Opposing effects of tumour necrosis factor α and hyperosmolarity on Na^+/myo -inositol co-transporter mRNA levels and *myo*-inositol accumulation by 3T3-L1 adipocytes

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Tumour necrosis factor α (TNF- α) regulates the transport of *myo*-inositol in 3T3-L1 adipocytes. Treating 3T3-L1 adipocytes with TNF- α decreases Na^+/myo -inositol co-transporter (SMIT) mRNA levels and *myo*-inositol accumulation in a concentration- and time-dependent manner. TNF- α decreases the V'_{max} for high-affinity *myo*-inositol transport with little change in the K'_m . Studies with actinomycin D suggest that RNA synthesis is required for the TNF- α -induced effect on SMIT mRNA levels. In contrast with the effect of TNF- α , hyperosmolarity increases SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes. Hyperosmolarity increases SMIT gene expression as evidenced by the inhibition of hyperosmotic induction of SMIT mRNA levels by actinomycin D, and of *myo*-inositol accumulation by actinomycin D and cycloheximide. TNF- α and osmotic stress have previously been shown to activate similar signal transduction pathways in mammalian cells. In 3T3-L1 adipocytes, both TNF- α and hyperosmolarity increase mitogen-

activated protein kinase pathway activity; however, with the possible exception of c-Jun N-terminal kinase, this pathway does not seem to regulate SMIT mRNA levels or *myo*-inositol accumulation. TNF- α activates nuclear factor κB (NF- κB) in 3T3-L1 adipocytes but, unlike the effect of TNF- α on cultured endothelial cells, NF- κB does not seem to contribute to the regulation by TNF- α of SMIT gene expression in 3T3-L1 adipocytes. Therefore other signal transduction pathways must be considered in the regulation by TNF- α of SMIT mRNA levels and activity. Thus TNF- α and hyperosmolarity have opposing effects on SMIT mRNA levels and activity in 3T3-L1 adipocytes. Because *myo*-inositol in the form of phosphoinositides is an important component of membranes and signal transduction pathways, the regulation of *myo*-inositol metabolism by TNF- α might represent another mechanism by which TNF- α regulates adipocyte function.

INTRODUCTION

We have previously reported that tumour necrosis factor α (TNF- α) selectively decreases levels of Na^+/myo -inositol co-transporter (SMIT) mRNA and *myo*-inositol accumulation in cultured large vessel and cerebral microvessel endothelial cells [1]. In contrast, TNF- α did not affect *myo*-inositol accumulation by a variety of other cultured mammalian cells [1], although an effect of TNF- α on SMIT mRNA levels and *myo*-inositol accumulation was observed in adipocytes. To explore further the effect of TNF- α on *myo*-inositol metabolism, we chose to study 3T3-L1 adipocytes. As in endothelial cells, TNF- α has a wide range of effects on adipocytes and has been linked to the development of insulin resistance [2–12].

TNF- α is synthesized and secreted from adipose tissue and acts in an autocrine-like fashion to mediate a wide range of biological effects [6–12]. Overexpression of TNF- α mRNA and increased production of TNF- α by adipose tissue have been described in many animal models of insulin resistance and obesity [11,13,14]. TNF- α is also overexpressed in the fat and muscle of obese humans [9,10]. In mammalian cells, TNF- α binds to two distinct receptors, p55 and p75 TNF receptors, that transmit signals to the cytoplasm and the nucleus [15]. In adipocytes, stimulation of the p55 TNF receptors is sufficient to inhibit insulin signalling [16]. Two second messengers, sphingosine and ceramide, are formed after the binding of TNF- α to its receptor [17]. In 3T3-L1 adipocytes, the exogenous addition

of cell-permeable ceramides duplicates the effect of TNF- α on insulin-stimulated glucose uptake [7,18].

Insulin resistance, a subnormal biological response to insulin, is a systemic phenomenon associated with several diseases, including chronic infection, cancer, obesity and non-insulin-dependent diabetes mellitus [19]. Several laboratories have shown that TNF- α is an important mediator of insulin resistance; however, the mechanism(s) by which TNF- α interferes with insulin action are not well understood [19]. Some of the effects of TNF- α on adipocytes include transcriptional repression of the gene for GLUT4, decreased GLUT4 mRNA stability, decreased insulin-stimulated autophosphorylation of the insulin receptor and phosphorylation of insulin receptor substrate 1, inhibition of lipoprotein lipase gene transcription and decreased DNA-binding activity and expression of the transcription factor CCAAT/enhancer binding protein [8,20–23]. Here we show that another effect of TNF- α on adipocytes is a decrease in SMIT mRNA levels and *myo*-inositol accumulation. Because *myo*-inositol uptake is important for maintaining phosphoinositide production in mammalian cells, and signalling pathways that utilize the phosphoinositide pool mediate insulin action, it is possible that a decrease in *myo*-inositol metabolism in adipocytes could have an impact on insulin signalling [1,24].

In the present study we also examined the effect of hyperosmolarity on SMIT mRNA levels and *myo*-inositol accumulation. Because stress factors such as hyperosmolarity have been shown to induce the same signal transduction pathways as those

Abbreviations used: ERK, extracellular signal-regulated protein kinase; IGF-I, insulin-like growth factor I; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF- κB , nuclear factor κB ; SMIT, Na^+/myo -inositol co-transporter; TGF- β , transforming growth factor β ; TLCK, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one; TNF- α , tumour necrosis factor α .

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of cytokines, the effect of hyperosmolarity was examined to compare and contrast its effect with TNF- α and to help elucidate the mechanism responsible for the TNF- α effect [25,26]. In endothelial cells and other cultured mammalian cells, hyperosmolarity has been shown to increase SMIT mRNA levels and *myo*-inositol transport [27–31]. However, the effect of hyperosmolarity on adipocytes has not been examined.

