Effect of Tumor Necrosis Factor-α on Basal and Insulin-Stimulated Glucose Transport in Cultured Muscle and Fat Cells

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It has been reported that tumor necrosis factor-α (TNF-α) inhibits insulin action in adipocytes and plays an important role as mediator of insulin resistance in non–insulin-dependent diabetes. The effect of this cytokine on insulin action in muscle, which is responsible for 80% of the glucose disposal in the body, has not been studied. Therefore, we examined the effect of TNF-α on basal and insulin-mediated transport of 2-deoxy[^{3}H]-glucose in L6 rat muscle cells. TNF-α treatment for 5 days up to a concentration of 20 ng/mL or 8 days at 10 ng/mL did not inhibit the insulin-stimulated increase in deoxyglucose transport in L6 cells. However, there was a significant increase in basal transport in TNF-α– treated cells. Comparative experiments with 3T3-L1 adipocytes showed that in cells cultured with insulin, TNF-α decreased basal transport but the insulin-stimulated increase was unaffected. In cells cultured without insulin, basal transport was slightly increased and the insulin-stimulated increase in transport was decreased by approximately 60% but the cell protein was decreased by approximately 60%, suggesting cytotoxicity. Cells cultured without insulin were more sensitive to inhibition of ^{14}C-alanine incorporation into proteins by low concentrations of TNF-α compared with cells cultured with insulin. These results suggest that TNF-α affects glucose metabolism, causing increased basal uptake in muscle cells and decreased uptake in adipocytes. TNF-α appears to affect general cell metabolism, including glucose transport in adipocytes, and not specifically insulin-stimulated glucose transport.

TUMOR NECROSIS factor-α (TNF-α), which is mainly produced by monocytes and macrophages, is one of the important pluripotent cytokines involved in cell growth and differentiation.[1-4] TNF-α mediates necrosis of several types of tumors and can be cytotoxic to several cancer cell lines in culture.[5] Nonmalignant cells are generally resistant to the cytotoxic action of TNF-α, and it has been shown that TNF-α can act as a powerful mitogen for normal fibroblasts in culture. TNF-α was found to be identical to cachectin, which is produced in significant quantities in several disease conditions caused by microbial infection and cancer.[6] It has been shown that administration of TNF-α to experimental animals can cause anorexia and weight loss.[7] However, the association between cachexia and TNF-α in humans has not been clearly established.

TNF-α affects glucose homeostasis in several tissues. Administration of TNF-α results in increased basal glucose turnover and decreased insulin-stimulated peripheral glucose utilization.[8,9] It has been reported that TNF-α plays an important role as a mediator of insulin resistance in non–insulin-dependent diabetes mellitus and obesity.[10] Adipose tissue of genetically obese and diabetic mice was found to produce high levels of mRNA for this cytokine compared with levels in lean control animals.[11] Further detailed studies have shown that TNF-α actually inhibits insulin-stimulated glucose uptake by cultured 3T3 adipocytes, and this inhibition was found to be at the level of autophosphorylation of the insulin receptor and phosphorylation of insulin receptor substrate-1.[11] A recent report from the same investigators has shown that tyrosine kinase activity of the insulin receptor is reduced in the fat and muscle of genetically obese-diabetic rats.[12] More than 80% of glucose disposal in the body is performed by the muscles. Although muscle tissue did not produce detectable amounts of TNF-α, it was proposed that TNF-α produced in fat tissue inhibited insulin receptor function in the muscle, probably through a paracrine effect.[13] However, it has been reported recently that TNF-α expression in human muscle can be detected by reverse transcriptase–polymerase chain reaction techniques, and muscle tissue from diabetic and insulin-resistant patients expressed more TNF-α than that from normal subjects.[14]

In the study reported herein, attempts were made to investigate the effect of TNF-α on insulin-stimulated glucose transport in cultured L6 rat muscle cells. These cells, which originate from neonatal rat leg skeletal muscle, are well described in the literature. They differentiate spontaneously in culture and express many metabolic and morphologic characteristics of skeletal muscle. These cells have been used in several investigations to study the insulin-mediated glucose transport system.[15,16] Our results indicate that TNF-α does not cause any impairment of insulin-stimulated transport of 2-deoxyglucose in these cells, even at high concentrations. Parallel experiments with 3T3-L1 adipocytes showed that TNF-α decreased total glucose transport in the presence of insulin. However, this was due to a decrease in basal glucose transport, and the degree of the insulin-stimulated increase in transport was unaffected by TNF-α treatment.

