Original Article

The Effect of Tumor Necrosis Factor- α on Tissue Specificity and Selectivity to Insulin Signaling

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Recent studies have indicated that tumor necrosis factor (TNF)- α plays a significant role in insulin resistance. It has been proposed that selective impairment of insulin signaling in glucose metabolism is related to the development of atherosclerosis, although the mechanisms are not clear. The aim of this study was to elucidate the effect of TNF- α on tissue specificity and selectivity to insulin signaling. L6 myotubes and rat aortic vascular smooth muscle cells (VSMC) were cultured. Cells were stimulated with insulin pretreated with or without TNF- α . The protein extracts were used for electrophoresis and immunoblotting studies to examine phosphorylation of insulin receptor (IR)- β , insulin receptor substrate (IRS)-1 and extracellular signal-regulated kinase (ERK). IR- β phosphorylation was not affected by TNF- α in L6 or in VSMC. TNF- α significantly ($p < 0.05$) inhibited IRS-1 phosphorylation by insulin but had no effect on ERK in L6. TNF- α had no effect on either IRS-1 phosphorylation or ERK in VSMC. Insulin induced ERK phosphorylation in a dose-dependent manner in VSMC. These results suggest that TNF- α plays a significant role in the tissue specificity and signal selectivity of insulin resistance. The pathway related to glucose metabolism is selectively impaired by TNF- α in skeletal muscle, and this impairment may induce compensatory hyperinsulinemia, which in turn would stimulate the pathway related to the cell proliferation in vascular tissues and possibly enhance the progression of atherosclerosis. (*Hypertens Res* 2003; 26: 389–396)

Key Words: insulin resistance, tumor necrosis factor- α , atherosclerosis

Introduction

Insulin resistance and hyperinsulinemia are common findings in patients with essential hypertension (1). Recent studies have indicated that insulin resistance and compensatory hyperinsulinemia may cause high blood pressure due to factors such as prompted sodium reabsorption (2), proliferation of vascular smooth muscle cells (3), and stimulation of the sympathetic nervous system and the renin-angiotensin system (4, 5). Imazu *et al.* have indicated that hyperinsulinemia is an independent predictor of the development of hypertension (6). Insulin resistance in glucose metabolism and hyperinsulinemia are associated with a high risk of cardiovascular diseases, and recent evidence indicates that they may play a

role in development of not only hypertension but also dyslipidemia and atherosclerosis (7, 8). Although evidence suggests that insulin resistance might be tissue specific, little is known about the possible mechanisms of this specificity. Skeletal muscle is a major site for insulin-dependent disposal of circulating glucose. Insulin, after binding to the insulin receptor (IR), stimulates a tyrosine autophosphorylation of IR, which activates the receptor kinase and allows transient interaction with intracellular protein substrates, including the insulin receptor substrate (IRS-1/2). The IRS-1/IRS-2 coupled IR activates metabolic events controlled by phosphatidylinositol (PI)-3 kinase (9). PI-3 kinase regulates metabolic effects of insulin, such as glucose uptake, gluconeogenesis, glycogen metabolism, and inhibition of lipolysis. On the other hand, insulin action in the vasculature

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mediated *via* both insulin and insulin-like growth factor (IGF)-I receptors has been proposed to contribute to atherosclerosis and the regulation of vascular tone (10). It has also been indicated that activation of the mitogen-activated protein kinase (MAPK) cascade, which is downstream of IRS-1 or Shc, is one of the major pathways for the regulation of proliferation and cell growth in various cultured cells (11, 12). It has been reported that activation of IRS-1 and Shc can lead to the activation of MAPK after insulin binds to the IR (13). Jiang *et al.* proposed that there is a selective impairment of insulin signaling not in MAPK cascades but in the PI-3 kinase pathway (14), and thus insulin resistance in glucose metabolism and compensating hyperinsulinemia may activate MAPK cascades and lead to atherosclerosis. However, the mechanisms of selective insulin resistance have not been clarified.