EXPERIMENTAL

Materials

Recombinant human TNF- α was obtained from Research & Development Systems (Minneapolis, MN, U.S.A.). The specific activity was 0.02 ng/ml, on the basis of a cytotoxicity assay with L-929 cells. The endotoxin level was less than 0.1 ng/ μ g of cytokine as determined by the *Limulus* amoebocyte lysate assay. Interleukin (IL)-1 α , IL-1 β , IL-2, genistein, *N*-oleoylethanolamine, staurosporin, sphingosine, C₆-ceramide, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (TLCK), pyrrolidine dithiocarbamate, PMA and other chemicals and reagents not specifically mentioned were obtained from Sigma (St. Louis, MO, U.S.A.). *myo*-[2-³H]inositol, [γ -³²P]ATP, [α -³²P]dATP and [³²P]UTP were obtained from Amersham (Arlington Heights, IL, U.S.A.). The anti-sense β -actin mRNA and 18 S anti-sense ribosomal RNA probe were obtained from Ambion (Austin, TX, U.S.A.). Chloroform, 3-methylbutan-1-ol, Corning 25 cm² and 75 cm² flasks and Falcon six-well plates were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). IL-6 was obtained from Bachem Bioscience (King of Prussia, PA, U.S.A.). Insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β) were obtained from Interger (Purchase, NY, U.S.A.). Wortmannin, rapamycin and calphostin C were obtained from Calbiochem (La Jolla, CA, U.S.A.). PD-98059 was a gift from Dr. Alan Saltiel (Department of Signal Transduction, Parke-Davis Pharmaceutical Division, Ann Arbor, MI, U.S.A.). SB-203580 was a gift from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). SDS was obtained from BDH (Poole, Dorset, U.K.). Ethidium bromide and Hepes were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Transcription buffer, dithiothreitol, RNasin, ATP, CTP, UTP, GTP, T7 RNA polymerase and DNase were obtained from Promega (Madison, WI, U.S.A.). Safety Solve, CsCl and scintillation vials were obtained from Research Products International (Mount Prospect, IL, U.S.A.). Cell culture medium was obtained from the Diabetes Endocrinology Research Center Cell Biology Core, University of Iowa (Iowa City, IA, U.S.A.). Synthetic oligonucleotides used for electrophoretic mobility-shift assays were provided by Genosys (Woodlands, TX, U.S.A.) through the Diabetes Endocrinology Research Center Molecular Biology Core, University of Iowa.

Cell culture

The murine 3T3-L1 fibroblasts used in this study were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured, maintained and differentiated as described previously [32].

myo-Inositol accumulation

For the determination of *myo*-inositol accumulation, 3T3-L1 adipocytes were incubated in Dulbecco's modified Eagle's medium (serum-free) containing 0.5% BSA for 1–24 h in the absence or presence of 0.1–10 ng/ml TNF- α or 50–300 mM raffinose to

induce hyperosmolarity as described previously [1,30]. For many of these studies *myo*-inositol accumulation was determined after a 30 min incubation. In these studies, '*myo*-inositol accumulation' represents the cellular accumulation of radioactivity derived from *myo*-[2-³H]inositol during the incubation and does not represent the initial rate of *myo*-inositol transport by the cells, nor does it account for any *myo*-[2-³H]-inositol taken up by the cells then secreted during the 30 min incubation period. TNF- α or hyperosmolarity did not cause an increase in the turnover of radioactivity after the metabolic labelling of the cells with *myo*-[2-³H]inositol for 24 h (results not shown). Therefore the secretion of *myo*-[2-³H]inositol during the 30 min incubation period probably had little effect on these results. A 1 h incubation of 3T3-L1 adipocytes with 10 ng/ml TNF- α or 150 mM raffinose did not increase ⁵¹Cr release as determined by the method of Thorne et al. [33], indicating that neither TNF- α nor hyperosmolarity was cytotoxic.

For reversion studies, adipocytes were preincubated, as described above, for 24 h in serum-free medium with 0.5% BSA containing 10 ng/ml TNF- α or 150 mM raffinose. The adipocytes were then washed with isotonic serum-free medium and incubated in this medium for 1–24 h. After selected periods of time, SMIT mRNA levels (see below) or *myo*-inositol accumulation was determined as described above.

We also determined the effect of C₆-ceramide, sphingosine, insulin, cytokines, growth factors, PMA or hyperglycaemia on *myo*-inositol accumulation or SMIT mRNA levels by 3T3-L1 adipocytes. For these studies, 3T3-L1 adipocytes were incubated as described in the figure and table legends. Afterwards *myo*-inositol accumulation was determined as described above. We also examined the effect of actinomycin D or cycloheximide on hyperosmolarity-induced changes in *myo*-inositol accumulation and on changes in SMIT mRNA levels induced by TNF- α or hyperosmolarity. For these studies, 3T3-L1 adipocytes were preincubated with 5 μ M actinomycin D or 25 μ M cycloheximide for 1 h before the addition of either 10 ng/ml TNF- α or 150 mM raffinose; the incubations were performed as indicated. Afterwards *myo*-inositol accumulation or SMIT mRNA was determined. In studies examining the effect of inhibitors on the changes in *myo*-inositol accumulation induced by TNF- α or hyperosmolarity, 3T3-L1 adipocytes were preincubated for 1 h in serum-free medium with 0.5% BSA in the absence or presence of each inhibitor at the concentrations indicated in Table 2. Afterwards 10 ng/ml TNF- α or 150 mM raffinose was added; the incubation continued for an additional 16 h. The accumulation of *myo*-inositol was then determined as described above.

Kinetic parameters for high-affinity and low-affinity *myo*-inositol transport were determined by incubating 3T3-L1 adipocytes for 5 min in Hepes buffer with or without NaCl and containing 5–1000 μ M *myo*-[2-³H]inositol as described previously [1].

To determine the effect of TNF- α or hyperosmolarity on the intracellular *myo*-inositol and sorbitol content, 3T3-L1 adipocytes were grown in 25 cm² flasks, then incubated in serum-free medium with 0.5% BSA in the absence or presence of 10 ng/ml TNF- α or 150 mM raffinose for 24 h. Afterwards the adipocytes were washed with glucose-free Hepes buffer, collected in water and sonicated. Aliquots of the cell suspension were taken for protein determination; derivatized samples were analysed as described previously [1].

RNA isolation and quantification of SMIT mRNA levels

RNA was prepared by using the guanidine isothiocyanate/CsCl method. RNA was quantified by measuring the absorbance at

260 nm, and the integrity of the RNA and accuracy of quantification were confirmed by size-separating the RNA by denaturing gel electrophoresis and comparing the intensity of the 18 S and 28 S ribosomal RNA bands after staining of the gel with ethidium bromide [1]. SMIT mRNA levels in 3T3-L1 adipocytes were quantified with a solution hybridization/RNase protection assay as described previously [1,30].