MATERIALS AND METHODS

Materials

Human recombinant TNF-α (1 to 10 × 10^7 IU/mg), deoxyglucose, 3-isobutyl 1-methylxanthine, and a triglyceride assay kit were obtained from Sigma Chemical (St Louis, MO). Dexamethasone phosphate was procured from American Regent Laboratories (Shirley, NY). 2-Deoxy[^{3}H]glucose was obtained from Dupont

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NEN (Boston, MA). Media, fetal bovine serum (FBS), and all reagents for tissue culture were purchased from GIBCO (Grand Island, NY). Human recombinant insulin (Novolin) was obtained from Novo Nordisk Pharmaceuticals (Princeton, NJ). All other reagents were of the best available grade.

Cell Culture

The L6 cell culture was a gift from Dr Amira Klip (The Hospital for Sick Children, Toronto, Ontario, Canada). These cells were maintained in minimum essential medium supplemented with 2% FBS and an antibiotic/antimycotic mixture containing penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin (250 ng/mL). The stock cultures were split when they were less than 80% confluent, and they were not allowed to differentiate and fuse. The medium was replenished every 2 or 3 days. For the experiments, the cells were seeded in 12-well culture dishes. TNF-α was added 5 days after seeding, when the cells began differentiating into myotubes.

3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and an antibiotic mixture containing penicillin and streptomycin. For the experiments, cells were cultured in 12-well dishes and differentiated into adipocytes using 3-isobutyl 1-methylxanthine, dexamethasone, and insulin, according to methods described by Clancy and Czech.[17] Briefly, confluent cultures were incubated with the differentiation medium containing dexamethasone (25 μmol/L), isobutyl methylxanthine (0.5 mmol/L), and insulin (100 nmol/L) in DMEM with 10% FBS for 48 hours. The cells were then maintained in a medium containing 10% FBS and insulin (10 nmol/L). TNF-α was added when greater than 90% of the cells were differentiated into adipocytes (7 to 10 days).

2-Deoxyglucose Transport

L6 cells. The culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). They were then incubated for 10 minutes with 0.5 μCi 2-deoxy[^{3}H]glucose (10 μmol/L) in 0.5 mL HEPES-buffered saline (NaCl 140 mmol/L, $CaCl_2$ 1 mmol/L, KCl 5 mmol/L, $MgSO_4$ 2.5 mmol/L, and HEPES 20 mmol/L, pH 7.4). Uptake of labeled 2-deoxyglucose was stopped by adding 100 μL 1-mol/L glucose solution. The medium was aspirated, and the cells were washed three times with PBS and dissolved in 0.5 mL 0.2-mol/L NaOH. Radioactivity in this lysate was determined by scintillation counting. Protein content in the lysate was determined using Bio-Rad reagent (Richmond, CA). To measure insulin-stimulated glucose transport, the cells were treated with insulin (100 nmol/L) for 30 minutes before the glucose transport assay. Non–carrier-mediated 2-deoxyglucose transport was determined in the presence of 10 μmol/L cytochalasin B, and it was always found to be 10% to 15% of basal transport. Correction for this non–carrier-mediated uptake did not make any difference in the results.

3T3-L1 adipocytes. Before glucose transport assay, the cells were preincubated with 1 mL serum-free DMEM for 3 to 4 hours. The cells were then washed twice with Krebs-Ringer phosphate buffer and incubated in the same buffer for 30 minutes. To determine insulin-stimulated glucose transport, insulin was added (10 nmol/L) and the incubation continued for another 30 minutes. 2-Deoxy[^{3}H]glucose was added to produce a final concentration of 0.1 mmol/L (1 μCi/100 nmol) and incubated for 10 minutes. The medium was aspirated, the cells were washed, and cell-associated radioactivity was determined as described for L6 cells.

