Effects of tumour necrosis factor-α and inhibition of protein kinase C on glucose uptake in L6 myoblasts

Divina PATIAG, Samuel GRAY, Iskander IDRIS and Richard DONNELLY
School of Medical and Surgical Sciences, Division of Vascular Medicine, University of Nottingham, U.K.

ABSTRACT
Clinical and experimental studies have implicated high circulating levels of the cytokine tumour necrosis factor-α (TNF-α) in the pathogenesis of insulin resistance, not only in obesity and diabetes, but also in clinical conditions associated with cachexia and sepsis. TNF-α impairs insulin-mediated glucose uptake in adipocytes, but because of lipolytic effects the interpretation of clinical studies and the extent to which TNF-α affects muscle insulin sensitivity are unclear. In addition, protein kinase C (PKC) has recently been implicated in the mechanism of TNF-α-induced insulin resistance. The present study investigated the effects of TNF-α and a PKC inhibitor (RO-318220) on basal and insulin-stimulated 2-[3H]deoxyglucose uptake in cultured L6 myoblasts. Reverse transcriptase–PCR analysis confirmed that L6 myoblasts express TNF-α receptors I and II (p60 and p80). Dose–response curves for glucose uptake were fitted to a quadratic function to derive $C_{I-150}$ values (concentration of insulin required to increase glucose uptake by 50%). Incubation with TNF-α at 1 or 10 ng/ml for 24 h had no significant effect on basal glucose uptake, insulin sensitivity or maximal insulin responsiveness. $C_{I-150}$ values (means ± S.E.M.) were as follows: basal, 91.2 ± 13 nM; 1 ng/ml TNF-α, 102 ± 12 nM; and basal, 70.8 ± 13 nM; 10 ng/ml TNF-α, 43.7 ± 40 nM. PKC inhibition markedly attenuated glucose uptake, but there was no difference in insulin sensitivity with RO-318220 alone compared with RO-318220 + TNF-α. In conclusion, although increased TNF-α expression and plasma concentrations have been implicated in the pathogenesis of insulin resistance in various clinical states, there is no evidence that TNF-α impairs insulin-stimulated glucose uptake in a skeletal-muscle-derived cell line.

INTRODUCTION
Tumour necrosis factor-α (TNF-α) is a potent cytokine that is secreted by activated macrophages. In addition to its cytotoxic and anti-tumour activity, TNF-α has important metabolic effects which have been strongly implicated in the development of insulin resistance in obesity, Type II diabetes and aging [1–3], as well as the insulin resistance related to severe catabolic states, e.g. septic shock, cachexia and trauma [4]. Expression of TNF-α is increased in adipose tissue and muscle from humans and animal models of insulin resistance [2,5,6], and systemic infusion of TNF-α reduces whole-body glucose disposal due to effects on both hepatic and peripheral insulin sensitivity, including that of skeletal muscle [7].

The interpretation of in vivo studies has not been straightforward. For example, since TNF-α also stim-
ulates lipolysis [8], its insulin-antagonist effects in adipose tissue (and adjacent muscle) may be due, in part, to local release of non-esterified fatty acids, rather than a direct action of TNF-α itself. In isolated adipocytes, there is consistent evidence showing that TNF-α reduces insulin-stimulated glucose uptake [8–11], although the underlying mechanisms have not been clearly established, e.g. decreased tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) [10], and/or reduced expression of mRNAs for GLUT-4, the insulin receptor and IRS-1 [11].

Phosphorylation events seem to be particularly important in the mechanism of TNF-α-induced insulin resistance in adipocytes. For example, exposure to TNF-α leads to serine phosphorylation of IRS-1, which inhibits the tyrosine kinase activity of the β-subunit of the insulin receptor [12], and increasing evidence suggests that the serine/threonine kinase protein kinase C (PKC) mediates the inhibitory effect of TNF-α on insulin signalling via effects on discrete PKC isoforms, e.g. PKC-ε [13] and/or PKC-β and PKC-ζ [14,15].