Tumor necrosis factor (TNF)- α is a multipurpose cytokine that modulates immune functions, induces apoptotic cell death (15) and lysis of tumor cells (16), and stimulates production of other cytokines (17). Recent studies have suggested that TNF- α mediates insulin resistance by suppressing insulin signal transduction; TNF- α induces enhancement of serine phosphorylation of IRS-1, resulting in inhibition of IRS-1 tyrosine phosphorylation by insulin. TNF- α is expressed in both adipose tissue and skeletal muscle, and many animal models of obesity and insulin resistance are associated with significantly higher levels of TNF mRNA and protein than corresponding lean controls (17). We recently reported that insulin resistance and hypertension in fructose-fed rats were concomitant with an increase in TNF- α in skeletal muscle (18). Although TNF- α in skeletal muscle may suppress glucose metabolism in a paracrine or autocrine manner (19), it is not clear what role TNF- α plays in vascular tissues or in the MAPK cascade. We hypothesize that TNF- α causes selective insulin resistance, and the aim of this study was to elucidate the effect of TNF- α on tissue specificity and selectivity to insulin signaling.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and a monoclonal antibody against smooth muscle α -actin (α -SMA) were obtained from Sigma (St. Louis, USA), and insulin was obtained from Lilly (Indianapolis, USA). Culture dishes were purchased from Becton Dickinson Labware (Two Oak Park, Bedford, UK). Polyclonal antibodies against extracellular signal-regulated kinases (ERK)1/2, phosphospecific ERK1/2 antibodies, anti-Akt, and phosphospecific Akt-antibodies were purchased from New England Biolabs (Beverly, USA). Antibodies against the insulin receptor β -subunit were obtained from Santa Cruz Biotechnologies (Santa Cruz, USA). Antibodies against IRS-1 were purchased from Upstate Biotechnology (Lake Placid, USA).

A monoclonal antibody against phosphotyrosine (α PY) was obtained from Transduction Laboratories (Venture Court, Lexington, USA). Rabbit anti-mouse immunoglobulin antibody was purchased from DAKO Corp. (Carpinteria, USA). Donkey anti-rabbit immunoglobulin horseradish peroxidase-linked antibody and an enhanced chemiluminescence kit were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, USA), and [125 I]protein A was from Life Sciences Products Inc. (Boston, USA). Recombinant rat TNF- α was from Pepro Tech (London, UK). Protein A-Sepharose 6MB was from Pharmacia Biotech AB (Uppsala, Sweden).

Cell Culture and Sample Extract

L6 myotubes derived from rat skeletal muscle were obtained from Cell Systems (Kirkland, USA) and cultured as described previously (20). Rat aortic vascular smooth muscle cells (VSMC) were prepared from the thoracic aortas of 4-week-old Sprague-Dawley (SD) rats (Charles River Japan Inc., Yokohama, Japan) and cultured as described previously (21). Cells from passages 3 to 8 were used for these experiments. Subconfluent cells were placed in serum-free DMEM for 16 h before the experiment. Cells were stimulated with insulin for 5 min or pretreated with TNF- α (20 ng/ml) for 6 h and then stimulated with insulin for 5 min. The cells were then incubated in lysis buffer containing 50 mmol/l HEPES (pH 7.4), 10 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l EDTA, 2 mmol/l Na_2VO_4 , 2 mmol/l PMSF, 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, 1.5 mg/ml benzamidine, 1% Triton X-100, 10% glycerol, and 137 mmol/l NaCl. Insoluble material was removed by centrifugation.

Immunoprecipitation and Western Immunoblotting

The supernatant was precipitated with antibodies against IR- β and IRS-1 and then incubated with protein A-Sepharose 6MB. Lysates were separated with 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to polyvinylidene difluoride (PVDF) membranes. To detect tyrosine phosphorylation of IR- β and IRS-1, the blots were first incubated with monoclonal antibody (mAb) α PY, then with rabbit anti-mouse antibody, and finally with [125 I]protein A. To detect protein levels of IR and IRS-1, blots were first incubated with polyclonal IR- β and IRS-1 antibodies, respectively, and then with [125 I]protein A. Finally, proteins were detected and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, USA). We confirmed that cultured cells were VSMC by using an mAb against vascular α -SMA (data not shown). Phosphorylation and protein levels of Akt and ERK1/2 were detected in the same manner as above.