Incorporation of ^{14}C-alanine Into Proteins

The cells in 12-well dishes were washed with PBS and incubated with 0.1 μCi ^{14}C-alanine in 1 mL PBS for 1 hour at 37°C. After this incubation, the medium was aspirated and the cells were washed three times with 2 mL PBS and lysed in 0.5 mL 0.2-mol/L NaOH. An aliquot of 10 μL was taken for protein determination, and the remaining lysate was treated with trichloroacetic acid to produce a final concentration of 15%. After 1 hour at room temperature, the samples were centrifuged and the protein pellets were washed with 10% trichloroacetic acid three times. The pellets were finally dissolved in 0.5 mL 0.1-mol/L NaOH, and radioactivity was determined in a scintillation counter.

Data Analysis

All experimental results are the mean of independent analyses from four or six wells as indicated in the figure legends. For statistical analysis, Student's *t* test was used.

RESULTS

Cultured Muscle Cells

Experiments were conducted to determine whether TNF-α had any inhibitory effect on the insulin-stimulated increase in the uptake of deoxyglucose in cultured L6 cells. Differentiated L6 cells were treated with TNF-α (5 ng/mL) for the indicated number of days, and basal and insulin-stimulated uptake of deoxyglucose was measured. The data presented in Table 1 indicate that TNF-α treatment for 5 days increased basal glucose transport by approximately 30% ($P < .001$). The results also show that insulin-stimulated enhancement of deoxyglucose transport was similar in control and TNF-α–treated cells. Experiments were performed to examine the effects of higher concentrations of TNF-α. The results presented in Table 1 show that treatment of L6 cells for 4 days with TNF-α at concentrations of 10 and 20 ng/mL increased basal glucose transport by nearly 30% ($P < .001$), but the insulin-stimulated increase in transport was similar in control and TNF-α–treated cells. When the cells were exposed to TNF-α at a concentration of 10 ng/mL for 8 days, basal glucose transport increased approximately 85%. These cells showed only about a 13% increase in glucose transport when exposed to insulin. This low degree of stimulation in response to insulin may be due to the fact that nearly

Table 1. Effect of TNF-α on Basal and Insulin-Stimulated Deoxyglucose Uptake by L6 Cells (pmol/mg cell protein)

Experiment No.	Additions	Basal	Insulin-Stimulated
1	None	56 ± 4	93 ± 8 (+62%)
	TNF-α 5 ng/mL × 5 days	73 ± 6*	120 ± 7 (+64%)
2	None	96 ± 4	146 ± 5 (+52%)
	TNF-α 10 ng/mL × 4 days	124 ± 12*	193 ± 5 (+55%)
	TNF-α 20 ng/mL × 4 days	129 ± 4*	189 ± 5 (+47%)
3	None	67 ± 3	98 ± 6 (+45%)
	TNF-α 10 ng/mL × 8 days	124 ± 14*	140 ± 12 (+13%)

NOTE. Results are the mean ± SD of independent determinations from 6 wells. Numbers in parentheses indicate the percent change in response to insulin.

*$P < .001$, control *v* TNF-α–treated cells.

maximal glucose transport has been reached under the influence of TNF-α in the absence of insulin. These results using cultured rat muscle cells are consistent with previous reports showing that TNF-α increases basal glucose transport in muscle.[8,9]

3T3-L1 Adipocytes

Experiments were conducted to study the effect of TNF-α on basal and insulin-stimulated glucose transport in differentiated 3T3 adipocytes for comparison to muscle cells. The results presented in Fig 1A show that when cells were treated with 5 ng/mL TNF-α for 5 days, basal glucose transport was decreased by 65% ($P < .001$). However, these cells responded to insulin treatment by a fourfold increase in glucose transport, similar to the control cells. Although total glucose uptake in the presence of insulin in TNF-α–treated cells was only about 50% of the uptake in control cells, the degree of insulin-stimulated increase from the basal level remained unchanged after TNF-α treatment.

