Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase

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The principal goal of the present study was to test the hypothesis that cytokines modulate glucose transport in skeletal muscle by increasing nitric oxide production. Cultured L6 skeletal muscle cells were incubated in the presence of tumour necrosis factor-α, interferon-γ or lipopolysaccharide (LPS) alone or in combination for 24 h. Neither cytokines nor LPS alone induced NO production, as measured by nitrite concentrations in the medium. However, when used in combination, the two cytokines significantly stimulated NO production, and this effect was synergistically enhanced by the presence of LPS. Reverse transcriptase-PCR (RT-PCR) analysis revealed that NO release was associated with the induction of inducible (macrophage-type) NO synthase (iNOS). The increase in iNOS expression was confirmed at the protein level by Western-blot analysis and NADPH/diaphorase histochemical staining. Cytokines and LPS markedly increased basal glucose transport in L6 myocytes. Insulin also stimulated basal glucose transport, but significantly less in cells chronically exposed to cytokines/LPS. The sensitivity of L6 muscle cells to insulin-stimulated glucose transport was also significantly decreased by cytokines/LPS treatment. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) inhibited nitrite production in cytokine/LPS-treated cells, and this prevented the increase in basal glucose transport and restored muscle cell responsiveness to insulin. Cytokines/LPS exposure significantly increased GLUT1 transporter protein levels but decreased GLUT4 expression in L6 cells. L-NAME treatment prevented the increase in GLUT1 protein content but failed to restore GLUT4 transporter levels. These results demonstrate that cytokines and LPS affect glucose transport and insulin action by inducing iNOS expression and NO production in skeletal muscle cells. The data further indicate that cytokines and LPS increase the expression of the GLUT1 transporter protein by an NO-dependent mechanism.

INTRODUCTION

It is well documented that septicaemia elicits profound changes in host metabolism, including increased energy expenditure and impaired whole-body glucose homoeostasis in both animals and humans [1]. Septic patients have an accelerated rate of glucose clearance in the basal state [2]. On the other hand, acute infection is also associated with a state of insulin resistance, as evidenced by diminished glucose tolerance, hyperinsulinaemia and impaired insulin action on peripheral glucose disposal [3,4]. Previous studies have focused on the role of cytokines in mediating the effects of infection on metabolism. Indeed, administration of the cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 to experimental animals has been reported to mimic the metabolic response to acute infection [5–7]. Sakurai et al. [6] have recently provided evidence that TNF-α directly stimulates glucose uptake in peripheral tissues of dogs. Conversely, *in vivo* infusion of endotoxin and cytokines has also been found to reduce insulin-stimulated glucose uptake by skeletal muscles, the main site of glucose disposal in the post-prandial state [7]. Other studies have shown that cytokines affect glucose metabolism in isolated muscles or cultured myocytes [8–10]. Thus cytokines appear to modulate glucose homoeostasis by a direct action on skeletal muscle cells.

The precise cellular mechanisms responsible for the action of cytokines on muscle glucose metabolism are not known. It has been recently shown that skeletal muscle expresses nitric oxide synthase (NOS), the enzyme that catalyses the formation of NO from L-arginine [11–14]. In resting muscle, both the constitutive neuronal-type (nNOS) and endothelial-type (eNOS) isozymes are expressed [11,13]. Whereas nNOS localization is restricted to type IIb fast-twitch glycolytic fibres, eNOS can be found both in the endothelial cells and within mitochondria-enriched (oxidative) fibres [11,13]. Moreover, experimental septicaemia induced by endotoxin treatment of rats has been reported to increase the expression of an inducible, calcium-independent NOS (iNOS) in skeletal muscle of rats [15,16]. iNOS expression and NO production have also been reported in cultured C2C12 myocytes challenged with cytokines and LPS [17]. Recent studies in our laboratory have shown that increasing NO concentrations with NO donors inhibit insulin-stimulated glucose transport in isolated rat soleus and extensor digitorum longus muscles [11]. This effect could be reproduced in cultured L6 myocytes, strongly suggesting that NO exerted its action by a direct action and not by diffusion to the muscle vasculature. Thus one possible mechanism by which cytokines and endotoxin could affect glucose metabolism in muscle cells is by induction of iNOS expression and exaggerated NO production. This hypothesis was directly tested in the present study using cultured L6 skeletal muscle cells.

MATERIALS AND METHODS

Materials

α-Minimum essential medium (α-MEM), foetal bovine serum and other tissue-culture products were obtained from Gibco

Abbreviations used: NOS, nitric oxide synthase; nNOS, neuronal-type NOS; eNOS, endothelial-type NOS; iNOS, inducible NOS; TNF-α, tumour necrosis factor-α; IF-γ, interferon-γ; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-PCR; L-NAME, N^G-nitro-L-arginine methyl ester; α-MEM, α-minimum essential medium; GAPDH, glyceraldehyde phosphate dehydrogenase.

