

Endothelin-1-stimulated glucose uptake is desensitized by tumor necrosis factor- α in 3T3-L1 adipocytes

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Rachdaoui, Nadia, and Laura E. Nagy. Endothelin-1-stimulated glucose uptake is desensitized by tumor necrosis factor- α in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 285: E545–E551, 2003. First published May 28, 2003; 10.1152/ajpendo.00160.2003.—Tumor necrosis factor- α (TNF- α) is a potent inducer of insulin resistance, and increased TNF- α expression is associated with impaired glucose disposal. Although insulin is the primary regulator of glucose transport in adipose, endothelin-1, a vasoconstrictor peptide that signals through the heterotrimeric G proteins $G_{\alpha_{q/11}}$, potently stimulates glucose uptake in 3T3-L1 adipocytes by a mechanism independent of phosphatidylinositol (PI) 3-kinase. Here, we report that exposure of 3T3-L1 adipocytes to TNF- α for 48 h dose-dependently decreased endothelin-1-stimulated glucose uptake and translocation of GLUT4 to the plasma membrane. TNF- α exposure had no effect on endothelin-1 receptor number at the cell surface. In contrast, TNF- α treatment reduced the quantity of $G_{\alpha_{q/11}}$ and proline-rich tyrosine kinase 2 (PYK2) and decreased endothelin-1-stimulated PYK2-Tyr⁴⁰² tyrosine phosphorylation. Taken together, these results suggest that TNF- α -induced desensitization of endothelin-1-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes is due, at least in part, to a decreased expression of $G_{\alpha_{q/11}}$, leading to a suppression in tyrosine phosphorylation of PYK2.

adipocytes; glucose homeostasis; G protein-coupled receptors; $G_{\alpha_{q/11}}$; proline-rich tyrosine kinase 2

ALTHOUGH INSULIN IS THE PRIMARY REGULATOR of glucose uptake in muscle and adipose tissue, endothelin-1, a vasoconstrictor peptide secreted primarily by endothelial cells (29), is also a potent stimulator of GLUT4 translocation and glucose transport in 3T3-L1 adipocytes (14, 22, 36). In adipocytes, endothelin-1 action is mediated by binding to the ET_A receptor, a G protein-coupled receptor expressed in many tissues and cultured cells including 3T3-L1 adipocytes (4, 36). Although the signal transduction cascade leading from the ET_A receptor to the activation of glucose transport is not completely understood, work from a number of laboratories has identified a critical role for $G_{\alpha_{q/11}}$ in the endothelin-1 pathway (14, 17, 22). $G_{\alpha_{q/11}}$ -stimulated glucose transport is independent of phosphatidylinositol (PI) 3-kinase (3, 18, 35, 36). Instead, activation of ET_A receptor or $G_{\alpha_{q/11}}$ stimulates GLUT4 transloca-

tion via a mechanism that requires tyrosine phosphorylation (6, 36) and involves ADP ribosylation factor-6, as well as F-actin polymerization (3, 19). Endothelin-1/ $G_{\alpha_{q/11}}$ activates the *src* family tyrosine kinase Yes (13), as well as proline-rich tyrosine kinase 2 (PYK2) (22), in 3T3-L1 adipocytes. Dominant inhibitory constructs of PYK2 prevent endothelin-1 stimulation of F-actin polymerization and GLUT4 translocation (22).

Tumor necrosis factor- α (TNF- α), a cytokine secreted by activated macrophages as well as adipose tissue, is an important mediator of insulin resistance (11). TNF- α production by adipose tissue increases during the development of insulin resistance and obesity in animal models (11), and adipose tissue is one of the major and immediate targets of TNF- α (26). Although it is clear that TNF- α interferes with glucose homeostasis and lipid metabolism, contributing to the development of insulin resistance in obesity and type 2 diabetes (11), its mechanism of action is not completely understood. Exposure to TNF- α leads to serine phosphorylation of insulin receptor substrate (IRS)-1, which inhibits the tyrosine kinase activity of the β -subunit of the insulin receptor and impairs insulin-stimulated signal transduction (12, 27). Increasing evidence suggests that serine/threonine kinases, including protein kinase C and p42/44 MAP kinases, mediate this inhibitory effect of TNF- α on insulin signaling (8, 20). Additional mechanisms of TNF- α -induced insulin resistance may include decreased GLUT4 expression (30), impaired myoinositol incorporation, and Ca^{2+} mobilization (37), as well as the induction of specific proteins by TNF- α such as suppressors of cytokine signaling-1, -3, and -6, which can negatively modulate insulin signaling (7, 21).