In these experiments, cells were cultured in the presence of insulin (10 nmol/L) from the day of differentiation, and insulin was removed from the medium for 3 to 4 hours before the measurement of deoxyglucose uptake with and without acute exposure to insulin. The enhancement of glucose transport in response to insulin in these cells was only twofold to fourfold, whereas in cells cultured in the absence of insulin, acute exposure to insulin caused a fivefold to 15-fold increase in glucose transport.[17,18] It seemed possible that the lower degree of stimulation by insulin could be due to a residual effect of insulin on the measured basal glucose transport. To rule out this possibility, ie, that the insulin effect was not completely reversed during the 3- to 4-hour incubation without insulin and serum, we compared glucose transport after 4 and 24 hours of insulin withdrawal. Both basal and acute insulin-stimulated glucose transport were nearly the same after 4 and 24 hours of insulin withdrawal, suggesting that the insulin effect may be completely reversed after 4 hours of insulin removal (data not shown).

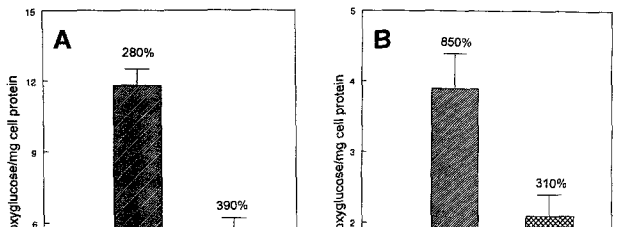

Fig 1. Effect of TNF-α on basal and insulin-stimulated glucose transport in 3T3-L1 adipocytes. Differentiated adipocytes cultured in the presence (A) or absence (B) of 10 nmol/L insulin were treated with 5 ng/mL TNF-α for 5 days. Results are the mean ± SD of 18 independent determinations from 3 experiments with 6 wells in each experiment. The percent increase in deoxyglucose uptake after acute insulin stimulation is indicated on top of the bar graph.

Chronic exposure to insulin can have complex effects on the cells, and it may be difficult to interpret the results on acute insulin response in these cells. Therefore, we decided to study the effect of TNF-α on the cells cultured in the absence of insulin except during the first 2 days of differentiation. In these cells, acute insulin treatment increased deoxyglucose transport by nearly ninefold, similar to other reported results[17,18] (Fig 1B). When these cells were exposed to TNF-α (5 ng/mL) for 5 days, basal deoxyglucose transport was slightly increased, but insulin-stimulated deoxyglucose transport was only threefold higher than the basal level, as opposed to the ninefold increase in control cells not treated with TNF-α. Thus, it appears that TNF-α blocks the insulin effect significantly in adipocytes cultured in the absence of insulin. However, TNF-α treatment resulted in a 40% to 70% decrease in cell protein, suggesting that TNF-α affected other metabolic processes when the cells were cultured without insulin. Insulin appears to have some protective effect against this "cytotoxic" effect.

Even in the presence of insulin, TNF-α treatment resulted in a significant decrease in cell protein in some experiments. (In cells cultured with insulin, the decrease in cell protein as a result of TNF-α treatment ranged from 10% to 25%, whereas in cells cultured without insulin TNF-α treatment resulted in a 35% to 65% decrease in cell protein.) Therefore, it appears that 5 ng/mL is close to the cytotoxic level. To determine if TNF-α has any deleterious effect on overall cell metabolism, the rate of incorporation of ^{14}C-alanine into protein was examined in cells cultured with and without insulin. The results shown in Fig 2 clearly indicate that the rate of incorporation of ^{14}C-alanine into proteins is more sensitive to inhibition by TNF-α in cells cultured without insulin versus cells cultured in the presence of insulin. In cells cultured with insulin, the inhibition was less than 20% ($P < .01$) at 2.5 ng/mL TNF-α, whereas in cells cultured without insulin the inhibition was approximately 35% ($P < .01$) at 0.5 μg/mL TNF-α and nearly 70% ($P < .001$) at 2.5 μg/mL.

To determine the effect of lower concentrations of TNF-α on glucose transport, a dose-response experiment was performed. The data presented in Fig 3 show that as little as 0.1 ng/mL TNF-α decreased basal glucose transport and 1 ng/mL inhibited it by approximately 50% ($P < .001$). Cell protein and triglyceride levels were only decreased by less than 20% at the highest concentration of TNF-α (data not shown). The time course of the TNF-α effect on basal glucose transport is shown in Fig 4. Within 24 hours of TNF-α treatment (5 ng/mL), basal glucose transport is decreased by about 25% ($P < .05$), and by day 5, it is decreased by nearly 80% ($P < .001$). The protein content of the cells after 5 days was decreased by approximately 20% ($P < .05$).

