Cyclin-dependent Kinase-5 Is a Key Molecule in Tumor Necrosis Factor-α-induced Insulin Resistance

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The mechanism of TNF-α-induced insulin resistance has remained unresolved with evidence for down-regulation of insulin effector targets effects or blockade of proximal as well as distal insulin signaling events depending upon the dose, time, and cell type examined. To address this issue we examined the acute actions of TNF-α in differentiated 3T3L1 adipocytes. Acute (5–15 min) treatment with 20 ng/ml (∼0.8 nM) TNF-α had no significant effect on IRS1-associated phosphatidylinositol 3-kinase. In contrast, TNF-α increased insulin-stimulated cyclin-dependent kinase-5 (CDK5) phosphorylation on tyrosine residue 15 through an Erk-dependent pathway and up-regulated the expression of the CDK5 regulator protein p35. In parallel, TNF-α stimulation also resulted in the phosphorylation and GTP loading of the Rho family GTP-binding protein, TC10α. TNF-α enhanced the depolymerization of cortical F-actin and inhibited insulin-stimulated glucose transporter-4 (GLUT4) translocation. Treatment with the MEK inhibitor, PD98059, blocked the TNF-α-induced increase in CDK5 phosphorylation and the depolymerization of cortical F-actin. Conversely, siRNA-mediated knockdown of CDK5 or treatment with the MEK inhibitor restored the impaired insulin-stimulated GLUT4 translocation induced by TNF-α. Furthermore, siRNA-mediated knockdown of p44/42 Erk also rescued the TNF-α inhibition of insulin-stimulated GLUT4 translocation. Together, these data demonstrate that TNF-α-mediated insulin resistance of glucose uptake can occur through a MEK/Erk-dependent activation of CDK5.

Adipose tissue is composed of adipocytes embedded in a loose connective tissue meshwork containing adipocyte precursors, fibroblasts, immune cells, and various other cell types. Adipose tissue was traditionally considered an energy storage depot with few interesting attributes. Because of the dramatic rise in obesity and its metabolic sequelae during the past decades, adipose tissue gained tremendous scientific interest. It is now regarded as an active endocrine organ that, in addition to releasing a large number of bioactive mediators (adipokines) modulating homeostasis, blood pressure, lipid and glucose metabolism, inflammation, and atherosclerosis. Among adipokines, resistin (1), retinol-binding protein 4 (2), interleukin-6 (3), and tumor necrosis factor-α (TNF-α) (4) are well known adipokines that can cause insulin resistance and diabetes mellitus.

Insulin resistance is a key condition for not only diabetes but also metabolic syndrome from the point of pathogenesis and as an important target of clinical treatment. Generally, insulin resistance is presented as a condition of impaired insulin-stimulated GLUT4 translocation and glucose uptake in adipose tissue and skeletal muscle (5). Currently, there are two major insulin signal pathways that are thought to regulate insulin-mediated GLUT4 translocation; one that is PI-3 kinase-dependent and another that is PI-3-kinase-independent (6). The former pathway regulates the docking and fusion step of GLUT4-containing vesicle at plasma membrane through either Akt-AS160 or Akt-Synip mechanism (7, 8). On the other hand, the latter pathway negatively regulates GLUT4 translocation through cortical the F-actin rearrangement through a C3G-TC10α mechanism (6, 9).