Measurement of 2-Deoxyglucose (2-DOG) Uptake

The procedure is described in a previous publication (22). In

Fig. 1. Effects of TNF- α on IR- β tyrosine phosphorylation and IRS-1 total protein in insulin-stimulated L6 myotubes (A) or VSMC (B). Serum-free starved cells were treated as indicated. Samples of cell lysate were immunoprecipitated with anti-IRS-1 antibody or anti- α PY antibody and then subjected to SDS-PAGE and transferred to PVDF membranes. IR- β tyrosine phosphorylation is expressed as a percentage of insulin-stimulated activity. Results are the means $\pm$ SEM of 4 to 6 independent experiments. * Increased ($p < 0.05$) relative to the basal level.

brief, cells in 12-well dishes were serum deprived for 16h and incubated with TNF- α (20 ng/ml) for 6h followed by insulin 100 nmol/l for 5 min at 37°C. Glucose uptake was initiated by addition of 50 μ l of reaction mixture containing 0.2 μ Ci of [3 H]2-DOG in 2 mmol/l 2-DOG. At the end of a 5-min incubation, cells were washed five times with PBS, then neutralized and assessed for radioactivity by scintillation counting.

Statistical Analysis

Unless indicated otherwise, data were compared using analysis of variance (ANOVA) and are presented as the means $\pm$ SEM. P values less than 0.05 were considered to be significantly different.

Results

Effects of TNF- α on IR- and IRS-1 Tyrosine Phosphorylation

Insulin significantly ($p < 0.05$) increased IR- β tyrosine phosphorylation in L6 myotubes (Fig. 1A; insulin vs. control, 100 vs. $21.7 \pm 6.0\%$) and in VSMC (Fig. 1B; insulin vs. control, 100 vs. $31.8 \pm 17.0\%$). TNF- α alone did not alter IR- β tyrosine phosphorylation compared with that in control cells (data not shown). TNF- α did not change IR- β tyrosine phosphorylation by insulin in L6 myotubes (Fig. 1A; insulin vs. TNF- α + insulin, 100 vs. $90.4 \pm 9.8\%$) or in VSMC (Fig. 1B; insulin vs. TNF- α + insulin, 100 vs. $100.8 \pm 9.1\%$). IR- β total protein content was not altered after insulin or TNF- α

treatment in either L6 myotubes or VSMC. Insulin significantly ($p < 0.05$) increased tyrosine phosphorylation of IRS-1 in both L6 myotubes and VSMC (Fig. 2). Pretreatment with TNF- α alone did not alter IRS-1 phosphorylation (data not shown). TNF- α significantly ($p < 0.05$) decreased IRS-1 tyrosine phosphorylation by insulin in L6 myotubes (Fig. 2A; insulin vs. TNF- α + insulin, 100 vs. $73.6 \pm 4.3\%$) but not in VSMC (Fig. 2B; insulin vs. TNF- α + insulin, 100 vs. $121.7 \pm 21.9\%$). Total protein content of IRS-1 was not different after insulin or TNF- α treatment in either L6 myotubes or VSMC.

Effects of TNF- α on Protein Kinase B (PKB)/Akt and ERK1/2 Phosphorylation

Insulin increased ($p < 0.05$) PKB/Akt phosphorylation in L6 myotubes (Fig. 3A; insulin vs. control, 100 vs. $30.6 \pm 4.7\%$) and in VSMC (Fig. 3B; insulin vs. control, 100 vs. $46.0 \pm 5.9\%$) to the same extent. TNF- α did not alter PKB/Akt phosphorylation by insulin compared with that in control cells in either L6 myotubes or VSMC. Total protein content (Fig. 3) of PKB/Akt was not different after insulin or TNF- α treatment in either L6 myotubes or VSMC. Insulin increased ($p < 0.05$) ERK phosphorylation in L6 myotubes (Fig. 4A; insulin vs. control, 100 vs. $73.8 \pm 5.3\%$) and in VSMC (Fig. 4B; insulin vs. control, 100 vs. $58.9 \pm 7.6\%$) to the same extent. TNF- α did not alter ERK1/2 phosphorylation by insulin compared with that in control cells in either L6 myotubes or VSMC. There was no difference in the total protein contents of ERK1/2 between either L6 myotubes or VSMC treated only with insulin and cells treated with both

Fig. 2. Effects of TNF- α on IRS-1 tyrosine phosphorylation and IRS-1 total protein in insulin-stimulated L6 myotubes (A) or VSMC (B). Serum-free starved cells were treated as indicated. Samples of cell lysate were immunoprecipitated with anti-IRS-1 antibody or anti- α PY antibody and then subjected to SDS-PAGE and transferred to PVDF membranes. IRS-1 tyrosine phosphorylation is expressed as a percentage of insulin-stimulated activity. Results are the means $\pm$ SEM of 4 independent experiments. * $p < 0.05$ relative to the basal level. # $p < 0.05$ relative to insulin treatment alone.