The aim of this study was to determine whether chronic TNF- α exposure inhibits endothelin-1-stimulated glucose transport and to define the components of the endothelin-1-signaling pathway involved in the response to TNF- α exposure. We found that chronic exposure of 3T3-L1 adipocytes to TNF- α decreased endothelin-1-stimulated glucose uptake. TNF- α exposure decreased the expression of $G_{\alpha_{q/11}}$ and PYK2, resulting in a reduced endothelin-1-stimulated PYK2-Tyr⁴⁰² tyrosine phosphorylation and a subsequent decrease in glucose uptake. These results suggest that

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impaired endothelin-1 signaling may contribute to the development of impaired glucose homeostasis after chronic exposure to TNF- α .

MATERIALS AND METHODS

Materials. The murine 3T3-L1 fibroblast cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell culture reagents were purchased from GIBCO (Grand Island, NY). Antibodies were obtained from the following sources: rabbit polyclonal anti-G $\alpha_{q/11}$ (carboxy-terminal) (Calbiochem, La Jolla, CA), rabbit polyclonal anti-GLUT4 (Biogenesis, Sandown, NH), rabbit polyclonal anti-GLUT1 (Chemicon International, Temecula, CA), anti-PYK2 (BD Transduction Labs, San Diego, CA, and Santa Cruz Biotechnology, Santa Cruz, CA), phosphospecific PYK2 antibodies (Biosource International, Camarillo, CA), and anti-*c-myc* (9E10; Developmental Hybridoma Studies Bank). Goat anti-rabbit and anti-mouse IgG coupled to horseradish peroxidase (HRP) were from Roche (Indianapolis, IN). 2-Deoxy-[3 H]glucose and 125 I-labeled endothelin-1 were from Amersham (Arlington Heights, IL). Porcine insulin and endothelin-1 were purchased from Sigma (St. Louis, MO). The *c-myc*-GLUT4 expression plasmid, containing a 14-amino acid sequence of the human *c-myc* epitope inserted into the first exofacial loop of GLUT4 (16), was from Drs. G. Hermann and R. Kelly (University of California at San Francisco). Endotoxin-free plasmid kits were from Qiagen (Valencia, CA).

Cell culture and treatments. 3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum (DMEM-FBS) at 37°C in a 10% CO $_2$ atmosphere for 4 days. 3T3-L1 cells were then cultured in DMEM-FBS containing 10 μ g/ml insulin, 0.25 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for 4 days to initiate differentiation. Medium was then changed to DMEM-FBS with insulin alone, cultured for 3 more days, and then changed to DMEM-FBS for 2 or 3 days before treatment. Under these conditions, >95% of the cell population exhibited the morphological characteristics of adipocytes. TNF- α treatments were initiated 9–10 days after the start of differentiation, at which time 3T3-L1 adipocytes were cultured for 48 h with 0–10 ng/ml TNF- α in DMEM-FBS.

Uptake of 2-deoxyglucose. Uptake of 2-deoxy-[3 H]glucose was measured in 3T3-L1 adipocytes differentiated in 24-well plates. After culture with or without TNF- α for 48 h, cells were washed once with serum-free DMEM and incubated in 1 ml of serum-free DMEM for 1 h at 37°C. Cells were then washed twice in PBS containing 0.1 mM CaCl $_2$, 0.1 mM MgCl $_2$, and 25 mM HEPES, pH 7.4, and then incubated in the same buffer in the presence or absence of insulin or endothelin-1 for 30 min at 37°C. In some experiments, 100 nM wortmannin was added 15 min before the addition of insulin or endothelin-1. After agonist treatment, uptake of 10 μ M 2-deoxy-[3 H]glucose was measured over 10 min. Reactions were terminated by rapidly washing the cells twice with ice-cold PBS. Cells were then extracted in 0.1 N KOH, and aliquots from the cell extracts were counted by liquid scintillation and used to determine protein concentration. Nonspecific uptake was measured in the presence of 10 mM phloretin, an inhibitor of equilibrative glucose transport.

Transient transfection of 3T3-L1 adipocytes and *c-myc*-GLUT4 cell surface assay. 3T3-L1 adipocytes differentiated in six-well plates were transfected with 2 μ g of the *c-myc*-GLUT4 expression vector using TransFast at a 3:1 ratio according to the manufacturer's instructions (Promega, Madison, WI) for 1 h. The transfection reagent was then removed, and cells were cultured in 2 ml DMEM-FBS for 2 h. Cells

were then washed with 1 ml of warm PBS, incubated with 2 mg/ml collagenase and 0.25% trypsin for 5 min to detach cells, and then replated in 96-well plates at 33,000 cells/well. Cells were allowed to attach for 2 h before treatment with or without TNF- α for 48 h.