Recently we have reported that CDK5 is involved in the latter pathway by regulating TC10α phosphorylation on Thr-197 residue that controls TC10α localization to caveolin-enriched plasma membrane domains (10). As mentioned above, TNF-α is a well established adipokine known to induce insulin resistance through increased phosphorylation of IRS-1 on Ser-636 and -639 residues leading to PI 3-kinase inhibition (11). However, other studies have suggested that TNF-α can function independent of reduced insulin-stimulated IRS1 tyrosine phosphorylation and association with the Class I PI 3-kinase. For example, TNF-α treatment of fetal brown adipocytes for 24 h inhibited insulin-stimulated tyrosine phosphorylation and PI 3-kinase association with IRS2 but not IRS1 (12). TNF-α treatment of 3T3L1 adipocytes was found to dedifferentiate adipocytes and down-regulate adipocyte-specific gene expression (13–16). More recently, PDGF signaling that occurs independent of IRS1 was unable to rescue TNF-α-induced insulin resistance in either 3T3L1 adipocytes or L6 myotubes (17). Based upon these previous studies, we speculated the presence of an alternative pathway mediating TNF-α-induced insulin resistance. In this study we demonstrate that TNF-α can acutely regulate CDK5-dependent phosphorylation of TC10α and thereby induce insulin resistance independent of IRS1-dependent signaling.

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2 The abbreviations used are: GLUT4, glucose transporter; PI, phosphatidylinositol; CDK, cyclin-dependent kinase; A.U., arbitrary unit; C, control.
**CDK5 and TNF-α**

**MATERIALS AND METHODS**

**Reagents**—Phosphotyrosine, IRS-1, phospho-IRS-1, phospho-Erk, Erk, CDK5, phospho-CDK5, p35, PI 3-kinase, and β-actin antibodies were from Santa Cruz Biotechnology. The 85-kDa regulatory subunit of PI3-kinase antibody was obtained from Millipore. Phospho-Thr MAPK/CDK substrate monoclonal antibody was from Cell Signaling. TC10α rabbit polyclonal antibody was purchased from Sigma. F-actin antibody was from Abcam. ProteoExtract Subcellular Proteome Extraction kit was from Calbiochem. CDK5 RNAi was obtained from Invitrogen. p44/42 MAPK (Erk1/2) siRNA was purchased from Cell Signaling. p35, PI 3-kinase, and/or TNF-α and/or TNF-α monoclonal or polyclonal-specific antibody as indicated in each figure. All of the other reagents were from Invitrogen. CDK5 and TNF-α were used in the study were purchased from Sigma.

**Cell Culture**—3T3-L1 preadipocytes were cultured in DMEM containing 25 mm glucose, 10% calf serum at 37 °C with 8% CO₂. Confluent cultures were induced to differentiate into adipocytes as previously described (18).

**PI 3-Kinase Activity Assay**—PI 3-kinase activity was determined using the PI3 kinase activity assay kit (Millipore). Briefly, 3T3-L1 adipocytes were treated with or without insulin and/or TNF-α, and cell extracts were immunoprecipitated with an antibody directed against the 85-kDa regulatory subunit of the Class I PI3-kinase. PI3 kinase activity in the immunoprecipitate was determined by addition of 5× reaction buffer and phosphatidylinositol diphosphate (50 μM) for 60 min at room temperature. After the reaction was stopped, biotinylated-PIP₃ solution was added. Then samples were transferred to a microplate containing the Grip1-PH domain. The plate was incubated for 60 min, and absorbance was measured at 450 nm after extensive washing. In this assay system the synthesized PIP₃ competes for the amount of bound biotinylated-PIP₃ analogous.

**Immunoprecipitation and Immunoblotting**—Scraped frozen cells were rocked for 30 min at 4 °C with Nonidet P-40 lysis buffer (25 mM Heps, pH 7.4, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMSF, 1% Nonidet P-40, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin). Insoluble material was separated from the soluble extract by centrifugation for 30 min at 4 °C, and the total protein amount in the supernatant was determined by BCA method. After the addition of 4.5 μg of antibody to the whole cell lysates, samples (typically 2–3 mg lysates) were incubated for 2 h at 4 °C. Then 50 μl of protein A/G-agarose was added, and samples were consistently rocked for 1 h at 4 °C. Then sample was centrifuged for 10 min at 7000 × g at 4 °C. The pellet was resuspended in Extraction Buffer IV, and this suspension was subjected to SDS-PAGE and transferred to PVDF membranes for Western blotting.

