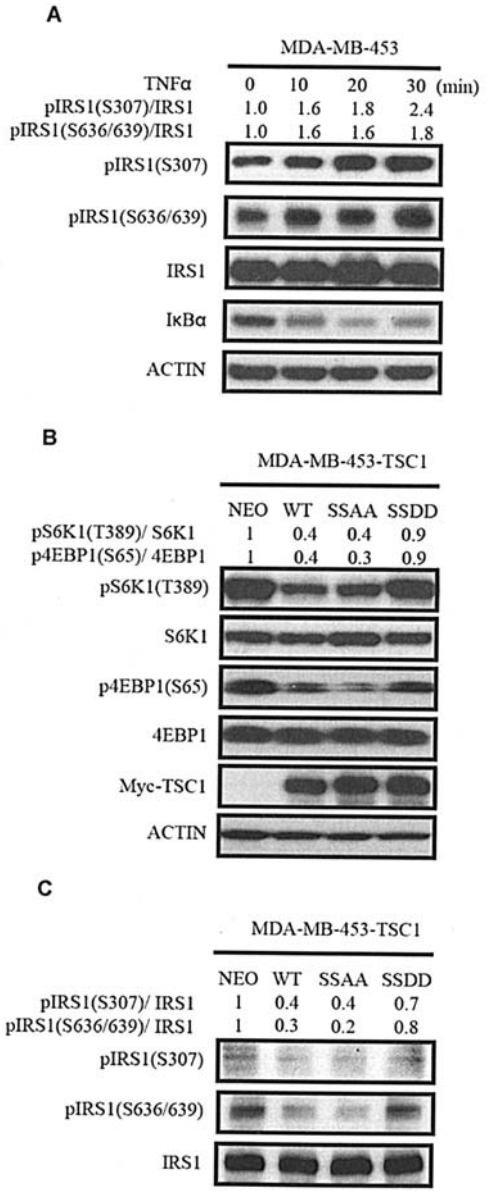

Figure 1. TNFα-mediated insulin resistance via inappropriate activation of the TSC1/mTOR/S6K1 signaling cascade. A) Phosphorylation of IRS1 Ser307 and Ser636/639 was induced by TNFα in MDA-MB-453 cells. Cells were serum-starved overnight and then treated with 20 ng/ml TNFα in a time-course study. Cell extracts were prepared and resolved using SDS-PAGE and immunoblotted with anti-pIRS1(S307), anti-pIRS1(S636/639), anti-IRS1, anti-IκBα, and anti-ACTIN antibodies. B) Endogenous expression levels of pS6K(T389) and p4EBP1(S65) were measured in MDA-MB-453 cells stably transfected with various TSC1 mutants or a vector control (NEO). MDA-MB-453-TSC1 stable transfectants were selected based on the equal amount of TSC1 expression in the transfectants. Transfectants were serum-starved overnight and lysed with RIPA-B buffer with protease inhibitors. Extracts of stable transfectants were resolved using SDS-PAGE and immunoblotted with anti-pS6K1(T389), anti-S6K1, anti-p4EBP1(S65), anti-4EBP1, anti-Myc, and anti-ACTIN antibodies. C) The expression levels of pIRS1(S307) and pIRS1(S636/639) were higher in MDA-MB-453-TSC1(SSDD) than in MDA-MB-453-TSC1(SSAA) stable transfectants. MDA-MB-453-TSC1 stable transfectants were serum-starved overnight and lysed with RIPA-B buffer with protease inhibitors. Extracts of stable transfectants were resolved using SDS-PAGE and immunoblotted with anti-pIRS1(S307), anti-pIRS1(S636/639), and anti-IRS1 antibodies.