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BRL. Human insulin (Humulin R) was purchased from Eli Lilly. 2-Deoxy-D-glucose, cytochalasin B, lipopolysaccharide (LPS) (from *Escherichia coli*) and NADPH were obtained from Sigma. 2-Deoxy-D-[^{3}H]glucose was purchased from NEN Dupont. N^G-Nitro-L-arginine-methyl ester (L-NAME) was obtained from Alexis (San Diego, CA, U.S.A.). Recombinant murine and human TNF-α were purchased from R & D systems and Boehringer Mannheim respectively. Murine interferon-γ (IF-γ) was kindly supplied by Dr. Martin Olivier (CHUL research center, Ste-Foy, QC, Canada). An antibody directed against a C-terminal peptide (amino acids 1131–1144) of mouse macrophage iNOS was purchased from Cedarlane Laboratories (Mississauga, ON, Canada). Polyclonal antibodies directed against rat GLUT1 and GLUT4 or mouse GLUT3 were from East Acres Biologicals (Southbridge, MA, U.S.A.). Monoclonal α1-Na/K-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts General Hospital, Boston, MA, U.S.A.).

Cell culture

A line of L6 skeletal muscle cells (kind gift of Dr. A. Klip, Hospital for Sick Children, Toronto, ON, Canada) clonally selected for high fusion potential was used in the present study. The L6 cell line was derived from neonatal rat thigh skeletal muscle cells and retains many morphological, biochemical and metabolic characteristics of skeletal muscle [18]. Fully differentiated L6 myotubes express several muscle-specific proteins, such as myosin ATPase, the sarcoplasmic reticulum Ca^{2+}-ATPase and the dihydropyridine receptor [19]. Cells were grown and maintained in monolayer culture in α-MEM containing 2% (v/v) foetal bovine serum and 1% (v/v) antibiotic/antimycotic solution (10000 units/ml penicillin, 10000 μg/ml streptomycin and 25 μg/ml amphotericin B) in an atmosphere of 5% CO_2 at 37 °C. L6 myoblasts were plated in 24-well plates, 6-well plates or 10 cm diameter dishes at 20000 cells/ml and were used after complete differentiation to myotubes (7 days post-plating).

Cell incubations and glucose-transport assay

L6 myotubes were incubated with or without TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml) for 24 h, followed by addition of insulin (0.6 μM) (or medium alone) for an additional 45 min. When present, the NOS inhibitor L-NAME was used at a concentration of 2 mM. Cells were rinsed once with glucose-free Hepes-buffered saline solution, pH 7.4 (140 mM NaCl, 20 mM Hepes/Na, 5 mM KCl, 2.5 mM $MgSO_4$ and 1 mM $CaCl_2$), and were subsequently incubated for 8 min with 10 μM 2-deoxy-D-glucose containing 0.3 μCi/ml 2-deoxy-D-[^{3}H]glucose in the same buffer. After the incubation in transport medium, cells were rinsed three times with ice-cold saline solution, and then disrupted by adding 50 mM NaOH. Cell-associated radioactivity was determined by scintillation counting. Protein concentrations were determined by the bicinchoninic acid method (Pierce), and the results were expressed in pmol/min per mg. Glucose uptake values were corrected for non-carrier-mediated transport by measuring hexose uptake in the presence of 10 μM cytochalasin B ($\sim$ 5–10% of total uptake).

Measurements of NO, glucose and lactate concentrations

The accumulation of nitrite in the incubation medium was used as an index of NO production. Nitrite was determined spectrophotometrically as previously described [20]. The Griess reagent [1.0% (w/v) sulphanilamide/0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride] was added to samples of the incubation medium, and the absorption was read at 540 nm. Glucose and lactate levels in the media were measured using a YSI type 2300 STAT Plus automatic glucose and lactate analyser (YSI, Yellow Springs, OH, U.S.A.), which uses the glucose oxidase and lactate dehydrogenase reactions.