**TC10 Activity Assay**—Cells were scraped with lysis/binding/wash buffer provided in the EZ-Detect™ CDC42 activation and transferred into Eppendorf tubes. After rocking for 5 min at 4 °C, sample was centrifuged for 15 min at 16,000 × g at 4 °C. 20 μg of GST-Pak1-p21 binding domain was applied to the supplied spin cup, and 700 μg of lysate was added. The sample was well mixed and rocked for 60 min at 4 °C. Then samples were washed with lysis/binding/wash buffer three times. After the TC10α bound to GST-Pak1-p21 binding domain was eluted, samples were applied on SDS-PAGE and transferred to PVDF membrane and immunoblotted with the TC10 antibody.

**Transfection of 3T3-L1 Adipocytes**—3T3-L1 adipocytes were suspended by mild trypsinization and electroporated with a total of 1 mg of plasmid under low voltage conditions (0.16 kV, 950 microfarads) as previously described (8, 10, 18). The cells were then allowed to adhere to collagen-coated tissue culture dishes for 30–48 h, and the adipocytes were then serum-starved for 4 h before incubation in the absence or presence of 100 nM insulin at 37 °C for the various time periods indicated in each figure.

**Quantification of GLUT4 Translocation**—Quantification of transfected GLUT4 translocation was determined previously described (8, 10, 18). Briefly, 3T3-L1 adipocytes were cotransfected with 400 μg of eGFP-cMyc-GLUT4 plus 600 μg of various other cDNAs as indicated in each figure. After basal or hormonal stimulation, the cells were cooled to 4 °C and incubated with an myc antibody followed by HRP-conjugated antibody bound to GST-Pak1-p21 binding domain was eluted, samples were applied on SDS-PAGE and transferred to PVDF membrane and immunoblotted with the TC10 antibody.

**Statistical Analysis**—All values are expressed as the mean ± S.E. Data were evaluated for statistical significance using the one-way analysis of variance and the Tukey-Kramer Multiple Comparison test. The minimum level of significance was set at p < 0.05. The InStat 2 program was used for statistical analysis.
RESULTS

Effect of TNF-α on IRS-1, Insulin Receptor Phosphorylation, and PI 3-Kinase Activity—It was previously reported that treatment of differentiated 3T3-L1 adipocytes with 20 ng/ml TNF-α for up to 6 h prevents secretion of other intrinsic adipokines (20). As expected, TNF-α treatment (20 ng/ml for 6 h) had no significant effect on insulin-stimulated insulin receptor β subunit autophosphorylation (Fig. 1A, upper panel). Although some studies have reported a TNF-α inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation whereas other studies have not observed any significant effect (21), consistent with the latter, we also did not detect any significant effect on insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 1A, middle panels). Consistent with these findings, we also did not detect any significant difference in insulin-stimulated PI 3-kinase activity in cells treated with or without TNF-α (Fig. 1B). However, there was a specific insulin induced increase in Ser-636 and -639 phosphorylation that corresponded to decreased IRS-1 electrophoretic mobility, although IRS-1 blotting did not show any significant difference with or without TNF-α treatment (Fig. 1A, lower two panels). Neither TNF-α nor insulin treatment had any effect on the total cellular amount of the p85 regulatory protein, as was the amount of p85 protein immunoprecipitated with the p85 antibody (Fig. 1C, lower panels). In addition, the total amount of loaded protein was identical, as determined by Ponceau S staining (data not shown). Simultaneously, the tyrosine-phosphorylated IRS-1 levels associated with PI 3-kinase were essentially identical with or without TNF-α treatment (Fig. 1C, upper panels), consistent with no significant difference in PI 3 kinase activity under these conditions (Fig. 1B).