Mitogen-activated protein kinase (MAPK) assays

MAPK assays were performed with an immune complex kinase assay as described previously [34]. In brief, adipocytes were treated as indicated and lysed in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1% (v/v) Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 0.12 mM PMSF and 0.7 µg/ml leupeptin. After a low-speed centrifugation (to remove the lipid plug) and clarification, 250 µg of cell lysate protein was incubated with anti-(extracellular signal-regulated protein kinase 1) (anti-ERK1), anti-ERK2, anti-(c-Jun N-terminal kinase 1) (anti-JNK1), anti-JNK2 or anti-(p38 kinase) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) that had been prebound to Protein A-agarose beads (Pierce Chemical Co., Rockford, IL, U.S.A.). The beads were collected and washed, then resuspended in kinase buffer [25 mM Hepes (pH 7.4)/10 mM MgCl₂/10 mM MnCl₂/1 mM dithiothreitol/15 µM ATP]. To the reaction mix were added the substrates myelin basic protein (Upstate Biotechnology, Lake Placid, NY, U.S.A.) for ERK1 and ERK 2 determination, c-Jun (1–81) (Santa Cruz Biotechnology) for JNK1 or JNK2 determination, or activating transcription factor (ATF)-2 (1–96) (Santa Cruz Biotechnology) for p38 kinase determination, at a final concentration of 167 µg/ml, together with 500 µCi/ml [γ -³²P]ATP. The reaction continued for 20 min at 22 °C and was terminated by the addition of Laemmli sample buffer. The phosphorylated reaction products were then separated by SDS/PAGE. The resulting gel was dried and exposed to X-ray film to determine the ³²P incorporation into each substrate by using a scanning densitometer. Each assay was conducted in duplicate with two separately prepared cell lysates.

Electrophoretic mobility-shift assay

3T3-L1 adipocytes were incubated for 30 min in serum-free medium containing 10 ng/ml TNF- α , 100 µM C₆-ceramide, 100 µM sphingosine or 150 mM raffinose. Afterwards the adipocytes were washed and harvested with PBS at 4 °C followed by a low-speed centrifugation. The adipocytes were resuspended in 1.5 ml of buffer A [10.0 mM Hepes (pH 8.0)/1.5 mM MgCl₂/10.0 mM KCl/0.5 mM dithiothreitol/300 mM sucrose/0.1% (v/v) Nonidet P40/1 µg/ml pepstatin/1 µg/ml antipain/1 µg/ml chymostatin/1 µg/ml aprotinin/0.1 µg/ml leupeptin/0.5 mM PMSF] and incubated on ice for 5 min. The crude nuclear pellet was then collected by microcentrifugation for 2 min at 4 °C. Afterwards the pellet was quickly washed with buffer A and resuspended in buffer B [20 mM Hepes (pH 8.0)/20% (v/v) glycerol/100 mM KCl/100 mM NaCl/0.2 mM EDTA/0.5 mM PMSF/0.5 mM dithiothreitol/1 µg/ml pepstatin/1 µg/ml antipain/1 µg/ml chymostatin/1 µg/ml aprotinin/0.1 µg/ml leupeptin]. The isolated nuclei were sonicated for 10 s at 4 °C and clarified by microcentrifugation. The protein concentration of the extract was determined; the extract was then stored at –70 °C until assayed. For gel mobility-shift assays, annealed oligonucleotides containing the consensus sequence for nuclear factor κ B (NF- κ B) (5'-TTTCGCGGGGACTTCCCGCGC-3'; 5'-TTTGCGCGGGAAAGTCCCGCGC-3') and the E-box of the adenovirus major late transcription factor promoter (5'-

ATAGGTGTAGGCCACGTGACCGGGTGT-3'; 5'-ACACCGGTCACGTG-3') were radiolabelled with [α -³²P]dATP and unlabelled dGTP, dCTP and dTTP by using Klenow DNA polymerase, then gel-purified as described previously [1]. Nuclear extract (15 µg) was preincubated for 10 min at 25 °C with 1 µg of poly(dI-dC)·poly(dI-dC) under ionic conditions. Radiolabelled probe (5 × 10⁴ c.p.m.; approx. 2 ng) was added to each 20 µl reaction and incubated for 15 min at 37 °C. Samples were analysed on a non-denaturing 5% (w/v) polyacrylamide gel in 0.5 × Tris/borate/EDTA [45 mM Tris/borate/1 mM EDTA (pH 8.0)] and subjected to electrophoresis at 115 V for 3 h at 25 °C. Gels were then dried and autoradiographs were exposed for the appropriate period at –70 °C with intensifying screens.

Data analysis

All results are expressed as means ± S.E.M. Results for myo-inositol accumulation and myo-inositol and sorbitol content are reported as nmol/mg of cell protein. Results for SMIT mRNA levels are reported as percentages of control. Statistical comparisons for significance were performed with ANOVA and an unpaired Student's *t*-test or Dunnett's analysis at a *P* value of 0.05.

RESULTS

Effect of TNF- α and hyperosmolarity on SMIT mRNA levels and myo-inositol accumulation

To determine the effect of TNF- α and hyperosmolarity on SMIT mRNA levels and myo-inositol accumulation, 3T3-L1 adipocytes were incubated for 16 h in serum-free medium containing 0.5%

Figure 1 Effect of TNF- α or hyperosmolarity on myo-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA containing no additions (control) or 10 ng/ml TNF- α or 150 mM raffinose (hyperosmolarity) for 16 h. Afterwards myo-inositol accumulation was determined by washing the cells with osmotically matched M199 serum-free medium containing 0.5% BSA and then incubating the cells in this medium containing myo-[2-³H]inositol as described in the Experimental section. For these studies, cells were incubated for 5–60 min. For SMIT mRNA determination, cells were incubated as described above and then RNA was isolated and SMIT and β -actin mRNA levels were determined as described in the Experimental section. Results are presented as nmol/mg of protein for myo-inositol accumulation. Each value is the mean ± S.E.M. for nine separate determinations. The inset shows a representative autoradiogram of SMIT and β -actin mRNA levels. **P* < 0.05, significant decrease compared with control; **P* < 0.05, significant increase compared with control.

Figure 2 Concentration and time-course effect of $\text{TNF-}\alpha$ on *myo*-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA containing 0–10 ng/ml $\text{TNF-}\alpha$ (concentration response; left panel) for 16 h, or 10 ng/ml $\text{TNF-}\alpha$ for 0–24 h (time course; right panel). Afterwards *myo*-inositol accumulation was determined by washing the cells with M199 serum-free medium containing 0.5% BSA and then incubating the cells in this medium containing *myo*-[2- ^3H]inositol for 30 min as described in the Experimental section. For SMIT mRNA determination, cells were incubated as described above and then RNA was isolated and SMIT mRNA levels were determined as described in the Experimental section. SMIT mRNA levels were standardized to 18 S rRNA levels. Results are presented as nmol/mg of protein for *myo*-inositol accumulation or as percentages of control for the SMIT mRNA levels, with the level of SMIT mRNA in control cells assigned a value of 1. Each value is the mean $\pm$ S.E.M. for nine separate determinations for *myo*-inositol accumulation, or four separate determinations for SMIT mRNA levels. * $P < 0.05$ compared with control.