RNA extraction and RT-PCR

Total cellular RNA was isolated using guanidium thiocyanate/phenol/chloroform extraction with the TRIzol Reagent (Life Technologies) based on the method developed by Chomczynski and Sacchi [21]. cDNA synthesis was performed with 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL), using 1 μg of total RNA in 20 μl of reverse transcriptase buffer (50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) containing 1 mM each dNTP and 8 pmol of iNOS or glyceraldehyde phosphate dehydrogenase (GAPDH) antisense primers. The reaction was performed at 42 °C for 1 h, and the enzyme was then denatured at 95 °C for 10 min. Samples were then supplemented with 3 μl of 10 $\times$ PCR buffer (1 $\times$ PCR buffer is 33.3 mM KCl and 3 mM $MgCl_2$)/8 pmol of iNOS or GAPDH sense primers/water to 30 μl. cDNAs were denatured for 5 min at 94 °C and cooled to 72 °C, and then 1 unit of *Thermophilus aquaticus* DNA polymerase (Boehringer Mannheim) was added to each sample. Amplification was performed as follows: 30 cycles of temperature (94 °C, 30 s; 60 °C, 1 min; 72 °C, 1 min) in a temperature cycler (DNA Thermal Cycler, Perkin–Elmer). Sequences of the antisense and sense oligonucleotides {based on rat iNOS and GAPDH (see [15])} were as follows: iNOS, 5′-TGGAACCACTCGTACTTGGGA-3′ and 5′-CAAGAGTTTGACCAGAGGACC-3′; GAPDH, 5′-AGATCCACAACGGATACATT-3′ and 5′-TCCCTCAAGATTGTCAGCAA-3′. The expected sizes of the amplification products were 653 bp for iNOS and 331 bp for GAPDH. The amplification products were run in 8% acrylamide gels and stained in ethidium bromide, and fluorescence associated with DNA bands was measured using a BioImage-Visage 110S scanner from Millipore.

Isolation of cellular proteins and membranes

Total cellular proteins were isolated from the same cells used for RNA isolation. The proteins were extracted from the phenol/ethanol supernatant obtained after precipitation of DNA with ethanol, as described by the manufacturer's instructions (TRIzol reagent; Life Technologies). These protein samples were used for determination of iNOS-protein content by Western blotting (see below). For membrane isolation, fully differentiated L6 myotubes were washed twice with ice-cold PBS (pH 7.4), scraped with a rubber policeman, collected and centrifuged at 700 ***g*** for 10 min. The 700 ***g*** cell pellet was resuspended in homogenizing buffer containing 250 mM sucrose, 2 mM EGTA, 5 mM NaN_3, 20 mM Hepes (pH 7.4), 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A and 10 μM E-64. Cells were homogenized using a Wheaton A (tight-fit) glass homogenizer (20 strokes), followed by centrifugation at 2000 ***g*** for 15 min. The resulting supernatant was centrifuged at 186000 ***g*** for 1 h at 4 °C. The pellet (total membranes) was resuspended in homogenizing buffer, and protein concentrations were determined by the bicinchoninic acid assay (Pierce), using BSA as standard. Membrane proteins were used for Western-blot analysis of glucose transporters (see below).

Western-blot analysis

Total or membrane protein samples (50 μg) were subjected to SDS/PAGE on 7.5% polyacrylamide gels as described by Laemmli [22] and were electrophoretically transferred (100 V, 2 h) to PVDF filter membranes for 2 h. Immunoblotting was

performed as previously described [23]. Briefly, PVDF membranes were incubated for 1 h at room temperature with buffer I (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl) containing 0.04 % (w/v) NP-40, 0.02 % (w/v) Tween-20 and 3 % (w/v) fatty acid-free BSA, followed by overnight incubation at 4 °C with primary antibodies. Dilutions of antibodies were: polyclonal anti-iNOS, 1:2500; polyclonal anti-GLUT1, 1:2000; -GLUT3, 1:1000; -GLUT4, 1:2000; and monoclonal anti-α1-Na/K-ATPase, 1:200. PVDF membranes were then washed for 30 min, followed by a 1 h incubation with either anti-mouse or anti-rabbit IgG (1:10000 dilution) conjugated to horseradish peroxidase (Amersham) in buffer I containing 1 % (w/v) BSA. The PVDF membranes were washed for 30 min in buffer I, and the immunoreactive bands were detected by the enhanced chemiluminescence method.

NOS histochemistry

NOS was selectively stained by reduced NADPH–diaphorase histochemistry [24]. L6 myotubes grown on cover slips in 6-well plates were incubated with or without cytokines + LPS for 24 h, washed three times in Tris buffer (0.1 M Tris/HCl, pH 8), and fixed in 4 % (v/v) paraformaldehyde followed by a 30 min incubation at 37 °C in 0.1 M Tris/HCl buffer containing 1 mM NADPH, 0.2 mM Nitro Blue Tetrazolium and 0.3 % (w/v) Triton X-100. Sections were rinsed in PBS (2 × 10 min) and mounted on slides for histochemistry. Cells were viewed using a high-power microscope (Nikon, optiphot). No staining was observed when NADPH was omitted from the reaction mixture (results not shown).