After 48-h treatment with TNF- α , cells were serum starved for 1 h and then stimulated with or without 100 nM insulin or 10 nM endothelin-1 for 30 min. Cell surface-accessible *c-myc*-GLUT4 were measured using a cell surface ELISA, as described by Wang et al. (34). Briefly, cells were fixed in 1% paraformaldehyde for 30 min and washed twice for 1 min in 25 mM glycine and twice in PBS. Wells were blocked for 90 min in PBS containing 2% BSA and 1% fish gelatin. Cells were then incubated for 2–3 h with antibody to *c-myc* (9E10, 2.4 μ g/ml) in blocking buffer, washed, and incubated for 1 h in anti-mouse IgG HRP (1:1,000). Cells were then washed and incubated with peroxidase substrate (Sigma) for 30 min, and the optical density at 450 μ m was measured. Nonspecific binding was measured in wells incubated without primary antibody.

Isolation of total membranes and subcellular fractions. 3T3-L1 adipocytes differentiated in 100-mm plates were cultured with 0 or 10 ng/ml TNF- α for 48 h. The cells were then serum starved for 1 h and then stimulated with or without 10 nM insulin or endothelin-1 for 30 min. Reactions were terminated by washing the 3T3-L1 adipocytes with ice-cold PBS. Plasma membrane fractions were then isolated as described previously (33) with slight modifications. Briefly, cells were homogenized in a buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, 255 mM sucrose, and protease inhibitors (Complete; Boehringer Mannheim). After a 20-min centrifugation at 16,000 g, the pellet was resuspended in the same buffer, layered on a 1.12 M sucrose pad, and centrifuged at 100,000 g for 70 min. The interface was then collected and centrifuged at 16,000 g for 15 min to pull down the plasma membrane fraction. In some experiments, total membranes were isolated by centrifuging the homogenate at 200,000 g for 60 min. Equal amounts of proteins (25–50 μ g/lane) were solubilized for 15 min at 37°C in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. Western blotting was carried out as described previously (23).

PYK2 tyrosine phosphorylation. After TNF- α treatments, 3T3-L1 adipocytes differentiated in six-well plates were stimulated with 10 nM endothelin-1 for 2 or 5 min, washed twice with ice-cold PBS, and then solubilized in 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 5 mM NaCl, 2% glycerol, 5 mM sodium fluoride, 1 mM EDTA, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, and protease inhibitors for 20 min on ice and sheared four times through a 22-gauge needle. The lysates were centrifuged at 12,000 g for 15 min to remove insoluble material. Samples (60 μ g of proteins) were then boiled in Laemmli buffer for 5 min and resolved by SDS-polyacrylamide gel electrophoresis.

Competitive 125 I-endothelin-1 binding. 3T3-L1 adipocytes differentiated in 24-well plates were cultured with or without 10 ng/ml TNF- α for 48 h. Specific 125 I-endothelin-1 binding was determined as described previously, with slight modifications (36). Briefly, the cells were placed on ice, washed, and preincubated in binding buffer (Hanks' buffered saline containing 25 mM HEPES, pH 7.4, 0.2% BSA and 0.1% glucose) with 20 pM 125 I-endothelin-1 with increasing concentrations of unlabeled endothelin-1 for 3 h at 4°C. 3T3-L1 adipocytes were then washed twice with ice-cold PBS to remove unbound radioligand and solubilized in 1 N NaOH. Bound 125 I-endothelin-1 was then counted by γ -spectrometry. Non-

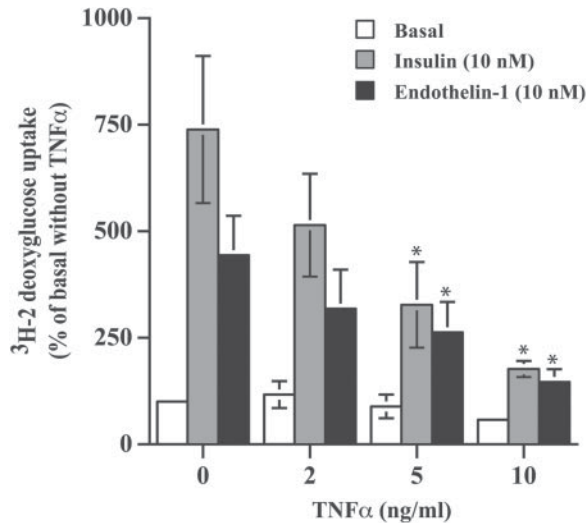


Fig. 1. Chronic TNF- α decreases insulin and endothelin-1-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured with 0–10 ng/ml TNF- α for 48 h, serum starved for 1 h, and then treated with or without 10 nM insulin or endothelin-1 for 30 min. 2-Deoxy-[3 H]glucose uptake was then measured over 10 min at 37°C, as described in MATERIALS AND METHODS. Values represent means \pm SE; $n = 4-7$. * $P < 0.05$ compared with cells not treated with TNF- α .

specific binding was determined in the presence of 1 μ M endothelin-1.

Statistical analysis. Data are presented as means \pm SE and are compared by Student's *t*-test or analysis of variance. Analysis of variance was carried out using the general linear model procedure on SAS for personal computers. Differences between treatment groups were then determined by least square means analysis. All data were tested for normal distribution.

