

Lipid mediators of insulin resistance: ceramide signalling down-regulates GLUT4 gene transcription in 3T3-L1 adipocytes

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We have previously demonstrated that chronic exposure of 3T3-L1 adipocytes to tumour necrosis factor- α (TNF) resulted in a marked decrease ($\sim 90\%$) in cellular GLUT4 (insulin-responsive glucose transporter) mRNA content as a result of a decreased transcription rate of the GLUT4 gene ($\sim 75\%$) and a reduced half-life of its mRNA (9 to 4.5 h). Investigation of the signalling mechanism responsible for this regulation demonstrated that in the 3T3-L1 adipocytes, sphingomyelin levels decreased to 50% of control levels within 40 min of exposure to TNF, consistent with activation of a sphingomyelinase. In the same manner as with TNF, treatment of the adipocytes with 1–3 μM C₆-ceramide, a membrane-permeable analogue of ceramide, decreased GLUT4 mRNA content by $\sim 60\%$. Subsequent investigations revealed

that transcription of the GLUT4 gene was reduced by $\sim 65\%$ in response to C₆-ceramide, demonstrating that the decrease in mRNA content is mediated by a reduction in the transcription of the gene. No effect on GLUT4 mRNA stability was observed after exposure of the adipocytes to C₆-ceramide. These observations are interesting in light of our previous data demonstrating that TNF affects both GLUT4 transcription and mRNA stability in the 3T3-L1 adipocytes. In conclusion, the effect of ceramide on GLUT4 gene expression is at the level of transcription, suggesting that another pathway controls mRNA stability. These data establish that ceramide-initiated signal transduction pathways exist within the adipocyte, and provide a potential mechanism for control of GLUT4 gene expression.

INTRODUCTION

The adipocyte is no longer viewed as a passive participant in the processes of energy storage and release, but rather as the centre of a key regulatory system for maintenance of energy homeostasis and body composition [1]. Previously, the adipocyte has been suggested to function as an endocrine cell capable of producing and secreting molecules with regulatory potential, including the product of the *ob* gene and the cytokine, tumour necrosis factor- α (TNF) [2–5]. The increased expression of TNF in the adipocytes of obese animals and humans is consistent with a role for TNF as a mediator of insulin resistance in the obese state [2,6].

Studies have suggested a role for a number of cytokines, especially TNF, as potential mediators of glucose homeostasis in various tissues [7]. Evidence from both whole-animal and cell-culture studies has demonstrated that TNF inhibits insulin-stimulated glucose uptake in adipose tissue and skeletal muscle [8–11]. The molecular mechanisms by which TNF induces insulin resistance in cell culture or in whole animals is not clear. Considerable attention is now being focused on the intrinsic catalytic activity of the insulin receptor and downstream signalling events. In murine 3T3-L1 or 3T3-F442A adipocytes, TNF treatment has been reported to result in a decrease in both the insulin-stimulated autophosphorylation of the insulin receptor and phosphorylation of insulin receptor substrate 1 [3,12]. This decrease in the intrinsic tyrosine kinase activity of the receptor was suggested to be an important component of the TNF-induced insulin resistance in obese models; however, it is not the only mechanism whereby TNF may mediate insulin resistance. A second model involves the TNF-induced suppression of the gene encoding the insulin-responsive glucose transporter, GLUT4. Previous work has shown that TNF-

treatment results in the down-regulation of GLUT4 mRNA and protein in cultured adipocytes and muscle cells [2,9,10,13]. Furthermore, GLUT4 protein is also known to be deficient in adipose tissues of certain obese animal models, as well as humans with non-insulin-dependent diabetes mellitus [14,15], suggesting that TNF-mediated insulin resistance may involve the regulation of GLUT4 gene expression. It remains to be determined whether the effects of TNF are due primarily to the modulation of GLUT4 gene expression or to broader effects on the insulin signalling pathway. Regardless of which model may explain the TNF-induced insulin resistance, it is important to define the signal transduction pathways through which the cytokine exerts its effects.