Statistical analysis

Values are means ± S.E.M. The effect of cytokines/LPS and insulin on glucose transport were compared by a two-way analysis of variance. The effects of different concentrations of insulin on glucose transport were analysed by two-way analysis of variance. The level of significance was $P < 0.05$.

RESULTS

The effects of individual cytokines and their combination on NOS activity in L6 myocytes were determined by measurements of nitrite levels in the medium, as shown in Table 1. Neither TNF-α nor IF-γ alone stimulated NO production over basal values. However, a significant increase in nitrite levels was observed when cells were incubated with both cytokines. Moreover, the addition of LPS synergistically enhanced the effects of the cytokines to promote NO production. LPS alone did not increase nitrite levels (results not shown). Addition of the NOS inhibitor L-NAME (2 mM) totally abrogated the effects of the cytokines and LPS on NO production.

Table 1 Effect of TNF-α, IF-γ, LPS and L-NAME on nitrite production by L6 myocytes.

L6 cells were treated or not with TNF-α (10 ng/ml), IF-γ (200 units/ml), LPS (10 μg/ml) and L-NAME (2 mM) for 24 h, and nitrite levels were measured in the incubation medium as indicated in the Materials and methods section. Values represent means ± S.E.M. from at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control values.

Conditions	Nitrite (μM)	
	− L-NAME	+ L-NAME
Control	0.27 ± 0.04	0.49 ± 0.14
TNF-α	0.09 ± 0.10	0.36 ± 0.26
IF-γ	0.22 ± 0.11	0.14 ± 0.10
TNF-α + IF-γ	1.60 ± 0.87	0.25 ± 0.07*
TNF-α + IF-γ + LPS	9.57 ± 0.85	0.93 ± 0.17**

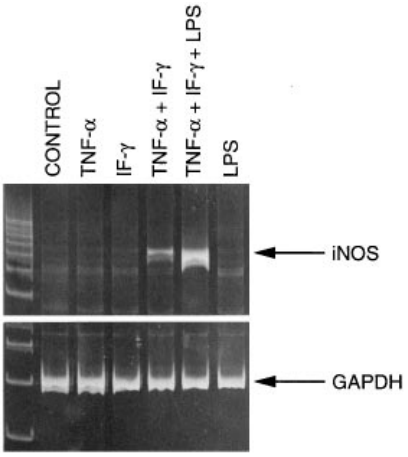

Figure 1 Effect of TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml) on the expression of iNOS mRNA in L6 myocytes.

L6 cells were treated or not with cytokines and LPS for 24 h before RNA isolation and RT-PCR analysis of iNOS mRNA. GAPDH mRNA levels were also evaluated in the same RNA samples. The sizes of the amplification products were, as expected, 653 bp for iNOS and 331 bp for GAPDH. The experiment shown is representative of three individual determinations with different cells.

We next investigated whether the increased production of NO was related to the induction of iNOS. L6 myocytes treated as above were used for extraction of RNA and iNOS mRNA detection by RT-PCR analysis (Figure 1). GAPDH mRNA levels assessed in the same samples were used as internal controls for the RT-PCR assay. iNOS and GAPDH were both amplified as single gene products migrating at the expected molecular size (iNOS, 653 bp; GAPDH, 331 bp) on agarose gels. iNOS mRNA was not detectable in control myocytes or in cells treated with either cytokines or LPS alone, but was observed in cells treated with the combination of TNF-α and IF-γ. As for nitrite levels, the combination of both cytokines and LPS markedly induced iNOS expression. The expression of GAPDH mRNA was not different among treatment groups. Moreover, neither the nNOS nor the eNOS isoforms could be detected in control or cytokine/LPS-treated L6 cells (results not shown).

The close parallelism between the action of cytokines and LPS to increase nitrite levels and iNOS mRNA strongly suggested that iNOS was responsible for the enhanced NOS activity. To establish that point, we also determined iNOS protein levels in these cells, using both immunoblotting and histochemical detection techniques. Using a specific polyclonal antibody against macrophage-type iNOS, we could detect iNOS induction at the protein level only in cells chronically treated with both cytokines and LPS (Figure 2). In contrast, the α1-Na/K-ATPase, used here as a control protein, was present in all samples. Moreover, this treatment did not affect total cellular proteins (μg per well) under the same conditions (results not shown). The lack of detection of iNOS protein in muscle cells treated with both cytokines in the absence of LPS may be related to the limited sensitivity of Western blotting as compared with RT-PCR analysis of iNOS mRNA. This is supported by the observation that nitrite pro-

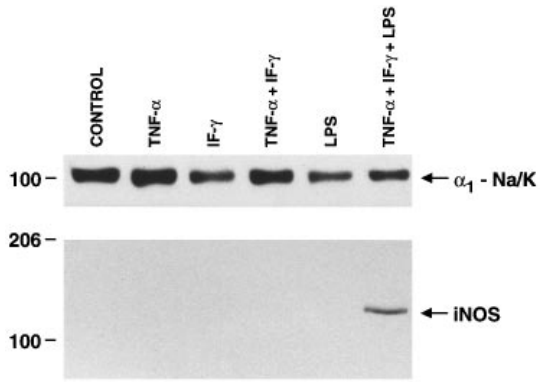

Figure 2 Effect of TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml) on the expression of iNOS protein in L6 myocytes.