RESULTS

TNF- α decreases endothelin-1-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes. Although many studies have demonstrated that TNF- α exposure decreases insulin-stimulated glucose transport (11), here we investigated whether chronic TNF- α interferes with endothelin-1-stimulated glucose uptake in 3T3-L1 adipocytes. In control 3T3-L1 adipocytes, endothelin-1 increased 2-deoxyglucose uptake 4.4-fold over basal (Fig. 1) compared with a 7.4-fold increase with insulin. Culture of 3T3-L1 adipocytes with 2, 5, or 10 ng/ml TNF- α for 48 h dose-dependently decreased both insulin- and endothelin-1-stimulated 2-deoxyglucose uptake (Fig. 1).

GLUT4 transporter quantity can be rate limiting for glucose uptake in adipocytes (31). We first asked whether TNF- α suppressed endothelin-1-stimulated glucose uptake by decreasing GLUT4 expression. However, chronic exposure of 3T3-L1 adipocytes to 10 ng/ml TNF- α for 48 h had no effect on total GLUT4 quantity (Fig. 2A). 3T3-L1 adipocytes also express GLUT1 transporter, and several reports have shown that TNF- α and other inflammatory cytokines increase GLUT1 expression (5). After treatment of 3T3-L1 adipocytes with 10 ng/ml TNF- α for 48 h, total GLUT1 quantity was increased 2.6-fold over control cells (Fig. 2A).

Endothelin-1 stimulates glucose uptake by promoting GLUT4 vesicle translocation to the plasma membrane. Therefore, we investigated whether the decrease in endothelin-1-stimulated glucose uptake after TNF- α treatment was associated with impaired GLUT4 translocation. In control cells, stimulation of 3T3-L1 adipocytes with 10 nM insulin or endothelin-1 increased

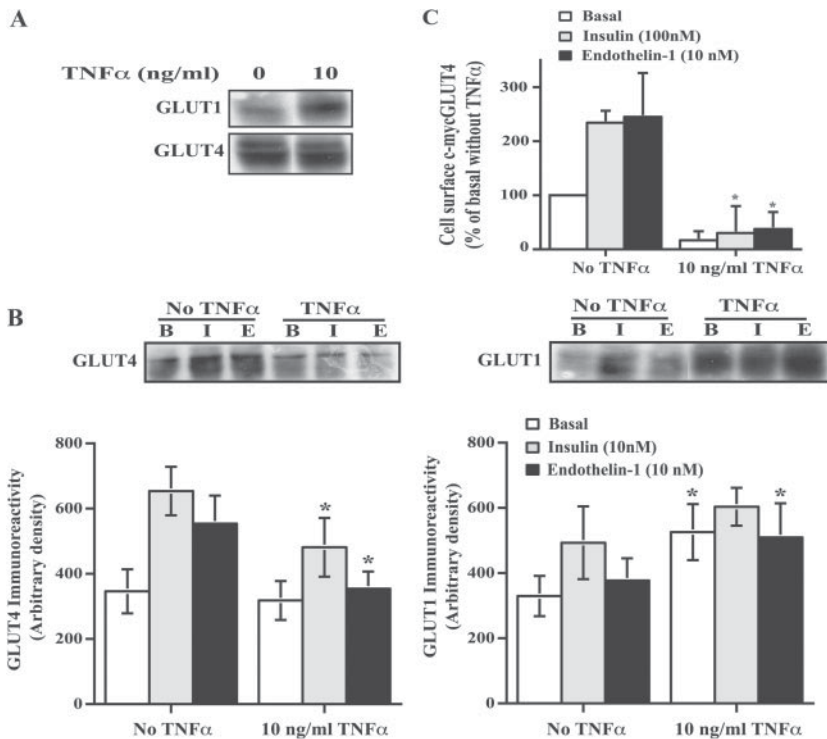


Fig. 2. Effect of TNF- α on insulin- and endothelin-1-stimulated GLUT1 and GLUT4 translocation to the plasma membrane of 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with or without 10 ng/ml TNF- α for 48 h and then serum starved for 1 h. **A:** total GLUT1 and GLUT4 content was measured by Western blotting. Images are representative of ≥ 3 experiments. **B:** 3T3-L1 adipocytes were stimulated for 20 min with or without 10 nM insulin or endothelin-1 and immunoreactive GLUT1 and GLUT4 in plasma membrane fractions measured by Western blotting. Values represent means \pm SE; $n = 3-7$. * $P < 0.05$ compared with cells not treated with TNF- α . **C:** 3T3-L1 adipocytes were stimulated for 20 min with or without 10 nM insulin or endothelin-1, and *c-myc*-GLUT4 translocation to the cell surface was assessed by cell surface ELISA, as described in MATERIALS AND METHODS. Values represent means \pm SE; $n = 3$. * $P < 0.05$ compared with cells not treated with TNF- α .