The effects of TNF-α on skeletal muscle are less clearly defined, yet muscle accounts for the majority of glucose disposal in vivo. It has been suggested that, in obese subjects, the peripheral insulin resistance in muscle is due to exposure to increased circulating levels of TNF-α released by hypertrophied fat cells [2], but in vitro studies have reported inconsistent results with respect to the effects of TNF-α on glucose uptake and metabolism in isolated cells. Paradoxically, it has been reported that TNF-α increases glucose uptake in muscle [15–17], while other in vitro studies have shown an inhibitory [18] or neutral [19,20] effect in isolated myocytes. The purpose of the present study was to define the effects of TNF-α, and PKC inhibition, on dose–response curves for insulin-stimulated glucose uptake in L6 cells, an established skeletal-muscle-derived cell line.

METHODS

Materials
Insulin and human recombinant TNF-α were purchased from Sigma Chemical Co., and cell culture reagents were obtained from Gibco BRL Life Technologies. 2-[1-3H]-Deoxy-D-glucose was purchased from Amersham Life Sciences. The bisindolylmaleimide PKC inhibitor, RO-318220 [21], was purchased from Roche Products Ltd.

Cell culture
L6 myoblasts, a rat skeletal-muscle-derived cell line (ECACC, Cambridge, U.K.), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum in an atmosphere of 5% CO₂ at 37 °C, to prevent the cells from undergoing further differentiation [22].

Reverse transcriptase-PCR (RT-PCR) analysis of TNF-α receptors I and II
Total RNA from rat L6 myoblasts was extracted with RNAStat60 (Biogenesis Ltd) and reverse-transcribed with MMLV (MoLoney-murine-leukaemia virus) reverse transcriptase, primed with oligo(dT)₁₂₁₈. The resulting cDNA was subjected to PCR amplification with two separate sets of oligonucleotide primers for rat TNF-α receptors I and II (p60 and p80) [23,24], as follows.

- p60: primer 1, 5’-GCTCCTGCTCGTCTGCTGAT-3’; primer 2, 5’-AACATTCTTTCTCCGACAT-3’; primer 3, 5’-CCACCCCCAATGGGGGACTG-3’; primer 4, 5’-CCGTGTTCTGTCTCTCCTTA-3’.
- p80: primer 1, 5’-GATGAGAATAATCCAGGATGCAGTGG-3’; primer 2, 5’-TGCAAGAAGCTGTCGAGG-3’; primer 3, 5’-TTCCGGAGTGGCCCGTCTGAGG-3’; primer 4, 5’-GCTGTTGTCAATAGGTGTCG-3’.

The expected product sizes for p60 were 288 bp (primers 1 and 2) [23] and 535 bp (primers 3 and 4) [24a], and those for p80 were 259 bp (primers 1 and 2) [24] and 527 bp (primers 3 and 4) [24a]. Glyceraldehyde-3-phosphate dehydrogenase primers (Stratagene) were used as a positive control. The PCR products were separated on a 1% (w/v) agarose gel and visualized with GelStar nucleic acid stain (FMC Bioproducts).

Measurement of insulin-stimulated 2-deoxy-glucose uptake
L6 cells were grown on 24-well plates, and then serum-starved by incubation in serum-free Dulbecco’s modified Eagle’s medium for 24 h. The following day, various concentrations of insulin (10⁻⁹–10⁻⁶ M), with or without TNF-α (1 and 10 ng/ml) and/or the PKC inhibitor RO-318220, were added to the quiescent cells, followed by

Figure 1 RT-PCR analysis of p60 and p80 subtypes of the TNF-α receptor in rat L6 myoblasts
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as a positive control.
incubation for a further 24 h (glucose concentration in the incubation 25 μM). Following this incubation period, cells were washed in Krebs buffer (114 mM NaCl, 5 mM KCl, 25 mM NaHCO$_3$, 1.18 mM MgSO$_4$ and 1.17 mM KH$_2$PO$_4$, pH 7.4) at room temperature and incubated with 1 ml of radioactive Krebs solution containing 2-[$^3$H]deoxyglucose (0.2 μCi; specific radioactivity 10 Ci/mmol) and 1 μM unlabelled 2-deoxyglucose for 10 min at room temperature. The radioactive Krebs solution was then washed off twice with ice-cold Krebs buffer at 4 °C. To determine the incorporated radioactivity, the cells were solubilized in 500 μl of 1 M NaOH, as described previously [16,25].