L6 cells were treated or not with cytokines and LPS for 24 h before protein extraction and Western-blot analysis with specific antibodies against iNOS and the α1-subunit of the Na/K-ATPase (α1-Na/K). The migrations of molecular-mass standards (in kDa) are shown on the left. The experiment shown is representative of three individual determinations with different cells.

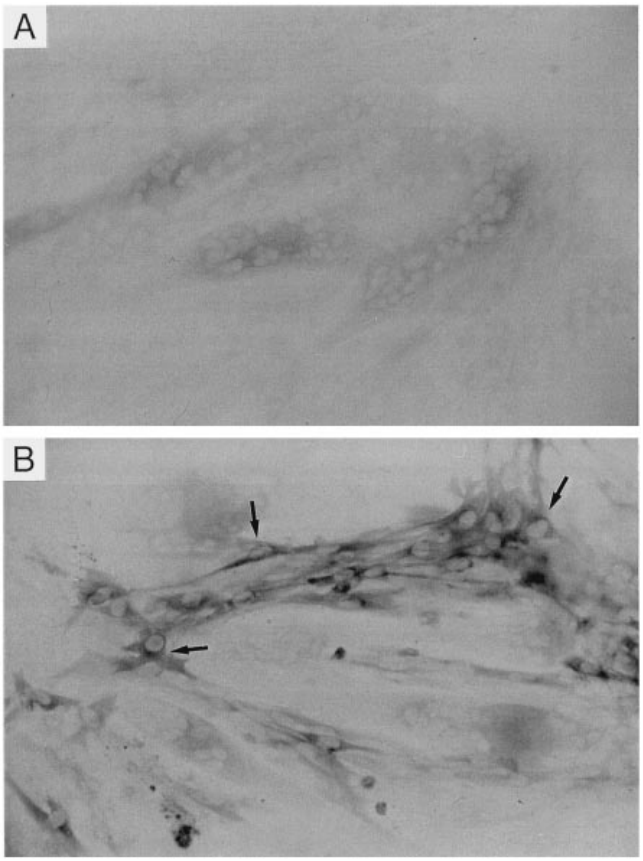

Figure 3 Histochemical detection of NOS in control (A) and cytokines/LPS-treated (B) L6 myocytes.

Cells grown on coverslips were incubated in the presence or not of the cytokines/LPS mixture for 24 h, and NOS was revealed by histochemical diaphorase staining, as described in the Materials and methods section. Arrows point to intense labelling located throughout the cytoplasm of multinucleated myotubes. Magnification: $\times$50. The experiment shown is representative of two separate experiments.

Table 2 Effects of chronic cytokines/LPS exposure on basal and insulin-stimulated glucose transport in L6 myocytes.

Cells were treated or not with a combination of TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml), with or without L-NAME (2 mM), for 24 h, followed by 0.6 μM insulin (or medium) for an additional 45 min incubation before glucose-uptake measurements. Values represent means $\pm$ S.E.M. of four individual experiments performed in triplicate. Insulin-mediated 2-deoxyglucose uptake values were calculated from the difference between insulin and non-insulin-stimulated values for each experimental conditions.

	2-Deoxyglucose uptake (pmol/min per mg)	
Conditions	$-$ L-NAME	$+$ L-NAME
Control	31.45 ± 1.93^{a}	27.85 ± 1.90^{a}
Insulin	53.10 ± 2.53^{b}	46.98 ± 3.67^{b}
Cytokines + LPS	70.66 ± 3.31^{c}	26.78 ± 0.81^{a}
Cytokines + LPS + insulin	82.14 ± 1.35^{d}	43.56 ± 3.81^{b}
	Insulin-mediated 2-deoxy-glucose uptake (pmol/min per mg)	
Conditions	$-$ L-NAME	$+$ L-NAME
Control	20.77 ± 3.47^{a}	19.13 ± 2.54^{a}
Cytokines + LPS	11.08 ± 2.46^{b}	16.78 ± 3.28^{a}

[a–d] Mean values not sharing a common superscript are significantly different at $P < 0.05$.