BSA in the absence or presence of 10 ng/ml $\text{TNF-}\alpha$ or 150 mM raffinose. Afterwards SMIT mRNA levels (Figure 1, inset) and *myo*-inositol uptake over a 5–60 min incubation were determined (Figure 1). *myo*-Inositol accumulation by 3T3-L1 adipocytes was linear over the 60 min incubation period. Treatment of adipocytes with $\text{TNF-}\alpha$ significantly decreased *myo*-inositol accumulation at all time points, whereas hyperosmolarity significantly increased *myo*-inositol accumulation. SMIT mRNA levels were decreased to 25% of control levels by $\text{TNF-}\alpha$. In contrast, hyperosmolarity stimulated an approx. 5-fold increase in SMIT mRNA levels. The results in Figure 2 show a concentration response and time course for the effect of $\text{TNF-}\alpha$ on *myo*-inositol accumulation and SMIT mRNA levels. $\text{TNF-}\alpha$ (1 ng/ml) caused a significant decrease in both SMIT mRNA levels and *myo*-inositol accumulation. Treatment of 3T3-L1 adipocytes with 10 ng/ml $\text{TNF-}\alpha$ for 3 h caused a significant decrease in both SMIT mRNA levels and *myo*-inositol accumulation, with maximal effects on *myo*-inositol accumulation after a 12 h incubation. The effect of hyperosmolarity on SMIT mRNA levels and *myo*-inositol accumulation was also concentration- and time-dependent (Figure 3). Exposure of 3T3-L1 adipocytes for 16 h to medium containing 50 mM raffinose, which increased the osmolarity of the medium from approx. 310 to 358 m-osM, significantly increased both SMIT mRNA levels and *myo*-inositol accumulation. Maximum effects were observed with 150 mM raffinose. The time-course study revealed that the hyperosmolarity-induced increase in SMIT mRNA levels preceded the increase in *myo*-inositol accumulation and that a 6 h exposure to medium containing 150 mM raffinose maximally stimulated SMIT mRNA levels.

To determine the effect of $\text{TNF-}\alpha$ and hyperosmolarity on the cellular content of *myo*-inositol or sorbitol in 3T3-L1 adipocytes, the cells were incubated for 24 h in serum-free medium containing

Figure 3 Concentration and time-course effect of hyperosmolarity on *myo*-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA containing 0–200 mM raffinose (concentration response; left panel) for 24 h, or 150 mM raffinose for 0–48 h (time course; right panel). Afterwards *myo*-inositol accumulation was determined by washing the cells with osmotically matched M199 serum-free medium containing 0.5% BSA and then incubating the cells in osmotically matched M199 serum-free medium containing *myo*-[2- ^3H]inositol for 30 min as described in the Experimental section. For SMIT mRNA determination, cells were incubated as described above and then RNA was isolated and SMIT mRNA levels were determined as described in the Experimental section. SMIT mRNA levels were standardized to β -actin mRNA or 18 S rRNA levels. Results are presented as nmol/mg of protein for *myo*-inositol accumulation or as percentages of control for the SMIT mRNA levels, with the level of SMIT mRNA in control cells assigned a value of 1. Each value is the mean $\pm$ S.E.M. for six separate determinations for *myo*-inositol accumulation, or three separate determinations for SMIT mRNA levels. * $P < 0.05$ compared with control.

0.5% BSA in the absence or presence of 10 ng/ml $\text{TNF-}\alpha$ or 150 mM raffinose. The *myo*-inositol content of 3T3-L1 adipocytes was decreased significantly from 183.6 ± 3.6 to 150.8 ± 6.2 nmol/mg of protein ($P < 0.05$) by $\text{TNF-}\alpha$. In contrast, the *myo*-inositol content was increased significantly (260.9 ± 14.8 nmol/mg of protein; $P < 0.05$) after exposure of 3T3-L1 adipocytes for 24 h to hyperosmotic conditions. If we assume that the intracellular volume of these cells is approx. 10 μl for 1 mg of cell protein, the intracellular concentration of *myo*-inositol in these cells would be approx. 18 mM and increased by approx. 40% after exposure of the cells to hyperosmotic conditions. Aldose reductase activity was apparently increased by hyperosmolarity in 3T3-L1 adipocytes because sorbitol levels were increased in 3T3-L1 adipocytes exposed to hyperosmotic medium (8.7 ± 2.2 nmol/mg of protein). Sorbitol was not detected in 3T3-L1 adipocytes incubated in isotonic medium.

A study of *myo*-inositol transport by 3T3-L1 adipocytes demonstrated that, like many other mammalian cells, 3T3-L1 adipocytes take up *myo*-inositol by high-affinity and low-affinity transport systems [35]. The high-affinity *myo*-inositol transport system in 3T3-L1 adipocytes has a K_m of $48.8 \pm 11.9 \mu\text{M}$ and a V_{max} of 171.1 ± 21.5 pmol/min per mg of protein, whereas the low-affinity system has a K_m of 2.47 ± 0.72 mM and a V_{max} of 969.2 ± 155.6 pmol/min per mg of protein. To determine whether $\text{TNF-}\alpha$ or hyperosmolarity affects the high-affinity *myo*-inositol transport system, 3T3-L1 adipocytes were incubated in serum-free medium containing 0.5% BSA for 16 h in the absence or presence of 10 ng/ml $\text{TNF-}\alpha$ or 150 mM raffinose. Neither $\text{TNF-}\alpha$ nor hyperosmolarity had a significant effect on the K_m for high-affinity *myo*-inositol transport in 3T3-L1 adipocytes (51.7 ± 5.7 , 65.6 ± 6.6 and $53.6 \pm 9.9 \mu\text{M}$ for control, $\text{TNF-}\alpha$ -treated and hyperosmolarity-treated cells respectively). In con-