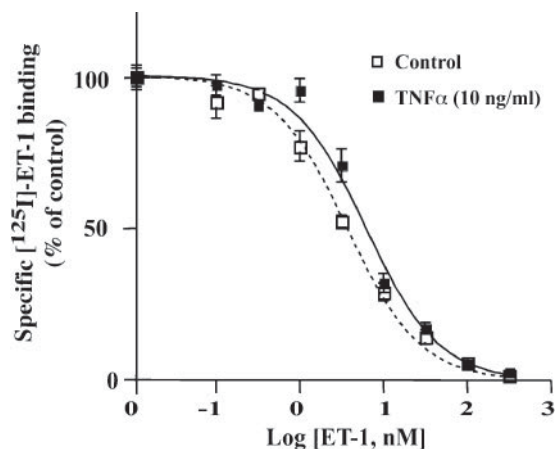


Fig. 3. Chronic TNF- α treatment did not change the endothelin (ET)_A receptor number or affinity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured with or without 10 ng/ml TNF- α for 48 h and then incubated with ¹²⁵I-labeled endothelin-1 and increasing concentrations of unlabeled endothelin-1 for 3 h at 4°C. Unbound ligand was removed, and cells were solubilized in 1 N NaOH. Bound ¹²⁵I-endothelin-1 was then measured by γ -scintillation counting. Values represent means \pm SE; $n = 6$.

GLUT4 protein at the plasma membrane 1.9- and 1.6-fold, respectively (Fig. 2B). However, after chronic exposure of 3T3-L1 adipocytes to 10 ng/ml TNF- α , GLUT4 translocation to the plasma membrane in response to insulin or endothelin-1 stimulation was reduced by 30–40% (Fig. 2B). GLUT1 translocation to the plasma membrane can also be increased by insulin in 3T3 adipocytes and contribute to insulin-stimulated glucose uptake (1). In control 3T3-L1 adipocytes, insulin increased GLUT1 quantity at the plasma membrane 1.5-fold, whereas endothelin-1 had no effect on GLUT1 translocation. Chronic treatment with TNF- α increased GLUT1 quantity at the plasma membrane at baseline, and insulin no longer stimulated GLUT1 translocation to the plasma membrane (Fig. 2B).

Chronic TNF- α exposure also decreased endothelin-1-dependent translocation of *c-myc*-tagged GLUT4 to the cell surface. The *c-myc* tag is inserted into the first exofacial loop of the GLUT4 peptide (16); immunological detection of the *c-myc* epitope at the cell surface is a measure of GLUT4 translocation (34). Stimulation of 3T3-L1 adipocytes with 100 nM insulin or 10 nM endothelin-1 increased surface-accessible *c-myc*-GLUT4 in control cells 2.6- and 3.3-fold, respectively (Fig. 2C). Chronic treatment of 3T3-L1 adipocytes with 10 ng/ml TNF- α decreased surface-accessible *c-myc*-GLUT4 in response to both insulin and endothelin-1 stimulation (Fig. 2C), consistent with the TNF- α -induced decrease in endothelin-1-stimulated 2-deoxyglucose uptake and GLUT4 translocation.

Chronic exposure to TNF- α did not change the ET_A receptor number or affinity in 3T3-L1 adipocytes. Endothelin-1 stimulates GLUT4 translocation and glucose transport via the ET_A receptor, which is the only endothelin-1 receptor isoform expressed in 3T3-L1 adipocytes (14, 35, 36). Therefore, we next asked whether suppression of endothelin-1-stimulated glucose uptake

in 3T3-L1 adipocytes in response to TNF- α was due to a decrease in the number of binding sites for endothelin-1 at the cell surface. Competition binding experiments were carried out to determine the levels of ET_A receptor density after treatment of 3T3-L1 adipocytes for 48 h with or without 10 ng/ml TNF- α . Neither the B_{max} (99.65 \pm 0.08% of control) nor the K_d (3.47 \pm 0.46 nM in control cells; 2.57 \pm 0.22 nM in TNF- α -treated cells) for ¹²⁵I-endothelin-1 binding was affected by chronic exposure of 3T3-L1 adipocytes to TNF- α (Fig. 3).