Effect of TNF-α on p35 Expression and CDK5 Phosphorylation—The p35 protein is an established positive regulator of CDK5 activity, and TNF-α was previously reported to up-regulate p35 expression in PC12 cells (22, 23). As shown in Fig. 2, TNF-α treatment of 3T3-L1 adipocytes increased p35 protein levels (top panels) without any significant change in the protein levels of CDK5 (middle panels). However, TNF-α stimulation resulted in increased CDK5 tyrosine phosphorylation (Tyr(P)-15) in both the presence and absence of insulin (bottom panels). As Tyr-15 phosphorylation of CDK5 is directly associated with up-regulation of p35, we conclude that TNF-α treatment of 3T3-L1 adipocytes stimulates CDK5 protein kinase activity via the up-regulation of p35.

Effect of TNF-α on TC10α Phosphorylation and GTP/GDP Exchange—Previous studies have demonstrated that insulin stimulates the activation of TC10α by increasing GTP binding...
through the exchange of GTP for GDP (25). We also reported that CDK5 phosphorylates Thr-197 residue in TC10 and lets TC10 localize in lipid raft microdomains (10). This will allow for TC10 to be shifted from GDP-TC10 to GTP-TC10 (10).

Therefore, we next determined whether TNF-α stimulation also increased GTP/GDP exchange of TC10 as well as TC10 phosphorylation. As we reported previously, in the basal state TC10 was phosphorylated as detected by TC10 immunoprecipitation followed by immunoblotting with a phospho-Thr MAPK/CDK substrate monoclonal antibody (Fig. 3, top left panels). Insulin stimulation for 5 min resulted in increased TC10 phosphorylation that declined to half of the maximum phosphorylation after 15 min of insulin stimulation. Although there was no significant effect on the basal state phosphorylation, TNF-α treatment resulted in a significant enhancement of insulin-stimulated TC10 phosphorylation (top right panel) with no change in TC10 protein levels (left middle panel). Quantification of insulin- and/or TNF-α-stimulated TC10 phosphorylation is presented in the bar graph (top right). To assess the GTP/GDP exchange of TC10, we took advantage of the Pak1 binding domain that recognizes only the GTP activated states of Rho family of small GTP binding proteins (10).

![FIGURE 2. Effect of TNF-α on p35 expression and CDK5 phosphorylation.](image1)

Differentiated 3T3L1 adipocytes were treated with and without TNF-α and subsequently stimulated without and with 100 nM insulin (I) for 5 and 15 min at 37 °C as described under "Materials and Methods." Whole cell extracts were prepared, and p35, CDK5, and β-actin were immunoblotted with either a rabbit p35 specific antibody (top panel; the left panel is in dark exposure, and the right panel is in short exposure) or a rabbit-specific CDK5 antibody (second panels). CDK5 was immunoprecipitated, and phosphorylated CDK5 was immunoblotted with the phospho-CDK5 specific monoclonal antibody (third panels). These are representative immunoblots independently performed three times and are expressed as the mean ± S.D. p35 band intensity and phosphorylated CDK5 band intensity were compared with the stimulated samples with vehicle (right panels); *, p < 0.05; **, p < 0.01.

![FIGURE 3. Effect of TNF-α on TC10 phosphorylation and GTP/GDP exchange.](image2)

Differentiated 3T3-L1 adipocytes were treated with and without TNF-α and subsequently stimulated without and with 100 nM insulin (I) for 5 and 15 min at 37 °C as described under "Materials and Methods." Whole cell extracts were prepared, and TC10 was immunoprecipitated (1/2 of whole cell lysates) with a polyclonal TC10 antibody. The immunoprecipitated TC10 protein (1/5) was subjected to TC10 immunoblotting with a polyclonal TC10 antibody (middle panels), and 1/5 of the immunoprecipitated TC10 protein was immunoblotted with a phospho-Thr (pT) MAPK/CDK substrate monoclonal antibody (top panels). These are representative experiments independently performed three times. The remaining half of the whole cell lysates was subjected to GST-Pak1-p21-binding domain pulldown and immunoblotted for TC10 (bottom panels). These are representative results independently performed three times and expressed as the mean ± S.D. The band intensity was compared with the stimulated samples with vehicle (right panels); *, p < 0.05; **, p < 0.01). C, control.
CDK5 and TNF-α