**Statistical analysis**

Dose–response curves were derived for insulin-stimulated 2-deoxyglucose uptake in the presence or absence of TNF-α and RO-318220. The curves were fitted to a quadratic function using the statistical software program GraphPad Prism to derive, where appropriate, C$_{150}$ values (concentration of insulin required to increase 2-deoxyglucose uptake by 50%). Results are expressed as means ± S.E.M.

### Table 1  C$_{150}$ values for L6 cells exposed to TNF-α

<table>
<thead>
<tr>
<th>Conditions</th>
<th>C$_{150}$ (nM)</th>
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<tbody>
<tr>
<td>Insulin $(n = 11)$</td>
<td>91.2 ± 13</td>
</tr>
<tr>
<td>Insulin + 1 ng/ml TNF-α $(n = 12)$</td>
<td>102 ± 12</td>
</tr>
<tr>
<td>Insulin $(n = 9)$</td>
<td>70.8 ± 13</td>
</tr>
<tr>
<td>Insulin + 10 ng/ml TNF-α $(n = 12)$</td>
<td>43.7 ± 40</td>
</tr>
</tbody>
</table>

### Figure 3  Effects of PKC inhibition on dose–response curves for insulin-stimulated 2-[$^3$H]deoxyglucose uptake in L6 myoblasts

- ■, Insulin alone;
- ●, insulin + 0.1 μM RO-318220;
- ▼, insulin + 0.1 μM RO-318220 + 10 ng/ml TNF-α.

### RESULTS

RT-PCR analysis showed that L6 myoblasts express mRNAs for both subtypes of TNF-α receptor, p60 and p80 (Figure 1). Co-incubation of L6 myoblasts with TNF-α for 24 h at concentrations of 1 ng/ml and 10 ng/ml had no significant effect on basal glucose uptake or the dose–response curve for insulin-stimulated 2-[$^3$H]deoxyglucose uptake (Figure 2). Neither insulin sensitivity nor maximal insulin responsiveness were affected by TNF-α. For example, the C$_{150}$ value for the higher dose of TNF-α was 43.7 ± 40 nM, compared with 70.8 ± 13 nM for control cells (Table 1).

Treatment with the PKC inhibitor RO-318220 (0.1 μM) markedly attenuated insulin-stimulated glucose uptake in L6 cells, but there was no further inhibitory effect on insulin sensitivity when 10 ng/ml TNF-α was combined with PKC inhibition (Figure 3).

### DISCUSSION

Although TNF-α has potent inhibitory effects on insulin action in isolated adipocytes [8–11], the extent to which this important cytokine affects glucose uptake and
metabolism in skeletal muscle remains unclear. The notion that TNF-α released from fat tissue circulates as a humoral antagonist, reducing muscle insulin sensitivity, has been central to the strongly held hypothesis that TNF-α mediates insulin resistance in obese patients [2], but clinical and experimental studies have been inconsistent. Systemic infusion of TNF-α decreases both peripheral and hepatic insulin sensitivity [7], but in in vivo studies it has been difficult to distinguish between direct effects of TNF-α on glucose uptake and indirect effects due to increased lipolysis and release of non-esterified fatty acids. TNF-α also causes dedifferentiation of adipocytes [26], which further complicates the interpretation of previous clinical studies.