Figure 4 Effect of TNF- α , C₆-ceramide, sphingosine, insulin or hyperosmolarity on *myo*-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA containing no additions (control; condition 1), 10 ng/ml TNF- α (condition 2), 100 μ M C₆-ceramide (condition 3), 100 μ M sphingosine (condition 4), 100 nM insulin (condition 5) or 150 mM raffinose (hyperosmolarity; condition 6) for 16 h. Afterwards *myo*-inositol accumulation was determined by washing the cells with osmotically matched M199 serum-free medium containing 0.5% BSA and then incubating the cells in osmotically matched medium containing *myo*-[2-³H]inositol for 30 min as described in the Experimental section. For SMIT mRNA determination, cells were incubated as described above, and then RNA was isolated and SMIT mRNA levels were determined as described in the Experimental section. SMIT mRNA levels were standardized to β -actin mRNA levels. Data are presented as nmol/mg of protein for *myo*-inositol accumulation or as percentages of control for the SMIT mRNA levels, with the level of SMIT mRNA in control cells assigned a value of 1. The inset shows a representative autoradiogram for SMIT and β -actin mRNA levels. Each value is the mean $\pm$ S.E.M. for five separate determinations. * P < 0.05, significant decrease compared with control. * P < 0.05, significant increase compared with control.

trast, exposure of 3T3-L1 adipocytes to TNF- α caused a significant decrease in the $V'_{\max}$ for high-affinity *myo*-inositol transport (188.5 ± 10.8 and 133.3 ± 7.8 pmol/min per mg of protein for control and TNF- α -treated cells respectively; P < 0.05), whereas exposure of cells to hyperosmotic conditions caused a significant increase in the $V'_{\max}$ for high-affinity *myo*-inositol transport (564.4 ± 49.4 pmol/min per mg of protein; P < 0.05 compared with control).

The effect of C₆-ceramide, sphingosine and insulin on SMIT mRNA levels and *myo*-inositol accumulation

Because ceramide and sphingosine are considered to be second messengers generated by mammalian cells after the binding of TNF- α to its receptor, we examined the effect of exogenous C₆-ceramide and sphingosine on SMIT mRNA levels and *myo*-inositol accumulation by 3T3-L1 adipocytes [36]. Neither C₆-ceramide nor sphingosine significantly altered SMIT mRNA levels or *myo*-inositol accumulation (Figure 4). Because insulin stimulates glucose transport in 3T3-L1 adipocytes, we also determined whether insulin alters *myo*-inositol uptake in these cells [7,8,18,23]. Results in Figure 4 show that insulin did not significantly change SMIT mRNA levels or *myo*-inositol accumulation in 3T3-L1 adipocytes [Figure 4 (inset) shows a representative autoradiogram of SMIT mRNA and β -actin mRNA levels]. In contrast, glucose uptake was significantly increased by more than 10-fold by insulin in these cells (results not shown).

Table 1 Effect of growth factors, cytokines, glucose and phorbol ester on *myo*-inositol accumulation by 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated for 16 h in serum-free medium containing 0.5% BSA and the different growth factors, cytokines, PMA or glucose at the concentrations shown. Afterwards the cells were washed and *myo*-inositol accumulation (nmol/mg of protein) was determined as described in the Experimental section. The results are means $\pm$ S.E.M. for six separate determinations. * P < 0.05 compared with control.

Condition	<i>myo</i> -Inositol accumulation (nmol/mg of protein)
Control	1.31 $\pm$ 0.22
TNF- α (10 ng/ml)	0.47 $\pm$ 0.07*
IL-2 (10 ng/ml)	1.10 $\pm$ 0.16
IL-6 (10 ng/ml)	1.09 $\pm$ 0.15
TGF- β (50 ng/ml)	0.91 $\pm$ 0.08
Insulin (100 nM)	1.28 $\pm$ 0.17
IGF-I (50 ng/ml)	1.36 $\pm$ 0.12
PDGF (50 ng/ml)	0.82 $\pm$ 0.08
IL-1 α (10 ng/ml)	0.96 $\pm$ 0.16
IL-1 β (10 ng/ml)	0.82 $\pm$ 0.12
PMA (100 nM)	0.94 $\pm$ 0.15
Glucose (50 mM)	1.66 $\pm$ 0.32

Effect of growth factors, cytokines, PMA and hyperglycaemia on *myo*-inositol accumulation by 3T3-L1 adipocytes

To determine the specificity of the effect of TNF- α and hyperosmolarity on *myo*-inositol accumulation we examined the effect of other growth factors, cytokines, PMA and hyperglycaemia on *myo*-inositol accumulation in 3T3-L1 adipocytes. As shown in Table 1, treatment of 3T3-L1 adipocytes for 16 h with insulin, IGF-I, platelet-derived growth factor or TGF- β did not affect *myo*-inositol accumulation. Similarly, exposure of 3T3-L1 adipocytes for 16 h to 50 mM glucose, the phorbol ester PMA or the cytokines IL-1 α , IL-1 β , IL-2 or IL-6 did not alter *myo*-inositol accumulation.

Effect of actinomycin D or cycloheximide on TNF- α - and hyperosmolarity-induced changes in SMIT mRNA levels and/or *myo*-inositol accumulation

To determine whether RNA or protein synthesis is required for the effect of TNF- α or hyperosmolarity on SMIT mRNA levels and/or *myo*-inositol accumulation by 3T3-L1 adipocytes, we preincubated the cells with either 5 μ M actinomycin D or 25 μ M cycloheximide before treatment with TNF- α or 150 mM raffinose. Actinomycin D blocked the TNF- α -induced decrease in SMIT mRNA levels (results not shown). Actinomycin D or cycloheximide pretreatment blocked the hyperosmolarity-induced increase in *myo*-inositol accumulation by 3T3-L1 adipocytes (results not shown). Furthermore actinomycin D pretreatment blocked the effect of hyperosmolarity on SMIT mRNA levels (results not shown).