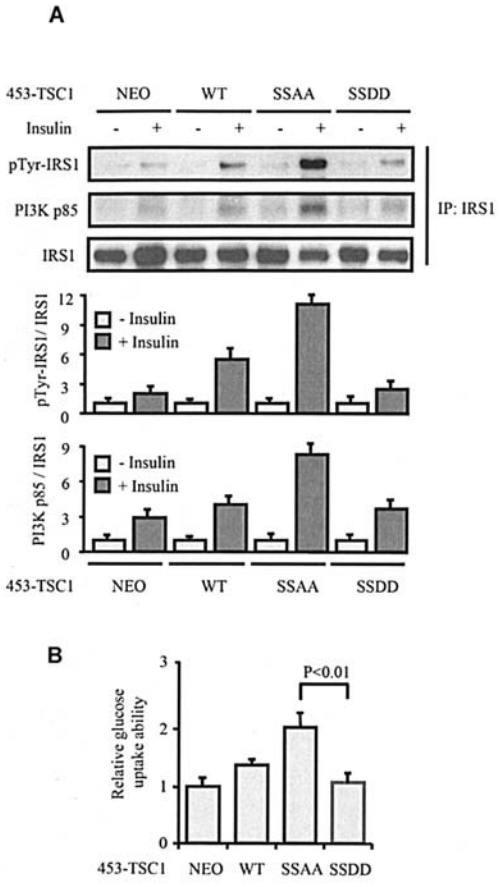

Figure 2. IKKß-mediated TSC1 phosphorylation impairs insulin action. A) Insulin-induced tyrosine phosphorylation of IRS1 and the association between IRS1 and PI3K p85 were significantly higher in MDA-MB-453-TSC1(SSAA) than in MDA-MB-453-TSC1(SSDD) stable transfectants. MDA-MB-453-TSC1 stable transfectants were serum-starved overnight and then treated with 10 μM insulin for 30 min. Cell extracts were prepared and immunoprecipitated with an anti-IRS1 antibody. The immunoprecipitated extracts were resolved using SDS-PAGE and immunoblotted with anti-pTyr, anti-p85, and anti-IRS1 antibodies. The experiment was quantified to yield the relative ratio of pTyr-IRS1 or PI3K p85 to total IRS1. The plots represent the mean of three independent experiments, and the error bars represent SD. B) Insulin-induced glucose uptake was higher in MDA-MB-453-TSC1(SSAA) than in MDA-MB-453-TSC1(SSDD) stable transfectants. 453-TSC1 stable transfectants were serum-starved overnight and treated with 10 μM insulin for 30 min followed by the addition of 2-deoxy-D-[2,6-^{3}H]glucose (1 μCi/ml) and 2-deoxy-D-glucose (0.1 mM) for an additional 5 min at 37˚C. PBS-washed transfectants were solubilized in 0.4 ml of 1% SDS, and the level of tritium radioactivity was measured using liquid scintillation counting. Nonspecific uptake was measured in the presence of cytochalasin B (20 μM) and subtracted from the total uptake to determine the specific glucose uptake level. The relative glucose uptake ability in response to insulin is shown as the average of three independent experiments. The Student's t-test was used for statistical analysis.

PBS; incubated with antibodies against pIKKß(S181), pTSC1(S511), pS6(S240/244), and pAKT(S473); incubated with goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate in the dark; and examined under a fluorescent microscope (Zeiss). The nuclei of liver cells were stained with 4,6-diamidino-2-phenylindole.

Results

TNFα-mediated insulin resistance via inappropriate activation of the TSC1/mTOR/S6K1 signaling cascade. Activation of the mTOR pathway functions as a feedback regulator of insulin action by increasing phosphorylation of IRS1 at Ser307 [pIRS(S307)] and Ser636/639 [pIRS (S636/639)] by S6K1

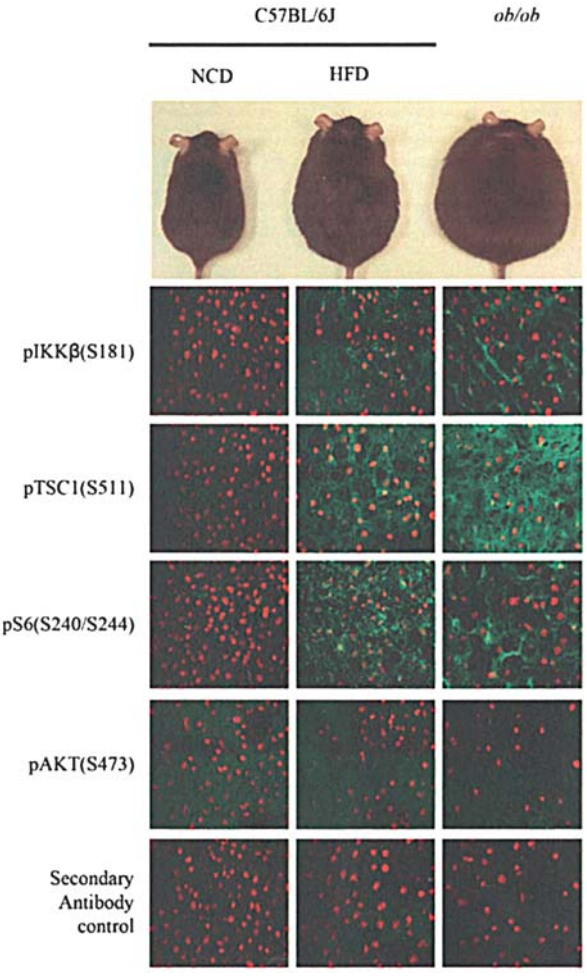

Figure 3. Up-regulation of IKKß/TSC1/mTOR/S6K1 signaling in C57BL/6J mice on an HFD and ob/ob mice. An immunostaining assay was performed to determine the pIKKß(S181), pTSC1(S511), pS6(S240/244), and pAKT(S473) status in livers obtained from the study mice. Male wild-type C57BL/6J mice were fed either an HFD or an NCD, whereas the ob/ob mice were fed an NCD only. After 16 weeks, the mice were sacrificed, and frozen liver sections obtained from them were fixed; stained with anti-pIKKß (S181), anti-pTSC1(S511), anti-pS6(S240/244), or anti-pAKT(S473) antibodies (green); and counterstained with TOPRO-3 dye (red) to visualize the nuclei of liver cells.