Endothelin-1 stimulation of glucose uptake in 3T3-L1 adipocytes is independent of PI 3-kinase. It is well established that insulin stimulation of GLUT4 translocation and glucose transport requires the activation of PI 3-kinase. TNF- α impairs insulin-stimulated glucose transport by inducing a serine phosphorylation of IRS-1, which impairs the ability of insulin to activate PI 3-kinase (12, 27). Endothelin-1-stimulated glucose uptake is independent of PI 3-kinase (17, 36). However, because one group (14) has reported that PI 3-kinase activity is required for endothelin-1 stimulation of glucose transport, we also examined whether PI 3-kinase is involved in the endothelin-1 stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Pretreatment of 3T3-L1 adipocytes with 100 nM wortmannin for 15 min reduced insulin-stimulated 2-deoxyglucose uptake to basal levels (Fig. 4). In contrast, wortmannin had no effect on endothelin-1-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes, consistent with other reports (17, 36) demonstrating that endothelin-1-stimulated glucose uptake in 3T3-L1 adipocytes is PI 3-kinase independent. These data suggest that the mechanisms by which TNF- α desensitizes endothelin-1-stimulated glucose uptake are independent of the effects of TNF- α on the IRS-1/PI 3-kinase pathway.

ET_A receptor couples to the G $\alpha_{q/11}$ family of heterotrimeric G proteins (4). G $\alpha_{q/11}$ is a necessary signaling

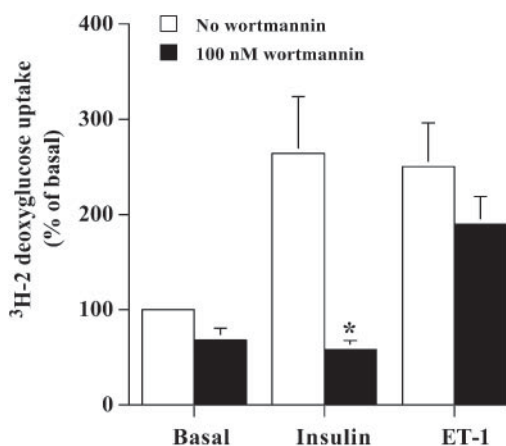


Fig. 4. Wortmannin inhibits insulin-stimulated, but not endothelin (ET)-1-stimulated, 2-deoxyglucose uptake in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were treated with or without 100 nM wortmannin for 15 min at 37°C and then stimulated with or without 10 nM insulin or endothelin-1 for 30 min. 2-Deoxy-[³H]glucose uptake was then measured over 10 min. Values represent means \pm SE; $n = 5$. * $P < 0.05$ compared with cells not pretreated with wortmannin.

intermediate in endothelin-1-induced GLUT4 translocation and glucose transport (3, 14). Because endothelin-1-stimulated glucose transport is independent of PI 3-kinase, we postulated that TNF- α -induced desensitization of endothelin-1-stimulated glucose uptake might result from decreased expression of $G_{\alpha_{q/11}}$. 3T3-L1 adipocytes were cultured with or without TNF- α for 48 h, and $G_{\alpha_{q/11}}$ quantity was measured by Western blot analysis. 3T3-L1 adipocytes expressed both G_{α_q} and $G_{\alpha_{11}}$ polypeptides (Fig. 5). Chronic exposure of 3T3-L1 adipocytes to 2 or 10 ng/ml TNF- α decreased G_{α_q} and $G_{\alpha_{11}}$ quantity by 50 and 70%, respectively (Fig. 5).

TNF- α reduced total PYK2 and endothelin-1-stimulated PYK2-Tyr⁴⁰² phosphorylation in 3T3-L1 adipocytes. Tyrosine phosphorylation is required for endothelin-1 stimulation of GLUT4 translocation and glucose transport. PYK2, a cytoplasmic tyrosine kinase known to be required for endothelin-1-stimulated glucose uptake (22), is tyrosine phosphorylated in response to endothelin-1, but not insulin, stimulation. Because chronic exposure of 3T3-L1 adipocytes to TNF- α decreased $G_{\alpha_{q/11}}$ expression, we hypothesized that endothelin-1 stimulation of PYK2 tyrosine phosphorylation would be decreased in TNF- α -treated adipocytes compared with controls. Although PYK2 can be tyrosine phosphorylated on four residues, activation of PYK2 is dependent on tyrosine phosphorylation on Tyr⁴⁰² (2). Using a PYK2-Tyr⁴⁰²-specific antibody, we found that endothelin-1 increased PYK2-Tyr⁴⁰² tyrosine phosphorylation in control cells, with a maximum phosphorylation observed after 5-min stimulation with endothelin-1 (data not shown). Chronic exposure of 3T3-L1 adipocytes to 10 ng/ml TNF- α decreased total PYK2 expression by 50% (Fig. 6). Endothelin-1