Reversion of the effect of TNF- α and hyperosmolarity on SMIT mRNA levels and *myo*-inositol accumulation

To determine the reversibility of TNF- α - and hyperosmolarity-induced changes in SMIT expression and *myo*-inositol accumulation, 3T3-L1 adipocytes treated with TNF- α or hyperosmotic medium for 24 h were returned to normal isotonic medium. The TNF- α -induced decrease in SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes was reversible after removing

Figure 5 Reversion of the effect of TNF- α on myo-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA in the absence or presence of 10 ng/ml TNF- α for 24 h. Afterwards some of the cells that had been exposed to medium containing TNF- α were washed three times with serum-free medium and then incubated in serum-free medium in the absence of TNF- α for up to 24 h. After this incubation, myo-inositol accumulation was determined by washing the cells with M199 serum-free medium containing 0.5% BSA and then incubating the cells in this medium containing myo-[2- 3 H]inositol for 30 min as described in the Experimental section. For SMIT mRNA determination, cells were incubated as described above, and then RNA was isolated and SMIT mRNA levels were determined as described in the Experimental section. SMIT mRNA levels were standardized to β -actin mRNA levels. Results are presented as nmol/mg of protein for myo-inositol accumulation or as percentages of control for the SMIT mRNA levels, with the level of SMIT mRNA in control cells assigned a value of 1. Each value is the mean $\pm$ S.E.M. for six separate determinations for myo-inositol accumulation and SMIT mRNA levels. * P < 0.05 compared with control; + P < 0.05 compared with the TNF- α treated cells.

the cells from the medium containing TNF- α (Figure 5). Recovery of SMIT mRNA levels preceded the recovery of myo-inositol accumulation and was complete within 6 h. Recovery of myo-inositol accumulation was complete after about 24 h. The hyperosmolarity-induced increase in SMIT mRNA levels and myo-inositol accumulation was also reversible (Figure 6). Incubation of hyperosmolarity-induced 3T3-L1 adipocytes for 3 h in isotonic medium resulted in a significant decrease in SMIT mRNA levels and myo-inositol accumulation compared with cells maintained in hyperosmotic medium. Complete normalization of SMIT mRNA levels and myo-inositol accumulation occurred within 24 h.

Effect of TNF- α - and hyperosmolarity on MAPK activity in 3T3-L1 adipocytes

To determine whether TNF- α and hyperosmolarity might be having different effects on signalling pathways in 3T3-L1 adipocytes, thereby explaining the opposing effects of TNF- α and hyperosmolarity on SMIT mRNA levels and myo-inositol accumulation, we examined MAPK kinase activity in control 3T3-L1 adipocytes (Figure 7, lanes A) and after a 10 min incubation with 150 mM raffinose (Figure 7, lanes B), 100 nM insulin (Figure 7, lanes C) or 10 ng/ml TNF- α (Figure 7, lanes D). Treatment of 3T3-L1 adipocytes with insulin was included in these studies for comparative purposes. Insulin increased ERK1 (8.2-fold), ERK2 (3.7-fold) and p38 kinase (1.5-fold) activity in 3T3-L1 adipocytes. Hyperosmolarity increased ERK1 (1.3-fold), JNK1 (3.6-fold), JNK2 (2.1-fold) and p38 kinase (2.2-fold)

Figure 6 Reversion of the effect of hyperosmolarity on myo-inositol accumulation and SMIT mRNA levels in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA in the absence or presence of 150 mM raffinose (Hyper) for 24 h. Afterwards some of the cells that had been exposed to hyperosmotic medium were washed three times with serum-free medium and then incubated in isotonic serum-free medium for up to 24 h. After this incubation, myo-inositol accumulation was determined by washing the cells with osmotically matched M199 serum-free medium containing 0.5% BSA and then incubating the cells in osmotically matched M199 serum-free medium containing myo-[2- 3 H]inositol for 30 min as described in the Experimental section. For SMIT mRNA determination, cells were incubated as described above and then RNA was isolated and SMIT mRNA levels were determined as described in the Experimental section. SMIT mRNA levels were standardized to β -actin mRNA levels. Results are presented as nmol/mg of protein for myo-inositol accumulation or as percentages of control for the SMIT mRNA levels, with the level of SMIT mRNA in control cells assigned a value of 1. Each value is the mean $\pm$ S.E.M. for six separate determinations for myo-inositol accumulation, or four separate determinations for SMIT mRNA levels. * P < 0.05 compared with control; + P < 0.05 compared with hyperosmolarity-treated cells.

activity, whereas TNF- α marginally increased only JNK1 (1.4-fold) and JNK2 (1.4-fold) activity in 3T3-L1 adipocytes.

Effect of TNF- α and hyperosmolarity on NF- κ B activation in 3T3-L1 adipocytes

We have previously shown that the activation of NF- κ B by TNF- α in cultured endothelial cells is associated with the TNF- α -induced decrease in myo-inositol accumulation [1]. We therefore examined the effect of TNF- α on NF- κ B activation in 3T3-L1 adipocytes. By electrophoretic mobility-shift assay we determined that TNF- α causes a concentration- and time-dependent activation of NF- κ B in 3T3-L1 adipocytes with maximal activity obtained after a 15 min incubation (results not shown). In contrast with the effect of TNF- α , neither C₆-ceramide, sphingosine nor hyperosmolarity activated NF- κ B in 3T3-L1 adipocytes (results not shown).

Effect of kinase inhibitors, ceramidase inhibitor, protease inhibitor and inhibitors of NF- κ B on TNF- α - or hyperosmolarity-induced changes in myo-inositol accumulation in 3T3-L1 adipocytes

TNF- α activates a variety of signal transduction pathways, including protein kinase C, phospholipase A₂ and protein phosphorylation of serine/threonine and tyrosine residues, whereas TNF- α and hyperosmolarity have both been shown to activate the MAPK pathway in mammalian cells [36–40]. In an attempt to determine the signal transduction pathway(s) responsible for the effects of TNF- α and hyperosmolarity on myo-inositol accumulation, we examined the effect of various

Figure 7 Effect of hyperosmolarity, insulin or TNF- α on mitogen-activated protein kinase activity in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in serum-free medium with 0.5% BSA containing no additions (control, lanes A), 150 mM raffinose (hyperosmolarity, lanes B), 100 nM insulin (lanes C) or 10 ng/ml TNF- α (lanes D) for 10 min. Afterwards the cells were washed, lysates were prepared and assays for ERK1, ERK2, JNK1, JNK2 and p38 kinase were performed as described in the Experimental section.

Table 2 Effect of kinase inhibitors, ceramidase inhibitor and inhibitors of NF- κ B activation on TNF- α - or hyperosmolarity-induced changes in *myo*-inositol accumulation by 3T3-L1 adipocytes

3T3-L1 adipocytes were preincubated in serum-free medium containing 0.5% BSA and the different inhibitors indicated for 1 h. Afterwards the medium was changed and some of the cells were treated with 10 ng/ml TNF- α or 150 mM raffinose (hyperosmolarity) in the absence or presence of the different inhibitors. This incubation was continued for an additional 16 h and then *myo*-inositol accumulation (shown as the percentage of control) was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for nine (TNF- α studies) or six (hyperosmolarity studies) separate determinations. * P < 0.05 compared with untreated cells. Abbreviation: n.d., not determined.