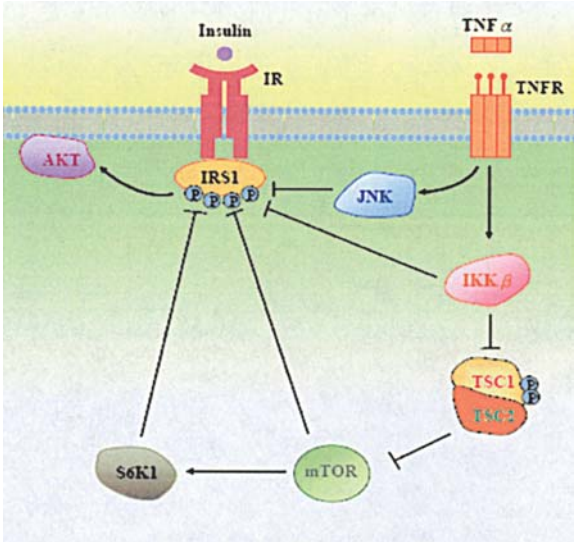

Figure 4. A model of the involvement of TNFα-mediated signaling pathways in insulin resistance. Multiple IRS1 serine kinases, including IKKß, JNK, mTOR, and S6K1, are involved in TNFα-induced serine phosphorylation and inactivation of IRS1, resulting in interference with insulin action.

(3) and mTOR (2), respectively. Also, studies showed that treatment with rapamycin prevents the development of insulin resistance by inhibiting the serine phosphorylation of IRS1 (3,10). Recently, we demonstrated that the pro-inflammatory cytokine TNFα activates the mTOR pathway and results in tumor angiogenesis (12,13). This raised the intriguing question of whether activation of the mTOR pathway contributes to TNFα-induced insulin resistance by increasing phosphorylation of IRS1 Ser307 and Ser636/639. In the present study, we found that treatment with TNFα substantially induced the expression of pIRS1(S307) and pIRS1(S636/639) in MDA-MB-453 cells within 10 to 30 min (Fig. 1A). To further address this issue, we used stable transfectants of the paired stable transfectants MDA-MB-453-TSC1(SSAA) and MDA-MB-453-TSC1(SSDD), which have constitutive suppression and activation, respectively, of the IKKß/TSC1/mTOR pathway, to determine whether suppression of TSC1 function by IKKß has a role in TNFα-mediated insulin resistance. In determining the phosphorylation status of S6K1 at T389 [pS6K1(T389)] and 4EBP1 at S65 [p4EBP1(S65)], which are two downstream phosphorylation sites of mTOR, we found higher expression of pS6K1(T389) and p4EBP1(S65) in 453-TSC1(SSDD) stable transfectants than in MDA-MB-453-TSC1(WT) or MDA-MB-453-TSC1(SSAA) stable transfectants (Fig. 1B), confirming our previous finding that IKKß phosphorylation of TSC1 activates the mTOR pathway (12,13). This result prompted us to examine whether IKKß-derived phosphorylation of TSC1 can serve as a switch for TNFα- and mTOR-induced phosphorylation of IRS1 at Ser307 and Ser636/639. Examination of the serine phosphorylation status of IRS1 [pIRS(S307) and pIRS(S636/639)] in various MDA-MB-453-TSC1 stable transfectants showed higher expression of pIRS(S307) and pIRS(S636/639) in MDA-MB-453-TSC1(SSDD) stable transfectants than in MDA-MB-453-TSC1(WT) or MDA-MB-453-TSC1(SSAA) stable transfectants (Fig. 1C), indicating that activation of mTOR signaling by IKKß-mediated phosphorylation of TSC1 increases phosphorylation of IRS1 at Ser307 and Ser636/639 through up-regulation of S6K1 and mTOR activity, respectively.