Previous studies have demonstrated that lipid raft targeting of overexpressed TC10α results in the disruption of cortical F-actin and inhibition of insulin-stimulated GLUT4 translocation (26). Therefore, we examined the effects of TNF-α stimulation on adipocyte cortical F-actin polymerization by phalloidin labeling. Although cortical F-actin organization of adipocytes treated with vehicle was essentially unaffected, pretreatment of TNF-α resulted in a marked disruption of cortical F-actin (Fig. 4A). Insulin stimulation in control cells had no observable effect on phalloidin labeling, but there was greater depolymerization of cortical F-actin after insulin stimulation in the TNF-α-treated adipocytes. To quantify these data, cytoskeletal and cytosolic fractions were isolated and immunoblotted for F-actin content as previously reported (10).

FIGURE 4. Effect of TNF-α on cortical F-actin polymerization state. A differentiated 3T3-L1 adipocytes were pretreated with or without TNF-α and subsequently stimulated without and with 100 nM insulin (I) for 5 and 15 min at 37 °C as described under “Materials and Methods.” The cells were fixed, and cortical F-actin was visualized by phalloidin staining. B, polymerized actin was isolated by differential centrifugation as described under “Materials and Methods” and quantified by actin immunoblotting. These are representative experiments independently performed three times and expressed as the mean ± S.D. The band intensity was compared with the stimulated samples with vehicle (lower right panel) *, p < 0.05. C, control.

In parallel, TNF-α treatment further increased insulin-stimulated TC10α GTP loading without significantly affecting the basal level of GTP bound GTP (bottom left panels). Quantification of insulin and/or TNF-α stimulated TC10α GTP loading are presented in the bar graph (bottom right). Effect of TNF-α on Cortical F-actin Polymerization State—Previous studies have demonstrated that lipid raft targeting of overexpressed TC10α results in the disruption of cortical F-actin and inhibition of insulin-stimulated GLUT4 translocation (26). Therefore, we examined the effects of TNF-α stimulation on adipocyte cortical F-actin polymerization by phalloidin labeling. Although cortical F-actin organization of adipocytes treated with vehicle was essentially unaffected, pretreatment of TNF-α resulted in a marked disruption of cortical F-actin (Fig. 4A). Insulin stimulation in control cells had no observable effect on phalloidin labeling, but there was greater depolymerization of cortical F-actin after insulin stimulation in the TNF-α-treated adipocytes. To quantify these data, cytoskeletal and cytosolic fractions were isolated and immunoblotted for F-actin content as previously reported (10). Insulin stimulation of control cells had no effect on the amount of F-actin present in the cytoskeleton fraction (Fig. 4B, left panels). Consistent with phalloidin labeling, after TNF-α treatment there was a decrease in the amount of F-actin present in the cytoskeleton fraction that was further reduced after insulin stimulation (Fig. 4B, right panel) and is quantified in the bar graph (Fig. 4B, right panel).

Effect of MEK Inhibitor/PD98059 on TNF-α-induced CDK5 Phosphorylation and Cortical F-actin Polymerization—To explore the mechanism of TNF-α increased CDK5 activity in 3T3-L1 adipocytes, we examined the potential involvement of the MEK/Erk signal pathway. As shown in Fig. 5, TNF-α pretreatment had no effect on the basal level of Erk1/2 phosphorylation but significantly increased the extent of insulin-stimulated Erk1/2 phosphorylation. The effect of TNF-α to enhance insulin-stimulated Erk1/2 phosphorylation was abrogated by the MEK-specific inhibitor PD98059 (Fig. 6A). Under these conditions, the TNF-α-induced increase in CDK5 Tyr-15 phosphorylation was also prevented and fully restored to the levels observed essentially identical to that of in the vehicle-treated cells (Fig. 6B). In parallel, the TNF-α-induced cortical F-actin depolymerization was also prevented after the pharmacological inhibition of MEK with PD98059 (Fig. 6C). Together, these data support a pathway by which TNF-α potentiates the insulin-stimulation of the MEK/Erk pathway, leading to CDK5 activation and CDK5-dependent phosphorylation of TC10α.