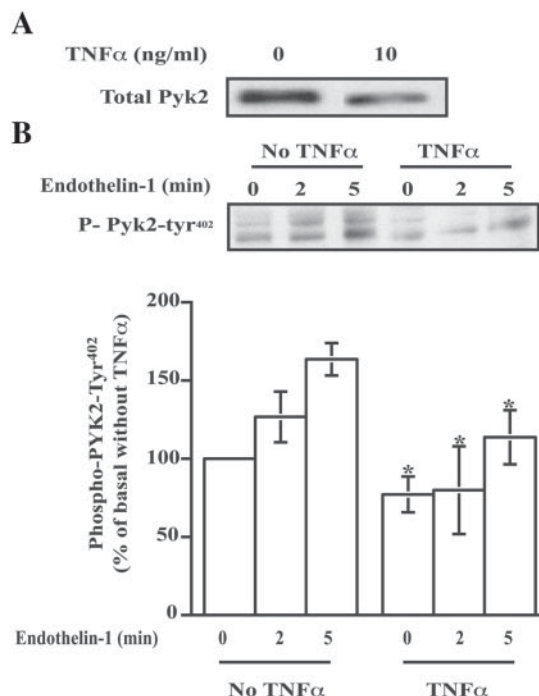


Fig. 6. Effect of chronic TNF- α treatment on total proline-rich tyrosine kinase 2 (PYK2) and endothelin-1-stimulated PYK2 tyrosine phosphorylation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured with or without 10 ng/ml TNF- α for 48 h, serum starved for 1 h, and then treated with or without 10 nM endothelin-1 for 0–5 min. Cells were then lysed and equal amounts of proteins (60 μ g) separated on an 8% SDS-PAGE, and total PYK2 (A) and tyrosine-phosphorylated (P)-PYK2-Tyr⁴⁰² (B) were measured by Western blotting. Total PYK2 quantity was decreased from 54 ± 14 arbitrary units of density in control cells to 25 ± 7 after TNF- α treatment ($n = 4$, $P < 0.05$). Values for phosphorylated PYK2-Tyr⁴⁰² represent means \pm SE; $n = 9$. * $P < 0.05$ compared with cells not cultured with TNF- α .

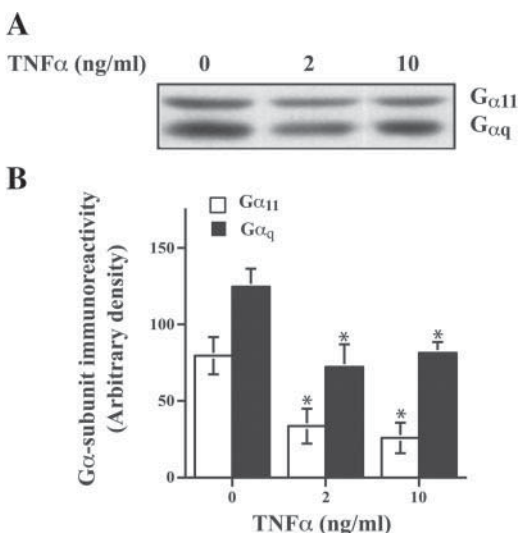


Fig. 5. Chronic TNF- α treatment decreased G_{α_q} and $G_{\alpha_{11}}$ subunit quantity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured with 0, 2, or 10 ng/ml TNF- α for 48 h. Plasma membrane-enriched fractions were then probed for immunoreactive G_{α_q} and $G_{\alpha_{11}}$ by Western blotting. Antibody to $G_{\alpha_{q/11}}$ recognized both $G_{\alpha_{11}}$ (apparent molecular mass of 43 kDa) and G_{α_q} (apparent molecular mass of 42 kDa). Values represent means \pm SE; $n = 5$. * $P < 0.05$ compared with cells not treated with TNF- α .

stimulated the phosphorylation of PYK2-Tyr⁴⁰² 1.6-fold over baseline in control adipocytes (Fig. 6). However, after chronic exposure to TNF- α , endothelin-1-stimulated tyrosine phosphorylation of PYK2-Tyr⁴⁰² was decreased to only 60–70% of the phosphorylation observed in controls (Fig. 6).

DISCUSSION

The key finding of the present investigation is the observation that chronic treatment of 3T3-L1 adipocytes with TNF- α induced a marked inhibition of endothelin-1-stimulated glucose uptake. Although TNF- α has potent inhibitory effects on insulin signaling, the extent to which this important cytokine can affect endothelin-1-stimulated glucose uptake in adipocytes has not been previously investigated. Here, we report that TNF- α exposure decreases endothelin-1-stimulated glucose uptake by impairing GLUT4 translocation to the plasma membrane. Although the signal transduction pathways leading from endothelin-1 receptor activation to GLUT4 translocation are not completely understood, it is clear that $G_{\alpha_{q/11}}$ and PYK2 are critical intermediates in this process (3, 14, 22). After chronic exposure to TNF- α , the expression of both $G_{\alpha_{q/11}}$ and PYK2 was decreased in 3T3-L1 adipocytes.

This reduction was associated with an impaired ability of endothelin-1 to stimulate the phosphorylation of PYK2-Tyr⁴⁰², demonstrating that TNF- α suppresses endothelin-1-stimulated glucose uptake by interfering with the signal transduction cascade required to activate GLUT4 translocation in 3T3-L1 adipocytes.