Table 3 Effects of chronic cytokines/LPS exposure on lactate and glucose concentrations in the culture medium of L6 myocytes.

Cells were treated or not with a combination of TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml), with or without L-NAME (2 mM), for 24 h. Medium was collected and used for measurements of lactate and glucose concentrations as described in the Materials and methods section. * $P < 0.05$ as compared with control values.

Conditions	Lactate (mM)	Glucose (mM)
Control	2.08 ± 0.24	4.16 ± 0.26
Cytokines/LPS	3.34 ± 0.42*	3.75 ± 0.14
L-NAME	1.79 ± 0.20	4.45 ± 0.22
Cytokines/LPS + L-NAME	2.21 ± 0.30	4.34 ± 0.25

duction was observed in cells treated with both cytokines (Table 1). Induction of iNOS was further confirmed by diaphorase staining (Figure 3B). Thus these results demonstrate that the induction of iNOS mRNA expression in cytokines/LPS-treated cells resulted in the synthesis of more iNOS protein and NO production.

We next investigated the effects of cytokines and LPS on basal and insulin-stimulated glucose transport in L6 myocytes (Table 2). As expected, acute insulin stimulation (45 min) significantly stimulated glucose transport in these cells. Chronic exposure (24 h) of L6 cells to the cytokines/LPS mixture markedly increased basal glucose uptake above that seen with insulin alone. As shown in Table 3, this treatment also induced a significant release of lactate in the medium. Lactate concentrations in the medium were not increased during the acute (45 min) stimulation with insulin (results not shown). In cells previously treated with cytokines/LPS, insulin produced a much smaller increase in glucose transport above that seen with cytokines/LPS treatment (Table 2). This stimulation (in

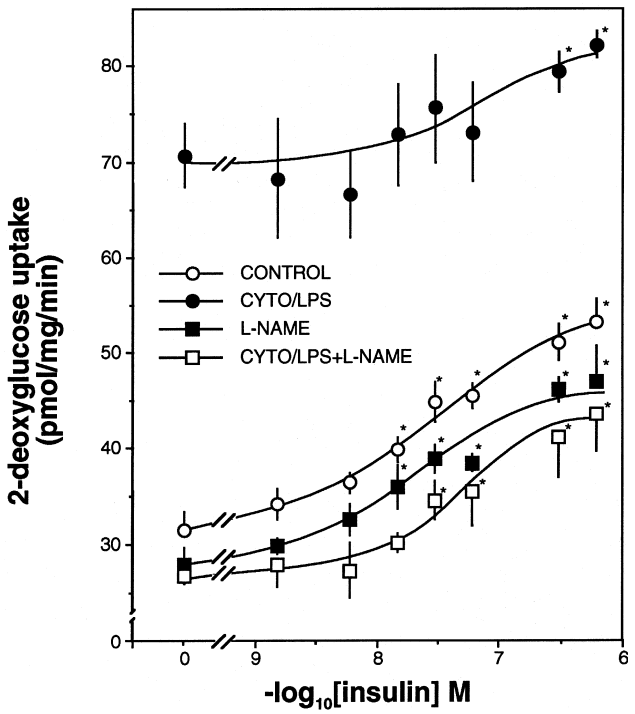

Figure 4 Dose–response curves of insulin-stimulated glucose transport in control and cytokines (cyto)/LPS-treated L6 myocytes.

Cells were treated as described in the legend to Table 3, followed by addition of indicated concentrations of insulin before glucose-uptake measurements. When present, L-NAME was used at a concentration of 2 mM. The EC_{50} values for the dose–response curves were as follows: control, 22 nM; cytokines/LPS, 70 nM; L-NAME, 20 nM; cytokines/LPS + L-NAME, 40 nM. Points represent means ± S.E.M. of three individual experiments performed in triplicate. $^*P <$ 0.05 compared with unstimulated (basal) 2-deoxyglucose-uptake values.

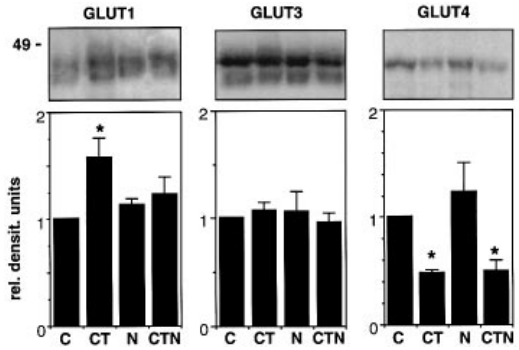

Figure 5 Effects of cytokines/LPS and L-NAME on the expression of GLUT1, GLUT3 and GLUT4 glucose transporter proteins in L6 myocytes.