Fig. 3. Effects of TNF- α on PKB/Akt phosphorylation and PKB/Akt total protein in insulin-stimulated L6 myotubes (A) or VSMC (B). Serum-free starved cells were treated as indicated. Samples of cell lysate were subjected to SDS-PAGE and transferred to PVDF membranes. PKB/Akt phosphorylation is expressed as a percentage of insulin-stimulated activity. Results are the means $\pm$ SEM of 4 to 6 independent experiments. * $p < 0.05$ relative to the basal level.

TNF- α and insulin. Also, insulin increased ERK1/2 phosphorylation in a dose-dependent manner in VSMC (Fig. 5).

Effects of TNF- α on Insulin-Stimulated 2-DOG Uptake in L6 Myotubes

Insulin significantly increased 2-DOG uptake and TNF- α significantly suppressed 2-DOG uptake by insulin to the control level in L6 myotubes (Fig. 6; 632 ± 17 , 977 ± 63 , 637 ± 36 DPM/mg protein/min for control, insulin, and insulin +

TNF- α , respectively; $p < 0.05$).

Discussion

It has been reported that skeletal muscles produce TNF- α , which can inhibit skeletal muscle glucose uptake in an autocrine or paracrine fashion, thereby causing insulin resistance (19). It has also been shown that TNF- α infusion resulted in a pronounced reduction in submaximal insulin-stimulated glucose disposal rate in SD rats (23). In the pres-

Fig. 4. Effects of TNF- α on ERK1/2 phosphorylation and ERK1/2 total protein in insulin-stimulated L6 myotubes (A) or VSMC (B). Serum-free starved cells were treated as indicated. Samples of cell lysate were subjected to SDS-PAGE and transferred to PVDF membranes. ERK1/2 phosphorylation is expressed as a percentage of insulin-stimulated activity. Results represent the means $\pm$ SEM of 4 to 6 independent experiments. * $p < 0.05$ relative to the basal level.

Fig. 5. Effects of different doses of insulin on ERK1/2 phosphorylation and ERK1/2 total protein in insulin-stimulated VSMC. Serum-free starved VSMC were treated as indicated. Samples of cell lysate were transferred to PVDF membranes. These experiments were repeated twice.

ent study, we demonstrated that TNF- α reduced glucose uptake stimulated by insulin in L6 myotubes. The mechanism of the first step stimulated by TNF- α is to an increase in serine phosphorylation of IRS-1, resulting in inhibition of tyrosine phosphorylation of IR- β or IRS-1 (24, 25). The first step in the mechanism by which TNF- α induces the above effects is to increase serine phosphorylation of IRS-1 would reduce the ability of IRS-1 to activate PI-3 kinase. In a variety of cell types, including C2C12 myotubes and adipocytes, TNF- α has been shown to decrease both insulin-induced IR- β and IRS-1 tyrosine phosphorylation (26). On the other hand, in Fao hepatoma cells, TNF- α reduced insulin-induced tyrosine phosphorylation of IRS-1 but did not significantly reduce IR kinase activity (27). We found in the present study that TNF- α impaired tyrosine phosphorylation of IRS-1 in L6 myotubes but not in VSMC, and that TNF- α did not affect IR- β phosphorylation in either L6 myotubes or VSMC. Impairment of tyrosine phosphorylation of IRS-1 in L6 myotubes is

Fig. 6. Effects of TNF- α on insulin-stimulated 2-deoxyglucose uptake in L6 myotubes. Serum-free starved cells were treated as indicated. Results represent the means $\pm$ SEM of 6 to 7 independent experiments.

one of the major causes of insulin resistance in glucose metabolism, which is regulated downstream of insulin signaling, glycogen synthesis, glucose uptake and glycogenesis, although tyrosine phosphorylation of IR- β was not affected by TNF- α . It thus appears that the insulin resistance in glucose metabolism is tissue specific. However, it is not clear where the different sites of insulin signaling impairment by TNF- α are located; these locations might be dependent on cell type.