Effect of CDK5 RNAi, MEK Inhibitor/PD98059, and p44/42 MAPK Erk1/2 RNAi on TNF-α-induced Impaired GLUT4 Translocation—If this model is correct, then the TNF-α inhibition of insulin signaling leading to GLUT4 translocation and glucose uptake should be dependent upon CDK5, TC10α, and MEK function. To address this, 3T3-L1 adipocytes were co-transfected with a cMyc-GLUT4-eGFP reporter and CDK5 RNAi to knock down CDK5 expression as previously reported.
As shown in Fig. 7, in control cells insulin stimulation resulted in an approximate 5-fold increase in GLUT4 translocation to the plasma membrane. In contrast, TNF-α treatment resulted in a near complete inhibition of insulin-stimulated GLUT4 translocation. Introduction of a CDK5 RNAi restored insulin-stimulated GLUT4 translocation in the TNF-α-treated...
CDK5 and TNF-α

DISCUSSION

TNF-α is an established proinflammatory cytokine that is secreted from both adipocytes and adipose tissue macrophages, playing an important contributing role in the development of insulin resistance in obesity (27). Previous studies have observed that TNF-α stimulation results in the phosphorylation of IRS-1 on Ser-636 and -639 residues uncoupling the ability of IRS-1 to activate PI 3-kinase and thus inhibiting PI 3-kinase-dependent downstream signaling events (11). However, over the past several years involvement of a PI 3-kinase-dependent signaling event in the TNF-α-induced adipocyte insulin resistance resulting from enhanced MEK/Erk activation leading to excessive CDK5 activation and TC10α GTP loading resulting in cortical F-actin depolymerization and impairment of insulin-stimulated GLUT4 translocation and glucose uptake.

FIGURE 7. Effect of CDK5 RNAi on TNF-α-induced impaired GLUT4 translocation. Differentiated 3T3-L1 adipocytes were co-transfected with the myc-GLUT4-eGFP reporter cDNA plus CDK5 RNAi as described under “Materials and Methods.” A, 48 h later, whole cell extracts were prepared, and CDK5 was immunoblotted (IB) with a rabbit CDK5-specific antibody. B, 48 h later, the whole cell extracts were treated with or without TNF-α and subsequently treated with and with 100 nM insulin (I) for 30 min as described under “Materials and Methods.” The amount of exofacial-exposed Myc epitope was quantified by the cell surface exposure of the myc epitope tag using an antibody-coupled colorimetric assay as described under “Materials and Methods.” These data were obtained from the average of five independent experiments and expressed as the mean ± S.D. (**, p < 0.01; ***, p < 0.001).

FIGURE 8. Effect of MEK inhibitor on TNF-α-induced impaired GLUT4 translocation. Differentiated 3T3-L1 adipocytes were co-transfected with the myc-GLUT4-eGFP reporter cDNA as described under “Materials and Methods.” A, 48 h later, whole cell extracts were prepared, and CDK5 was immunoblotted with a rabbit-specific CDK5 antibody or the phospho-CDK5-specific monoclonal antibody. These are representative immunoblots independently performed three times and expressed as the mean ± S.D. (**, p < 0.01; ***, p < 0.001).