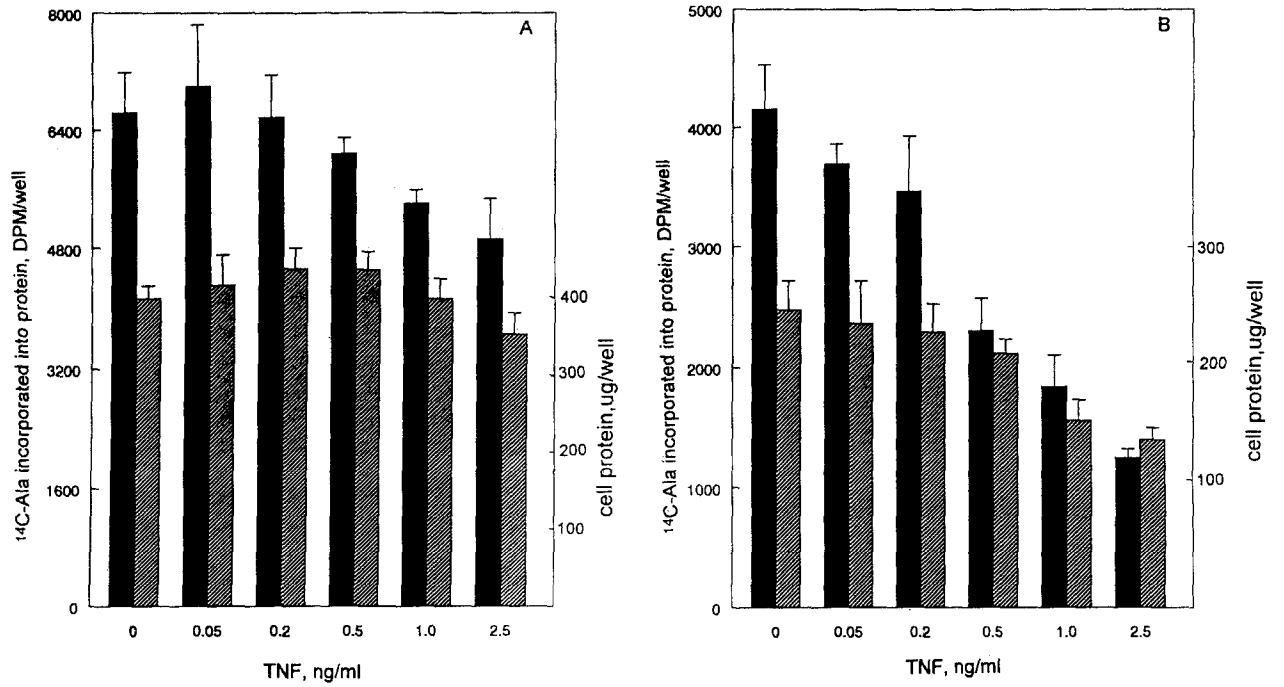

Fig 2. Effect of TNF-α on incorporation of ^{14}C-alanine into protein. Differentiated 3T3-L1 adipocytes were cultured with (A) or without (B) 10 nmol/L insulin in the medium and treated with indicated amounts of TNF-α for 4 days. The cells were then washed, and incorporation of ^{14}C-alanine (0.1 μCi/well for 1 hour at 37°C) into trichloroacetic acid–precipitable proteins was determined. Values are the mean ± SD of independent measurements from 4 dishes. (■) ^{14}C-Ala incorporation; (▨) cell protein.

The effect of TNF-α on undifferentiated 3T3-L1 fibroblasts was studied to investigate if this cytokine had any similar effect on glucose transport in other types of cells. It was found that TNF-α (5 ng/mL) stimulated basal glucose transport approximately eightfold in 3T3-L1 fibroblasts (530 ± 16 dpm control *v* 4,240 ± 420 dpm TNF-α–treated), suggesting that TNF-α has opposite effects in different cell types. It decreased basal glucose transport in fat cells, but increased it in nonfat tissue (muscle and fibroblasts).