In vitro studies have also been inconclusive, reporting stimulatory [15–17], inhibitory [18] and neutral [19,20] effects of TNF-α on glucose uptake and metabolism in skeletal muscle. The explanation for these different results is not entirely clear, but the duration of exposure to TNF-α may be important. Stimulatory effects on glucose uptake were reported after brief exposure to TNF-α (5 ng/ml for 90 min) [15], whereas inhibitory effects on glucose transport and glycogen synthase activity were more pronounced when the incubation period was 6–12 h [18]. In addition, some studies have used primary cultures of human muscle cells, while others have examined insulin-responsive muscle cell lines. L6 myoblasts are highly insulin-responsive, are derived from rat skeletal muscle, and provide a sensitive and reliable in vitro model for detecting changes (either positive or negative) in insulin-stimulated glucose uptake [27], although effects seen in cultured cells do not necessarily correlate with responses in intact muscle tissue.

The strengths of the present study include, firstly, a longer duration of exposure to TNF-α (24 h), in order to investigate possible metabolic effects under conditions that mimic clinical practice scenarios where there is known to be short-to-medium term overproduction of TNF-α, e.g. in patients with acute inflammation or sepsis, or when metabolic stress occurs at times of trauma or acute myocardial infarction. Since there was no direct effect on insulin-stimulated glucose uptake in muscle cells, it seems unlikely that increased circulating levels of TNF-α play a significant role in the pathogenesis of insulin resistance and hyperglycaemia under clinical circumstances of metabolic stress. Secondly, whereas previous studies have measured 2-[^3H]deoxyglucose uptake under basal conditions and after one fixed dose of insulin, the present study characterized incremental dose–response curves for insulin-stimulated glucose uptake, which were then fitted to a quadratic function. This has the advantage of being able to potentially identify different effects of TNF-α on insulin sensitivity (C_{1/2max}) and maximum insulin responsiveness (C_{max}). In addition, since exposure to TNF-α had no effect on basal glucose uptake, it can also be concluded that TNF-α does not influence non-insulin-dependent pathways of glucose transport.

The mechanism of the insulin-antagonist effect of TNF-α, at least in adipose tissue, is still not clearly understood, but binding to TNF-α receptors, which are widely expressed in different tissues [28], results in activation of several second messenger systems, including formation of diacylglycerol via phospholipase C [29]. Circulating levels of the soluble TNF-α receptor I (p55 or sTNFRI) and receptor II (p80 or sTNFRII) subtypes also correlate with metabolic variables [30], and it is possible that TNF-α exerts metabolic effects only when bound to one of these soluble TNF receptor proteins. RT-PCR analysis in the present study showed that L6 cells express mRNAs for both receptor subtypes. In addition, it is possible that, in vivo, other endocrine changes associated with metabolic stress serve as essential cofactors for TNF-α-induced insulin resistance.

Intracellular formation of diacylglycerol as a result of TNF-α binding activates the serine/threonine kinase PKC, and there is evidence that increased serine phosphorylation of IRS-1 is an important step in TNF-α-induced insulin resistance in hepatocytes [12,31]. More specifically, Kellerer et al. [13] have shown that activation of the ε isoform of PKC is an essential step in TNF-α-mediated phosphorylation and down-regulation of insulin signalling, while other groups have shown that increased expression of PKC-ε in skeletal muscle contributes to insulin resistance in diabetes and obesity [32,33]. In the present study, incubation of L6 cells with a specific PKC inhibitor markedly attenuated glucose uptake, but there was no additional inhibitory effect when TNF-α was combined with RO-318220.

PKC inhibition reduces glucose uptake in adipocytes [34], but the effects in isolated muscle cells have not been reported previously. Compared with some of the early PKC inhibitors (e.g. staurosporine) the bisindolylmaleimides are much more selective for PKC, but still lack the isoenzyme specificity needed to explore functional differences between the 12 PKC isoforms. Activation of PKC-ε, -β and -ζ has been particularly implicated in TNF-α-induced insulin resistance [13,14], but in the absence of isoform-selective PKC inhibitors it is difficult to explore these pathways pharmacologically.

REFERENCES

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