FIGURE 6. Effect of MEK inhibitor/PD98059 on TNF-α-induced phosphorylation of CDK5 and cortical F-actin polymerization. A, differentiated 3T3-L1 adipocytes were treated without (left panels), with TNF-α (middle panels), or TNF-α plus PD98059 (right panels) and subsequently stimulated without and with 100 nM insulin (I) for 5 and 15 min at 37 °C as described under “Materials and Methods.” Whole cell extracts were prepared, and phosphorylated Erk and total Erk were immunoblotted with either a mouse phospho-p44/42 MAPK (Thr-202/Tyr-204)-specific antibody or a rabbit p44/42 MAPK-specific antibody. These are representative experiments independently performed three times and expressed as the mean ± S.D. The band intensity was compared with the stimulated samples with vehicle and PD98059 (bottom panels). ***, p < 0.01; B, differentiated 3T3-L1 adipocytes were treated without (left panels) or with TNF-α (middle panels) or TNF-α plus PD98059 (right panels) and subsequently stimulated with and with 100 nM insulin for 5 and 15 min at 37 °C as described under “Materials and Methods.” Whole cell extracts were prepared, and CDK5 and phosphorylated CDK5 was immunoblotted with a rabbit-specific CDK5 antibody or the phospho-CDK5-specific monoclonal antibody. These are representative immunoblots independently performed three times and expressed as the mean ± S.D. Phosphorylated CDK5 band intensity was compared with the stimulated samples with vehicle (bottom panel); *, p < 0.05; **, p < 0.01. C, differentiated 3T3-L1 adipocytes were treated without (left panels) or with TNF-α (middle panels) or TNF-α plus PD98059 (right panels) and subsequently stimulated without and with 100 nM insulin for 5 and 15 min at 37 °C as described under “Materials and Methods.” Polymerized actin was isolated by differential centrifugation as described under “Materials and Methods” and quantified by actin immunoblotting. These are representative results independently performed three times and expressed as the mean ± S.D. The band intensity was compared with the stimulated samples with vehicle (bottom panel); *, p < 0.05.
CDK5 and TNF-α

Effect of p44/42 MAPK (Erk1/2) RNAi on TNF-α-induced impaired GLUT4 translocation. Differentiated 3T3-L1 adipocytes were co-transfected with the myc-GLUT4-eGFP reporter cDNA plus p44/42 MAPK (Erk1/2) RNAi as described under “Materials and Methods.” A, 48 h later whole cell extracts were prepared, and p44/42 MAPK (Erk1/2) was immunoblotted (IB) with a rabbit p44/42 MAPK (Erk1/2)-specific antibody. B, 48 h later the cells were treated with or without TNF-α and subsequently treated without and with 100 nM insulin (I) for 30 min. The amount of exofacial-exposed Myc epitope was quantified by the cell surface exposure of the myc epitope tag using an antibody-coupled colorimetric assay as described under “Materials and Methods.” These data were obtained from the average of five independent experiments and are expressed as the mean ± S.D. **, p < 0.01; ***, p < 0.001.

Previously, we have demonstrated that TC10α is a substrate for activated CDK5, resulting in the localization of TC10α to lipid raft microdomains where it becomes accessible to the guanine nucleotide exchange factor C3G (10). Consistent with these data, TNF-α not only increased TC10α phosphorylation on the CDK5 phosphorylation site (Thr-197) but also resulted in enhanced TC10α GTP loading. Several studies have also demonstrated that overexpression of constitutively active TC10α has marked effects to depolymerize cortical F-actin in adipocytes (9). In agreement with the ability of TNF-α to activate TC10α through MEK/Erk and CDK5 pathway, TNF-α stimulation resulted in cortical F-actin depolymerization that was prevented by pharmacological inhibition of MEK activity.