To further investigate the potential role played by TNF- α in insulin resistance, we evaluated the effects of TNF- α on

PKB/Akt, which is downstream of insulin signaling. With respect to PKB/Akt, there are a number of papers reporting both positive and negative evidence for its role in glucose transport stimulation. In a human study, phosphorylation of PKB/Akt by insulin was not changed in NIDDM (28). In addition, constitutively active PKB α has been shown to promote GLUT4 translocation or increased glucose uptake in rat adipocytes and L6 myotubes (29). On the other hand, a kinase inactive PKB α mutant inhibited insulin-stimulated GLUT4 translocation when transfected into rat adipocytes, and this same construct had no significant effect in L6 myotubes (30). Our results demonstrated that TNF- α reduced tyrosine phosphorylation of IRS-1 stimulated by insulin in L6 myotubes but had no effect on phosphorylation of PKB/Akt, which lies downstream of PI-3 kinase. Although it is not clear why there was no effect of TNF- α on phosphorylation of PKB/Akt in the present study, it is known that PKB/Akt plays a role not only in glucose metabolism but also in protein synthesis (31), lipid metabolism (32), apoptosis (33), and production of nitric oxide (34). It has recently been reported that insulin activates atypical forms of protein kinase C (PKC); *i.e.*, PKC ζ and/or PKC λ can stimulate glucose uptake in 3T3L1 adipocytes (35) and L6 myotubes (36). The increases in atypical PKC enzyme activity caused by insulin appear to be largely dependent on activation of PI-3 kinase (30, 37). On the other hand, it has also been reported that the G α_q subunit of heterotrimeric G proteins exists in the insulin signaling pathway, that its action is dependent on PI-3 kinase, and that G α_q can transmit signals that lead to GLUT4 translocation in 3T3 L1 adipocytes (35). The results of these studies suggest that PKB/Akt might not play a significant role in insulin resistance by TNF- α , and that other signals might be responsible for insulin resistance in L6 myotubes.

In VSMC, it has been demonstrated that pretreatment with TNF- α led to a transient suppression of ERK1/2 activation induced by insulin through inhibition of Shc phosphorylation with exposure to TNF- α for 120 min, and then ERK1/2 activation in response to insulin was restored (38). In the present study, pretreatment with TNF- α for 6 h did not affect tyrosine/threonine phosphorylation of ERK1/2 stimulated by insulin in either L6 myotubes or VSMC. In L6 myotubes, pretreatment with TNF- α for 30 min led to a transient suppression of insulin-induced ERK1/2 activation, but pretreatment with TNF- α for more than 1 h did not suppress insulin-induced ERK1/2 activation (data not shown). Our results showed that TNF- α inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 but did not affect tyrosine/threonine phosphorylation of ERK1/2 in L6 myotubes and also did not affect IRS-1 or ERK1/2 in VSMC. Thus, it appears that there is a selective resistance, not in ERK1/2 but in IRS-1, in insulin signaling by TNF- α in L6 myotubes. It has been demonstrated that point mutations in the insulin receptor that prevent phosphorylation of IRS-1 and IRS-1-growth factor receptor bound protein 2 (Grb2) formation have no effect on

tyrosine phosphorylation of Shc or MAPK systems in rat fibroblasts (39). It is possible that the Shc-Ras system might be more sensitive than *via* IRS-1 pathway in L6 myotubes. It was also demonstrated in the present study that ERK tyrosine/threonine phosphorylation, which is related to DNA synthesis and migration, is induced by insulin in a dose-dependent manner in VSMC. Insulin also has biological actions in arterial tissue, including stimulation of smooth muscle cell proliferation and migration (40) and enhancement of lipoprotein metabolism (41). Recently, hyperinsulinemia has been shown to occur in advance of the development of some risk factors for atherosclerosis (42). Tsuchihashi *et al.* reported that hyperinsulinemia is a risk factor for coronary artery disease and emphasized the danger of coronary atherosclerosis in normal glucose-tolerant subjects (43).

In conclusion, our results indicate that TNF- α plays a significant role in the tissue specificity and signal selectivity of insulin resistance. TNF- α impaired the insulin-stimulated IRS-1-PI3-kinase pathway in L6 myotubes, but the insulin-stimulated MAPK pathway was not impaired in VSMC. The IRS-1-PI3-kinase pathway, which is related to glucose metabolism, is selectively impaired by TNF- α in skeletal muscle tissues, and this impairment might cause hyperinsulinemia. Compensating hyperinsulinemia stimulates the MAPK pathway related to the stimulation of smooth muscle cell proliferation and migration, possibly resulting in progression of atherosclerosis. To our knowledge, this is the first report on the effects of TNF- α on tissue specificity and selectivity to insulin signaling *in vitro*.

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