Cells were treated or not with a combination of TNF-α (10 ng/ml), IF-γ (200 units/ml) and LPS (10 μg/ml), with or without L-NAME (2 mM), for 24 h. Upper panels: total cell membranes were isolated, and equivalent amounts (50 μg) of proteins were subjected to SDS/PAGE and immunoblotting with isoform-specific antibodies against GLUT1, GLUT3 and GLUT4, as described in the Materials and methods section. The migration of the 49 kDa molecular-mass standard is indicated to the left. Lower panels: results are expressed as changes in the amounts of transporter proteins relative to control values. Values represent means ± S.E.M. of three or four individual determinations with different batches of cells. Abbreviations: C, control; CT, cytokines/LPS; N, L-NAME; CTN, cytokines/LPS + L-NAME; rel. densit., relative densitometric units. $^*P < 0.05$ compared with the control.

pmol/min per mg of protein) was about half that seen in control untreated myocytes. Cells treated with TNF-α and IF-γ either alone or in combination failed to affect basal or insulin-stimulated glucose transport in the same conditions (results not shown). Noticeably, preventing NO production by addition of L-NAME to the incubation medium of cells treated with cytokines and LPS blocked the stimulation of basal glucose uptake and lactate release, and restored the responsiveness of L6 myocytes to insulin (Tables 2 and 3). Similar results were obtained when NO production was abolished by L-N^6-(1-iminoethyl)lysine (0.1 mM), a more selective inhibitor of iNOS [25] (results not shown).

We also determined the effects of cytokines/LPS on insulin sensitivity by measuring glucose uptake at different concentrations of insulin (Figure 4). Cells chronically exposed to the cytokines/LPS mixture were more resistant to the action of insulin, as reflected by the lack of significant effect of lower doses (up to 60 nM) of the hormone to activate glucose transport in cytokines/LPS-treated cells (see the EC_{50} in the legend to Figure 4). L-NAME improved insulin sensitivity in cytokines/LPS-treated myocytes, as shown by the restored ability of lower concentrations to stimulate glucose transport. The NOS inhibitor alone slightly decreased absolute glucose transport rates in L6 cells. However, both basal and insulin-stimulated glucose transport were similarly affected, and, thus, L6-cell insulin responsiveness and sensitivity were not significantly different from control myocytes.

Glucose transport is mediated by the GLUT1, GLUT3 and GLUT4 transporters in L6 muscle cells, and insulin has been shown to stimulate glucose transport by inducing the translocation of these transporter proteins to the plasma membrane [26]. The effect of cytokines/LPS treatment on the cellular expression of these glucose transporter isoforms is shown in Figure 5. The cytokines/LPS mixture significantly increased (by $\sim 60\%$) the expression of the GLUT1 transporter protein. In marked contrast, the expression of the GLUT4 glucose transporter was markedly decreased in the same cells. Inhibition of NO production by L-NAME treatment reversed the increasing effect of cytokines/LPS on GLUT1 protein expression but failed to restore GLUT4 protein levels. The NOS inhibitor did not alter GLUT1 or GLUT4 expression. On the other hand, the expression of the GLUT3 transporter protein was not affected by these treatments (Figure 5). As expected, acute exposure (45 min) of the cells to insulin did not affect the total levels of any GLUT isoforms (results not shown).

DISCUSSION

The present study shows that chronic exposure of L6 myocytes to cytokines induced iNOS expression and NO production in these cells. Moreover, the cytokine effect was markedly enhanced by the presence of LPS. These results in L6 cells are in good agreement with previous studies showing iNOS mRNA induction in skeletal muscle of endotoxin-treated rats and in C2C12 mouse myocytes exposed to cytokines [15,17]. Our findings further demonstrate that iNOS expression is increased at the protein level, as determined by immunoblotting and histochemical techniques.

This induction of iNOS expression was associated with important changes in glucose transport in muscle cells. First, cytokines and LPS markedly increased basal glucose-transport activity in L6 myocytes. This metabolic effect has been recently observed in another study with muscle cells [27], and the present study suggests that the extra glucose taken up by the cells was mainly used for glycolysis, since lactate concentrations significantly rose in the medium. These results are in good agreement with the known effects of cytokines such as TNF-α *in vivo*.

Indeed, TNF-α has been reported to increase skeletal muscle glucose disposal in rats and even cause hypoglycaemia at high doses [28,29]. The increase in glucose utilization was more easily observed at high concentrations of the cytokine [29]. It has been suggested that this metabolic response is not readily observed at lower doses of TNF-α because of the concomitant elevation of counter-regulatory hormones (glucagon, catecholamines and cortisol). Indeed, the hypoglycaemic effects of TNF-α are observed at lower doses of the cytokine in adrenalectomized rats [29]. More recently, Sakurai et al. [6] have provided evidence that TNF-α directly stimulates peripheral glucose uptake in peripheral tissues by preventing any changes in insulin and glucagon during TNF-α infusion.