Moreover, as previous studies have demonstrated that overexpressed TC10α inhibits insulin-stimulated GLUT4 translocation (30), we hypothesized that CDK5 is involved in insulin resistance induced by TNF-α stimulation, and thus, by blocking CDK5 function, we should restore impaired insulin action. To prove this, we took several different approaches. When CDK5 expression was knocked down using CDK5 RNAi gene silencing (data not shown), it is possible that TNF-α activates other CDK5-tyrosine kinases, inhibits CDK5 phosphatases, or that the p35 regulatory subunit either enhances CDK5 as a kinase substrate or reduces it as phosphatase substrate in a manner analogous to the 5′-adenosine monophosphate-activated protein kinase (29). In any case, the TNF-α-induced CDK Tyr-15 phosphorylation was dependent upon the MEK/Erk pathway, as the specific MEK inhibitor (PD98059) prevented the TNF-α-induced increase in p35 expression and CDK tyrosine phosphorylation. These data are fully consistent with a study in PC12 cells demonstrating that the TNF-α receptor/MEK/Erk/Egr1 signal pathway mediates p35 up-regulation and increased CDK5 activity (22).

Consistent with this model, we initially found that TNF-α stimulation enhanced expression of p35, a regulator of CDK5 and CDK5 phosphorylation on the Tyr-15 residue compared with vehicle-treated cells. Although Src family and Abl-tyrosine kinases are potential upstream kinases responsible for CDK5 Tyr-15 phosphorylation (28), we have been unable to detect any TNF-α-dependent increases in these kinases (data not shown). Alternatively, it is possible that TNF-α activates other CDK5-tyrosine kinases, inhibits CDK5 phosphatases, or that the p35 regulatory subunit either enhances CDK5 as a kinase substrate or reduces it as phosphatase substrate in a manner analogous to the 5′-adenosine monophosphate-activated protein kinase (29). In any case, the TNF-α-induced CDK Tyr-15 phosphorylation was dependent upon the MEK/Erk pathway, as the specific MEK inhibitor (PD98059) prevented the TNF-α-induced increase in p35 expression and CDK tyrosine phosphorylation. These data are fully consistent with a study in PC12 cells demonstrating that the TNF-α receptor/MEK/Erk/Egr1 signal pathway mediates p35 up-regulation and increased CDK5 activity (22).

Taken together, these data support a model in which MEK/Erk/p35/CDK5/TC10α/cortical F-actin pathway plays an important role in mediating insulin resistance induced by TNF-α stimulation. These data also address a physiological relevance of the PI 3-kinase-independent pathway to negatively regulate insulin stimulated GLUT4 translocation. Although many studies have reported a correlation between TNF-α-induced insulin resistance and an inhibition of IRS-1-dependent signals (11), several studies have demonstrated dissociation between insulin resistance and IRS-1. For example, in cultured
rat aortic vascular smooth cells, TNF-α treatment had no effect on IRS-1 tyrosine phosphorylation (32), and in rat skeletal muscle, acute TNF-α stimulation had no effect on either PI 3-kinase activity or IRS-1 tyrosine phosphorylation (33). Moreover, bypassing IRS-1 signals through activation of the PDGF receptor was unable to correct TNF-α-induced inhibition of glucose uptake, and insulin resistance occurs independently of IRS in 3T3-L1 adipocytes (17). Similarly insulin-treated brown adipocytes did not inhibit insulin-stimulated IRS-1-associated PI 3 kinase activity (12). Thus, TNF-α appears to have pleiotropic effects that depend on the dose, time, and specific cellular conditions examined. Nevertheless, in our experimental system we have observed a rapid TNF-α-dependent regulation of CDK5 signaling that occurs independent of IRS-1-associated PI 3 kinase. Although the observed differences between IRS-1-dependent and independent events as the proximal cause of insulin resistance remains unresolved, our data are consistent with the possibility that at least an additional pathway that is independent of IRS-1-PI 3 kinase is involved.

Recently Choi et al. (31) revealed that PPARγ is a CDK5 substrate and CDK5 negatively regulates PPARγ function. Thus, we propose that CDK5 can serve as a novel therapeutic target for the treatment of insulin resistance and type 2 diabetes mellitus from the point of improving not only by enhancing PPARγ function but also by preventing cytokine signaling that impairs insulin-stimulated GLUT4 translocation.

Acknowledgment—We thank Atsuko Miura (Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Japan) for excellent technical assistance.

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