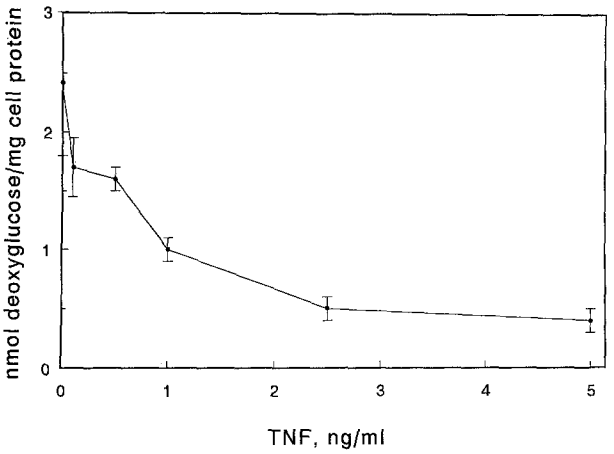

Fig 3. Dose-response of TNF-α on basal glucose transport in 3T3-L1 adipocytes. Differentiated adipocytes were cultured with 10 nmol/L insulin and treated with increasing concentrations of TNF-α for 4 days. The cells were washed and then incubated with insulin-free medium for 4 hours, and basal deoxyglucose transport was determined. Values are the mean ± SD of individual analyses from 4 dishes. Mean protein and triglyceride contents were 252 and 220 μg in the controls and 207 and 181 μg in dishes treated with 5 ng/mL TNF-α, respectively.

DISCUSSION

Our studies with L6 cells clearly show that TNF-α has no inhibitory effect on insulin-stimulated glucose transport. The data indicating that TNF-α increases basal glucose transport are in agreement with previous reports.[19,20] Although cultured cells from rat and human muscle may not be representative of muscle tissue in vivo, the effects

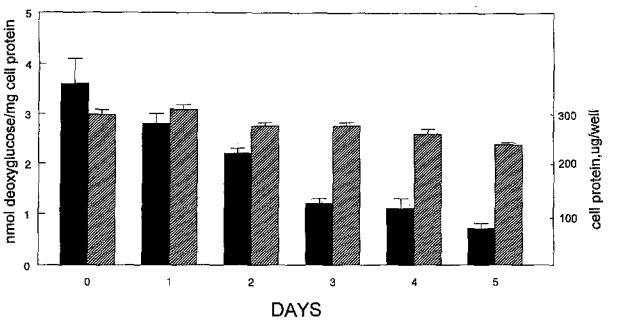

Fig 4. Time course of the effect of TNF-α on basal glucose transport. 3T3-L1 adipocytes cultured in the presence of 10 nmol/L insulin were treated with 5 ng/mL TNF-α for the indicated periods, and basal deoxyglucose uptake was measured. Values are the mean ± SD from 4 wells. (■) Deoxyglucose uptake; (▨) cell protein.

observed with these cells appear to agree well with the results obtained from infusing TNF-α in whole animals. Lang et al[9] have found that infusion of TNF-α in rats increased basal glucose disposal in the whole body and in isolated muscles. The insulin-stimulated enhancement of glucose disposal in TNF-α–treated rats was not as high as that in control animals. They have concluded that TNF-α interferes with insulin action, but this conclusion is questionable in light of the enhanced basal glucose disposal, which was subtracted from total glucose disposal to yield insulin-stimulated glucose disposal. Evans et al[21] also have observed enhanced glucose uptake by peripheral tissues in dogs infused with TNF-α.[21]

The effect of TNF-α on 3T3-L1 adipocytes varies depending on the presence or absence of insulin in the culture medium during TNF-α treatment. Hotamisligil et al[11] found that stimulation of glucose transport by insulin was inhibited in TNF-α–treated cells. These cells had been cultured in the presence of insulin, which was removed 2 hours before acute stimulation by insulin. In the present studies, when experiments were conducted with insulin in the culture medium, it was found that TNF-α caused a large decrease in basal glucose transport. However, the insulin-stimulated enhancement of glucose transport was unaffected, strongly suggesting that TNF-α interferes with metabolism in the adipocytes, resulting in decreased basal glucose transport. It was also observed in the present studies that there was a small but significant decrease in cell protein in TNF-α–treated cells, suggesting cytotoxicity. This is further substantiated by the data showing a decreased rate of incorporation of ^{14}C-alanine into protein, possibly due to decreased protein synthesis or accelerated protein degradation.