Condition	<i>myo</i> -Inositol accumulation (% of control)			
	TNF- α		Hyperosmolarity	
	–	+	–	+
Control	–	57 $\pm$ 10*	–	310 $\pm$ 37*
Pyrrolidine dithiocarbamate (100 μ M)	109 $\pm$ 33	63 $\pm$ 12*	n.d.	n.d.
TLCK (100 μ M)	101 $\pm$ 26	52 $\pm$ 16*	n.d.	n.d.
Calphostin C (1 μ M)	88 $\pm$ 9	60 $\pm$ 7*	55 $\pm$ 7*	202 $\pm$ 29*
<i>N</i> -Oleylethanolamine (0.5 mM)	81 $\pm$ 28	52 $\pm$ 3*	n.d.	n.d.
Genistein (100 μ M)	90 $\pm$ 12	59 $\pm$ 11*	127 $\pm$ 19	365 $\pm$ 57*
Wortmannin (100 nM)	96 $\pm$ 13	54 $\pm$ 9*	133 $\pm$ 22	392 $\pm$ 31*
Rapamycin (10 μ M)	93 $\pm$ 9	51 $\pm$ 2*	118 $\pm$ 14	335 $\pm$ 51*
PD-98059 (10 μ M)	105 $\pm$ 10	66 $\pm$ 6*	144 $\pm$ 13	455 $\pm$ 63*
SB-203580 (100 μ M)	90 $\pm$ 6	58 $\pm$ 7*	86 $\pm$ 13	278 $\pm$ 36*

inhibitors of phosphorylation, protease degradation, NF- κ B activation and ceramidase activity on TNF- α - and/or hyperosmolarity-induced changes in *myo*-inositol accumulation (Table 2). Inhibitors of NF- κ B activation, pyrrolidine dithiocarbamate, TLCK (a protease inhibitor used to inhibit I- κ B- α degradation) and genistein [a protein tyrosine kinase inhibitor used to block the phosphorylation of the inhibitor of NF- κ B (I- κ B- α)], did

not prevent the TNF- α -induced decrease in *myo*-inositol accumulation. In separate studies, we showed that each of these compounds prevented, to a different degree (50–100 %), the TNF- α -induced activation of NF- κ B in 3T3-L1 adipocytes (results not shown). Genistein did not prevent the hyperosmolarity-induced increase in *myo*-inositol accumulation. Inhibitors of ERK1 (PD-98059), p38 kinase (SB-203580), p70 S6 kinase (rapamycin) or phosphatidylinositol 3-kinase (wortmannin) did not affect either the TNF- α - or hyperosmolarity-induced changes in *myo*-inositol accumulation. Inhibitors of protein kinase C, including calphostin C, staurosporin and H7, were also found to have no effect on the TNF- α - or hyperosmolarity-induced changes in *myo*-inositol accumulation (results not shown). Lastly, *N*-oleoylethanolamine, a ceramidase inhibitor, had no effect on the TNF- α -induced decrease in *myo*-inositol accumulation.

DISCUSSION

myo-Inositol is acquired by mammalian cells through an active, Na⁺-dependent co-transporter, and the internal concentration of *myo*-inositol is generally 5–500-fold higher than the level of *myo*-inositol in plasma or extracellular fluid. *myo*-Inositol is a component of cellular membranes and, in the form of phosphoinositides, is an integral part of several signal transduction pathways, including phosphatidylinositol 3-kinase and the phosphatidylinositol cycle. In addition, *myo*-inositol is an organic osmolyte, and the regulation of its transport is important for maintaining an osmotic balance during periods of osmotic stress [27–31]. In the present study we have shown that 3T3-L1 adipocytes, like other mammalian cells, acquire *myo*-inositol by way of the SMIT, and that SMIT activity is mediated by hyperosmolarity and TNF- α [15,16,18,19]. Because TNF- α and hyperosmolarity are well-known cellular stress factors and activate similar signal transduction pathways, we postulated that they would have similar effects on SMIT mRNA levels and activity [13,14]. However, in cultured endothelial cells and 3T3-L1 adipocytes, TNF- α and hyperosmolarity have opposite effects on SMIT mRNA levels and *myo*-inositol accumulation [1].

Hyperosmolarity has been shown to increase the uptake of *myo*-inositol by a variety of mammalian cells, including renal, neural and endothelial cells [27–31]. Studies by Yamauchi et al. [28] have shown that this is due to an increase in transcription of the SMIT gene. Previously, investigation of the regulation in the metabolism of two other organic osmolytes, sorbitol and betaine, led to the identification of osmotic response elements on the aldose reductase gene and betaine transporter gene, suggesting that increased transcription of these genes in response to hyperosmolarity was responsible for increasing sorbitol production and betaine uptake respectively [41,42]. Recently the SMIT gene has also been characterized and an osmotic response element identified [43]. Because actinomycin D blocked the induction by hyperosmolarity of SMIT mRNA levels and activity, and cycloheximide blocked the hyperosmolarity-induced increase in *myo*-inositol accumulation without affecting the increase in SMIT mRNA levels, it is likely that the effect of hyperosmolarity on *myo*-inositol uptake by 3T3-L1 adipocytes is due to the regulation of SMIT gene transcription. 3T3-L1 adipocytes were very sensitive to changes in osmolarity, as demonstrated by the increase in SMIT mRNA levels and *myo*-inositol accumulation in response to a small change in osmolarity (50 mM raffinose), and the rapid increase in SMIT mRNA levels 3 h after exposure to 150 mM raffinose that preceded an increase in *myo*-inositol accumulation. Because the hyperosmolarity-induced increase in

myo-inositol levels in 3T3-L1 adipocytes greatly exceeded the increase in sorbitol levels, it seems likely that *myo*-inositol is the major osmolyte in adipocytes for regulating changes in osmotic stress.

TNF- α has been shown to decrease GLUT4 mRNA levels in 3T3-L1 adipocytes; in adipocytes this is thought to contribute to insulin resistance [7,8,16,18,23]. The TNF- α -induced decrease in GLUT4 mRNA levels and insulin-stimulated glucose transport in 3T3-L1 adipocytes were caused by a decrease in GLUT4 gene transcription and mRNA stability [7,8,18,23]. We observed a similar effect of TNF- α on SMIT mRNA levels and *myo*-inositol accumulation. Studies with actinomycin D to block mRNA synthesis suggest that the effect of TNF- α on SMIT mRNA levels requires mRNA synthesis, although an additional effect of TNF- α on SMIT mRNA stability cannot be excluded. In bovine aortic endothelial cells, TNF- α is known to modulate the expression of a protein that regulates the stability of endothelial nitric oxide synthase mRNA levels [44]. Studies are currently being conducted to determine whether TNF- α might be regulating SMIT mRNA stability by a similar mechanism.