Prolonged exposure to TNF- α , especially at periods longer than 72 h, induces dedifferentiation of fully differentiated adipocytes, including a decrease in GLUT4 expression (8). Several reports have demonstrated that TNF- α -induced insulin resistance is associated with decreased GLUT4 expression (30, 32). However, under the conditions of TNF- α exposure used in our model system, no changes in the 3T3-L1 adipocyte morphology and lipid content were observed (data not shown). Furthermore, suppression of endothelin-1-stimulated glucose transport by TNF- α was not associated with a decrease in total GLUT4 protein quantity. Instead, decreased insulin and endothelin-1-stimulated glucose uptake after TNF- α exposure was associated with impaired GLUT4 translocation to the plasma membrane. Although chronic TNF- α exposure for 48 h had no effect on GLUT4 expression, the quantity of GLUT1 was increased. Cytokines, including TNF- α and interleukin-1, have been shown to upregulate GLUT1 and increase basal glucose uptake through GLUT1 (5). However, this increase in GLUT1 quantity in response to TNF- α exposure was not sufficient to compensate for the loss of insulin- and endothelin-1-stimulated glucose uptake.

3T3-L1 adipocytes contain a single class of endothelin-1-binding sites identified as the ET_A receptor subtype (4, 36). One possible consequence of chronic exposure to TNF- α could be a decrease in endothelin-1 receptor number and affinity leading to less endothelin-1 stimulation of glucose transport. However, competition binding experiments showed that the B_{max} and K_d for binding of endothelin-1 to the ET_A receptor were not changed by chronic exposure of 3T3-L1 adipocytes to TNF- α , suggesting that TNF- α exposure affected endothelin-1-dependent signaling downstream of the ET_A receptor.

Insulin and endothelin-1 have additive effects on glucose uptake in adipocytes (25, 36). Endothelin-1-stimulated glucose uptake in 3T3-L1 adipocytes is independent of PI 3-kinase. Instead, numerous studies have demonstrated a critical role of G $\alpha_{q/11}$ proteins in the endothelin-1-stimulated (14), as well as insulin-stimulated (15), GLUT4 vesicle translocation and glucose transport (17). Overexpression of constitutively active forms of G α_q (Q209L) or G α_{11} (Q209L) in 3T3-L1 adipocytes stimulates F-actin polymerization, GLUT4 translocation, and glucose uptake (3, 18). Conversely, microinjection of RGS2 (regulator of G protein signaling 2), a protein that inhibits G $\alpha_{q/11}$ activity, or microinjection of antibodies against G $\alpha_{q/11}$ inhibits GLUT4 translocation (14). Prolonged exposure to TNF- α has been reported to modulate the expression of heterotrimeric G proteins in different cell types (9, 10, 24, 28). In the present study, we show that chronic exposure of 3T3-L1 adipocytes to TNF- α decreased G $\alpha_{q/11}$ expres-

sion. This decrease in G $\alpha_{q/11}$ quantity likely contributes to the TNF- α desensitization of endothelin-1-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. Moreover, because G $\alpha_{q/11}$ has also been implicated in insulin-stimulated GLUT4 translocation, TNF- α -induced decrease in G $\alpha_{q/11}$ expression may also contribute, in conjunction with impaired insulin receptor signaling (11), to the effects of TNF- α on insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

Insulin and endothelin-1/G $\alpha_{q/11}$ utilize distinct signaling pathways to stimulate GLUT4 translocation and F-actin polymerization (22). PYK2, a Ca²⁺-sensitive protein tyrosine kinase, is a downstream effector in the endothelin-1/G $\alpha_{q/11}$, but not the insulin, stimulation of GLUT4 vesicle translocation to the plasma membrane (22). Dominant inhibitory constructs of PYK2 (CADTK-related nonkinase, CRNK) inhibited endothelin-1 but not insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (22). Because TNF- α -induced desensitization of insulin-stimulated glucose uptake is primarily due to impaired insulin signaling via the PI 3-kinase pathway (11), we hypothesized that the effects of TNF- α on endothelin-1-stimulated glucose uptake would reveal an additional signaling pathway susceptible to regulation by TNF- α in 3T3-L1 adipocytes. Here, we show that endothelin-1 stimulates the phosphorylation of PYK2-Tyr⁴⁰² in 3T3-L1 adipocytes. Importantly, when 3T3-L1 adipocytes were exposed to 10 ng/ml TNF- α for 48 h, expression of total PYK2 was decreased and endothelin-1 stimulation of PYK2-Tyr⁴⁰² phosphorylation was impaired. These data identify PYK2 expression and phosphorylation as new targets for regulation by TNF- α and suggest that TNF- α -induced decreases in G $\alpha_{q/11}$ -mediated signaling, in combination with decreased PYK2 protein quantity, together contribute to TNF- α -induced desensitization of endothelin-1-stimulated glucose transport.

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DISCLOSURES

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