When the experiments were performed in the absence of insulin in the culture medium, the cytotoxic effect of TNF-α was more prominent. In these cells, TNF-α also inhibited insulin-stimulated glucose transport, but this was at the expense of greatly diminished cell protein possibly due to cytotoxicity. Insulin appears to offer some protective effect against TNF-α toxicity. These results also suggest that TNF-α may be inhibiting some vital functions of insulin in cell growth and metabolism, and high concentrations of insulin in the medium may be helping to overcome this effect. However, Stephens and Pekala[22,23] have not observed any cytotoxic effects of TNF-α on 3T3-L1 adipocytes even at a concentration of 85 ng/mL. Fraker et al[24] have reported that rats treated with TNF-α showed negative nitrogen balance and decreased body weight. TNF-α treatment also resulted in histopathologic abnormalities in the liver, heart, lungs, and spleen. Most of these changes were reversed when these rats were treated with insulin, suggesting a protective action of insulin against TNF-α toxicity, similar to our findings from in vitro studies.

Another important finding from our studies is that the effect of TNF-α is not same in all cells. In L6 muscle cells and 3T3-L1 fibroblasts, TNF-α stimulated basal glucose transport, whereas in 3T3-L1 adipocytes, it inhibited basal glucose transport. Cornelius et al[25] have also reported that TNF-α stimulated Glut-1 gene expression and hexose transport in 3T3-L1 fibroblasts. These results suggest that TNF-α can initiate mitogenic events in the same manner as serum-derived growth factors. Liao and Floren[26] have shown that TNF-α can augment the expression of low-density lipoprotein receptors in the human hepatoma cell line HepG2 by stimulation of de novo synthesis. There was no change in protein and DNA synthesis in the TNF-α–treated cells. Hotamisligil et al[11] have reported that TNF-α inhibited insulin signaling in 3T3 adipocytes, requiring 3 days of TNF-α treatment before inhibition could be detected. In rat hepatoma Fao cells, TNF-α inhibited insulin receptor and insulin receptor substrate-1 phosphorylation within 1 hour, according to a report by Feinstein et al.[27] Another recent report has shown that TNF-α causes a rapid loss of expression of Glut4 gene in cultured human adipocytes, resulting in decreased insulin-stimulated glucose transport.[28]

All these data from in vitro experiments suggest that TNF-α has varied effects on different cells, and it is difficult to relate these effects to the pathogenesis of insulin resistance. Even if TNF-α blocks 50% of the insulin signaling action, as demonstrated in 3T3 adipocytes,[11] can it impair the stimulation of glucose transport significantly? For many of insulin's acute biological actions, only a small percent of receptors need to be occupied.[29,30] It has also been shown that in muscle, the rate of glucose disposal is nearly maximal when the receptor kinase is activated to 50% of the maximum level.[31] These observations demonstrate the presence of "spare receptors." Therefore, 50% inhibition of insulin receptor function alone is not likely to cause any significant impairment of glucose transport.

In conclusion, the data presented herein show that TNF-α does not affect insulin-stimulated glucose transport in L6 muscle cells, whereas it inhibits insulin action in 3T3-L1 adipocytes. TNF-α also caused a decrease in cell protein content in 3T3-L1 adipocytes, but no such effect was observed in L6 muscle cells even at high concentrations and after long periods of incubation with TNF-α. It appears that there is some tissue specificity for TNF-α action on cells.

Although evidence for the association of TNF-α and insulin resistance in obesity appears to be strong, it may be difficult to conclude that insulin resistance under these conditions is caused primarily by inhibition of the initial reactions of insulin signaling by TNF-α. Our studies do not provide support for a paracrine effect of TNF-α produced by adipose tissue on muscle, the tissue responsible for the great majority of insulin-stimulated glucose disposal. Further studies are needed to understand the precise role of TNF-α in the perturbation of carbohydrate metabolism in muscle and fat cells.

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