We have shown previously that TNF- α decreases SMIT mRNA levels and *myo*-inositol accumulation in cultured endothelial cells [1]. As in endothelial cells, the effect of TNF- α on 3T3-L1 adipocyte SMIT mRNA levels and *myo*-inositol accumulation was reversible and caused a decrease in the $V'_{\max}$ without affecting the K'_m for high-affinity *myo*-inositol transport. The latter observation suggests that the effect of TNF- α on *myo*-inositol metabolism in 3T3-L1 adipocytes and endothelial cells might be due to a decrease in the amount of *myo*-inositol transporter present on the plasma membrane. Our SMIT mRNA results are consistent with this, but confirmation of this finding will require Western blot analysis of the SMIT protein with an antibody that is presently not available.

Our studies showed that several different growth factors (IL-1 α , IL-1 β , IL-2, IL-6), PMA and hyperglycaemia did not affect *myo*-inositol accumulation by 3T3-L1 adipocytes. In cultured aorta and pulmonary artery endothelial cells, IL-1 β and TGF- β were found to mimic the effects of TNF- α on *myo*-inositol accumulation [1]. However, in 3T3-L1 adipocytes, decreased *myo*-inositol accumulation was observed only after treatment with TNF- α , indicating a highly specific and sensitive mechanism of regulation of *myo*-inositol metabolism in 3T3-L1 adipocytes by TNF- α . In studies of cultured renal cells, PMA has been shown to decrease *myo*-inositol uptake [45]. However, such a mechanism does not seem to be present in 3T3-L1 adipocytes.

Two second messengers, sphingosine and ceramide, are formed after the binding of TNF- α to its receptor [17]. In 3T3-L1 adipocytes, the exogenous addition of cell-permeable ceramides duplicates the effects of TNF- α on insulin-stimulated glucose uptake [7,18]. In cultured endothelial cells, TNF- α and the exogenous addition of C₆-ceramide were shown to decrease SMIT mRNA levels and *myo*-inositol accumulation [1]. However, in 3T3-L1 adipocytes, treatment with exogenous C₆-ceramide or sphingosine did not duplicate the effects of TNF- α on SMIT mRNA levels or *myo*-inositol accumulation. Therefore there seems to be a primary difference in the mechanism by which TNF- α exerts its effects on (1) SMIT mRNA levels and *myo*-inositol accumulation in endothelial cells and 3T3-L1 adipocytes, and (2) GLUT4 mRNA levels, insulin-stimulated glucose transport, SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes. The reasons for these differences are currently not clear. In cultured endothelial cells, the effect of TNF- α on SMIT mRNA levels and *myo*-inositol accumulation was associated with the activation of NF- κ B [1]. Treatment of cultured endothelial cells with pyrrolidine dithiocarbamate, TLCK,

genistein or calphostin C, compounds that can block the activation of NF- κ B, inhibited the effect of TNF- α on *myo*-inositol accumulation [1]. TNF- α also activates NF- κ B in 3T3-L1 adipocytes; however, treatment of 3T3-L1 adipocytes with the same compounds, which to differing degrees inhibited the TNF- α -induced activation of NF- κ B in 3T3-L1 adipocytes, did not alter the effect of TNF- α on *myo*-inositol accumulation. Therefore, in addition to the different effect from C₆-ceramide on SMIT mRNA levels and *myo*-inositol accumulation in cultured endothelial cells and 3T3-L1 adipocytes, the activation of NF- κ B by TNF- α in 3T3-L1 adipocytes does not seem to be directly associated with the effect of TNF- α on *myo*-inositol accumulation, as it was in cultured endothelial cells [1].

In mammalian cells, hyperosmolarity and TNF- α induce three MAPK pathways: ERK, JNK and p38 kinase [25,26,46]. In 3T3-L1 adipocytes, hyperosmolarity induced all three of these kinase pathways to differing degrees, whereas TNF- α had a small effect on JNK activity and no effect on ERK or p38 kinase activity. Inhibitors of ERK or p38 kinase did not block the effect of hyperosmolarity or TNF- α on *myo*-inositol accumulation. This suggests that in 3T3-L1 adipocytes the effects of hyperosmolarity or TNF- α on SMIT activity are not mediated by ERK or p38 kinase. MAPK activation by osmotic stress is a consistent finding in many mammalian cells, but it is not essential for betaine or *myo*-inositol transporter gene transcription [47]. In Madin-Darby canine kidney cells the p38 kinase inhibitor SB-203580 prevented the osmotic induction of heat-shock protein mRNA [48]. However, SB-203580 did not prevent the hyperosmolarity-induced increase in *myo*-inositol accumulation in 3T3-L1 adipocytes. This suggests that a cellular difference in the signal transduction pathways mediating the effects of hyperosmolarity, and perhaps TNF- α , might exist.

In summary, our studies have shown that hyperosmolarity and TNF- α have opposing effects on SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes. The effect of TNF- α on SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes and endothelial cells is similar; however, unlike in endothelial cells, activation of the transcription factor NF- κ B by TNF- α in 3T3-L1 adipocytes does not seem to be directly associated with its effects on SMIT activity [1]. The signalling pathways responsible for the effects of hyperosmolarity and TNF- α on SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes remain to be elucidated. TNF- α -induced hydrolysis of sphingomyelin does not seem to be necessary for the regulation of SMIT mRNA levels and *myo*-inositol accumulation in 3T3-L1 adipocytes. In 3T3-L1 adipocytes, but not in endothelial cells, C₆-ceramide did not mimic the effects of TNF- α on SMIT mRNA levels or activity or activate NF- κ B. In Jurkat T cells, C₃-ceramide mimicked the effects of TNF- α on cell growth but was unable to induce the activation of NF- κ B [49]. Thus TNF- α signalling involves multiple second-messenger pathways that function independently or co-ordinately to transduce distinct functions of TNF- α . In 3T3-L1 adipocytes, other signalling pathways activated by TNF- α include the activation of Janus tyrosine kinases (Jak) and the subsequent phosphorylation of members of the STAT family of transcription factors [50]. Further studies will be necessary to determine whether these or other pathways are responsible for mediating the effects of TNF- α on SMIT activity in 3T3-L1 adipocytes.

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