IKKß-mediated TSC1 phosphorylation impairs insulin action. Since serine phosphorylation of IRS1 has a significant role in counteracting insulin action (IR-induced IRS1 tyrosine phosphorylation and association with PI3K p85) (1,18,19), increased serine phosphorylation of IRS1 by IKKß/TSC1/mTOR signaling likely inhibits insulin response. To further validate the physiological relevance of our observations described above, we studied the effect of IKKß-mediated phosphorylation of TSC1 on insulin-induced tyrosine phosphorylation of IRS1, the association between IRS1 and PI3K p85, and the level of glucose uptake by insulin stimulation. As expected, we observed less of a response to insulin-induced tyrosine phosphorylation of IRS, less of an association between IRS1 and PI3K p85 (Fig. 2A), and a lower level of glucose uptake (Fig. 2B) in MDA-MB-453-TSC1(SSDD)

stable transfectant than in MDA-MB-453-TSC1(WT) or MDA-MB-453-TSC1(SSAA) stable transfectants. Thus, activation of the mTOR/S6K1 signaling pathway by IKKß-induced phosphorylation and inactivation of TSC1 may contribute to TNFα-mediated insulin resistance by increasing serine phosphorylation of IRS1.

Up-regulation of IKKβ/TSC1/mTOR/S6K1 signaling in C57BL/6J mice on an HFD, and in ob/ob mice. Obesity is significantly associated with insulin resistance and is a state of chronic inflammation as indicated by increased plasma concentrations of TNFα in obese humans and animals (8,9). Protection against obesity-induced insulin resistance by either knockout or neutralization of TNFα by a soluble TNFα receptor provides solid evidence supporting the concept that TNFα has an essential role in obesity-mediated insulin resistance (9). To further determine whether suppression of TSC1 function by TNFα-activated IKKß is related to obesity-induced insulin resistance, we sought to determine whether the TNFα/IKKß/TSC1/mTOR/S6K1 pathway is activated in mice on an HFD and in genetically obese mice. We measured the pIKKß(S181), pTSC1(S511), and pS6(S240/244) (which is phosphorylated by S6K1 and used as an indicator of S6K1 activity) status in frozen section of livers obtained from C57BL/6J mice that were fed the HFD and genetically obese C57BL/6J ob/ob mice, in which leptin deficiency leads to hyperglycemia, hyperinsulinemia, and insulin resistance (20). We found that the expression of pIKKß(S181), pTSC1(S511), and pS6(S240/244) was significantly higher in livers obtained from 16-week-old HFD C57BL/6J mice and ob/ob mice than in those obtained from NCD C57BL/6J mice (Fig. 3). Since insulin resistance impairs the PI3K/AKT signaling pathway, we determined the pAKT(S473) status in these livers. We observed a lower expression of pAKT(S473) in the livers obtained from HFD C57BL/6J mice and C57BL/6J ob/ob mice than in those obtained from NCD C57BL/6J mice (Fig. 3). These observations indicated that up-regulation of mTOR and S6K1 activity via suppression of TSC1 by IKKß may contribute to diet- and obesity-induced insulin resistance.

Discussion

TNFα has an important role in mediating diet- and obesity-induced insulin resistance by increasing serine phosphorylation of IRS1, which inhibits insulin action. Several studies have suggested that IKKß and JNK are central coordinators in the regulation of TNFα-induced insulin resistance (16,21,22), whereas the cellular and molecular mechanisms, by which TNFα impairs IRS1 function, are not fully elucidated. The findings of the present study of diet- and obesity-induced insulin resistance in murine models suggest that suppression of TSC1 by IKKß activates mTOR and S6K1, which in turn phosphorylate IRS1 at Ser636/639 and Ser307, thereby inhibiting IRS1 function). Based on these and previous findings, herein we propose a model in which the high levels of TNFα secreted by adipocytes and infiltrating macrophages (23) decrease the insulin response of cells to insulin through IKKß-, JNK-, mTOR-, and S6K1-mediated phosphorylation and inactivation of IRS1 in obese populations (Fig. 4). This in turn prevents glucose uptake by GLUT4 and increases glucose concentrations in the blood, symptoms of diabetes. In addition to TNFα, interleukin-6 (IL-6) is a pro-inflammatory cytokine involved in obesity-derived insulin resistance via the signal transducer and activator of transcription 3/suppressor of cytokine signaling 3 (STAT3) pathway. Notably, HFD- and obesity-derived increases in IL-6 expression may also occur due to transcriptional up-regulation by TNFα/ IKKß-induced nuclear factor κB (NF-κB) activation (22). Collectively, these findings emphasize the vital role of TNFα/IKKß signaling in diet- and obesity-induced insulin resistance.

Importantly, dysregulation of TNFα contributes not only to obesity-mediated insulin resistance but also to cancer development. We recently demonstrated that the upregulation of the IKKß/TSC1/mTOR/S6K1 signaling pathway enhances angiogenesis and culminates in breast cancer development. Clinical studies further suggest that dysregulation of this pathway is associated with poor clinical outcome of breast cancer. Findings of our present study and previous work (12,13) suggest that the interaction between IKKß and TSC1 is a molecular switch for triggering both cancer and obesity-mediated type 2 diabetes and offers a rationale for the role of obesity as a risk factor for both diseases (24).

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