Another finding of the present study is that cytokines/LPS treatment impaired insulin stimulation of glucose transport in L6 muscle cells. This impairment was observed despite the elevation of basal glucose transport by cytokines/LPS exposure. These results are consistent with previous reports that septic patients are insulin resistant [3] and that exogenous challenge with cytokines impairs insulin-mediated glucose uptake in skeletal muscle [7]. It is likely that the insulin-resistant effects of cytokines are better appreciated *in vivo*, since, as mentioned above, the direct action of TNF-α to enhance muscle glucose disposal is over-ruled by counter-regulatory hormones such as glucagon. Importantly, in the conditions used in the present study (24 h), TNF-α alone was not able to influence muscle cell glucose uptake, thus suggesting that it interacts with other cytokines and/or endotoxin during infection *in vivo*. These results are in accordance with the recent work of Ranganathan and Davidson [27], who also found that insulin action on glucose transport is not affected by TNF-α alone. However, these data are at odds with another study in which the same concentration of TNF-α was found to inhibit insulin-stimulated glucose uptake within minutes and for up to 12 h in L6 cells [30]. The reasons for these discrepant findings are not known. They cannot be attributed to the type (murine or human) of TNF-α used and, thus, the type of TNF-α receptors activated (murine TNF-α binds to both p55 and p75 receptors but human TNF-α only binds to the p55 receptor [31]), as we found similar effects of murine or human TNF-α on iNOS expression and glucose transport in this study (results not shown).

An important goal of the present study was to determine the cellular mechanism by which cytokines and endotoxin affect glucose transport in muscle cells. The fact that cytokines/LPS increased NO production and modulated glucose transport in the same cells suggests that these effects are related, but a causal relationship remained to be shown. Our findings that the NOS inhibitor L-NAME inhibited NO production and fully prevented the effects of the cytokines/LPS challenge on basal and insulin-stimulated glucose transport provide convincing evidence that both the stimulatory and insulin-resistant actions of cytokines are linked to the induction of iNOS and the production of NO by muscle cells.

Since chronic alterations in glucose-transport activity may be linked to changes in the expression of glucose transporters, we have determined the cellular protein levels of the three transporter isoforms known to be present in L6 muscle cells. Our results strongly suggest that cytokines/LPS increase basal glucose transport at least in part by augmenting the expression of the GLUT1 glucose transporter. It should be noted that the effect of cytokines/LPS on GLUT1 expression (60%) was smaller than their enhancing action on basal glucose transport (120%). However, it is possible that most of the biosynthesized GLUT1 proteins are localized in the plasma membrane (where glucose transport takes place) in cytokine-treated cells, thus explaining the greater increases in basal glucose transport. Another possibility is that GLUT1 intrinsic activity is activated by cytokines. Whatever the mechanism, it was found that GLUT1 overexpression is blocked by L-NAME treatment, indicating that NO production was responsible for the effects of cytokines and LPS on both basal glucose transport and GLUT1 protein levels.

In contrast to their effects on GLUT1 expression, cytokines and LPS markedly reduced GLUT4 protein levels in L6 muscle cells. These findings suggest that the impaired insulin action in cytokine-treated L6 cells is associated with a decreased expression of GLUT4, an hypothesis that is in accordance with previous studies in which chronic treatment with cytokines was found to decrease GLUT4 expression in adipocytes [32–34]. However, the finding that NOS blockade with L-NAME re-established insulin action without restoring GLUT4 content indicates that the cytokine effect is not only related to the cellular expression of the GLUT4 protein. Insulin stimulates glucose transport in L6 myocytes by translocation of glucose transporters (GLUT1, GLUT3 and GLUT4) to the plasma membrane [26]. Thus it is possible that cytokines and LPS decrease insulin action by impairing the translocation of glucose transporters and that this effect is NO dependent, as it can be reversed by L-NAME treatment. This hypothesis is consistent with our recent observations that NO inhibits insulin-stimulated glucose transport in isolated rat skeletal muscles and in L6 myocytes [11].

In summary, the present study demonstrates that the cytokines TNF-α and IF-γ, when added in combination, induce iNOS expression and stimulate nitrite production in L6 myocytes, and that LPS synergistically enhances these effects. Moreover, cytokines and LPS markedly increase basal glucose transport but decrease insulin action to stimulate glucose uptake, and these effects are mediated by induction of iNOS and NO production. Our results further show that cytokines and endotoxin increase GLUT1 expression by an NO-dependent mechanism.

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