The sphingomyelin (SM) cycle has been demonstrated to play a major role in signal transduction pathways initiated by TNF [16–18]. Initiation of the cycle has been suggested to occur through activation of a C-type phospholipase associated with the plasma membrane known as the neutral sphingomyelinase (SMase) [19]. In response to ligand occupation of its receptor, the SMase is activated and catalyses the hydrolysis of membrane SM, generating phosphocholine and ceramide. Ceramide then serves as a second messenger, activating specific phosphatases and kinases. The ceramide-activated protein phosphatase (CAPP), a group-2A protein phosphatase that is inhibited by okadaic acid, is a potential target [20,21]. This enzyme translocates to the plasma membrane on activation by ceramide and is involved in the induction of *c-myc*. A second target has been shown to be the ceramide-activated, protein-directed, membrane-bound serine/threonine protein kinase. This kinase may also involve communication through Raf, MEK [mitogen-activated protein (MAP) kinase kinase] and MAP kinases prior to activation of gene transcription [17,22,23]. Through these pathways, ceramide has been shown to play a role in activation of the

Abbreviations used: TNF, tumour necrosis factor- α ; GLUT4, insulin-responsive glucose transporter; GLUT1, basal, growth-related glucose transporter; SM, sphingomyelin; SMase, sphingomyelinase; AA, arachidonic acid; ETYA, eicosatetraynoic acid, the acetylenic analogue of arachidonic acid; cPLA₂, cytosolic phospholipase A₂; PKC ζ , the ζ -isoform of protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; MAP, mitogen-activated protein.

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transcription factor NF- κ B [17,24,25], and prostaglandin release, through the induction of both phospholipase A₂ (PLA₂) and cyclo-oxygenase [25].

Studies by Hannun and colleagues have suggested that arachidonic acid (AA) mediates the TNF-stimulated SM hydrolysis through prior activation of cytoplasmic PLA₂ (cPLA₂) [26]. Examination of the kinetics of SMase activation has provided evidence that the TNF-induced activation of cPLA₂, and release of AA from the *sn*-2 position of phosphatidylcholine, precede TNF-stimulated SM hydrolysis. Thus, after TNF binds to its receptor, the proposed pathway includes an initial activation of cPLA₂ generating free AA, which in turn could activate the SMase. Jayadev et al. [26] further demonstrated that other fatty acids (18:1, 18:2, 18:3, 16:0 and 14:0) and the non-metabolizable analogue of AA, eicosatetraenoic acid (ETYA) can induce SM turnover with potencies similar to AA, suggesting that free AA and not a metabolite was responsible for SMase activation.

Our laboratory has been studying the mechanism by which TNF can alter expression of the insulin-responsive glucose transporter (GLUT4). Previously we have demonstrated that exposure of fully differentiated 3T3-L1 adipocytes to either TNF or AA results in attenuation of GLUT4 gene transcription (50% decrease) and decreased stability of the GLUT4 mRNA (*t*_{1/2} decreased from 9 h to 4.5 h) [13,27]. These observations suggested that both AA and TNF may alter GLUT4 gene expression by similar mechanisms. We recently demonstrated that AA may act via two independent mechanisms to suppress the GLUT4 gene in 3T3-L1 adipocytes [28]; one of these involves the oxidation of AA to prostaglandin E₂, which functioned in an autocrine fashion to increase cAMP levels. We [13], as well as Kaestner et al. [29], have demonstrated that cAMP analogues are capable of down-regulation of GLUT4 gene transcription. The second pathway, non-oxidative in nature, can be activated by ETYA, a non-metabolizable analogue of AA, as well as other fatty acids that cannot be converted into E-series prostaglandins. These observations were similar to those of Jayadev et al. [26] described above and led us to suggest that fatty acids may function to suppress GLUT4 gene expression through activation of the neutral SMase. However, in this study we demonstrate that TNF activates SMase independently of AA and, while ceramide is demonstrated to be a principal mediator of TNF-induced down-regulation of GLUT4 gene expression, it does not appear to be generated by the neutral SMase described by Jayadev et al. [26].

EXPERIMENTAL

Materials

Recombinant human TNF was generously provided by Biogen (Cambridge, MA, U.S.A.). The specific activity was 9.6×10^6 units/mg of protein, based on a cytotoxicity assay using L929 cells. The endotoxin contamination was 0.12 ng/mg of protein, based on a *Limulus* amoebocyte lysate assay (Sigma). C₆-ceramide was obtained from Biomol. Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies, Inc. Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, U.S.A.) and used at a 1:10 dilution in DMEM. Based on specifications provided by HyClone, the mean AA content of culture medium containing 10% (v/v) FBS would be $\sim 3 \mu\text{M}$. Radiolabelled compounds were obtained from DuPont NEN. Hybond-N blotting membrane was purchased from Amersham Co. Deoxyribonucleotides were obtained from Pharmacia LKB Biotechnology, Inc. Klenow fragment and TRIzol Reagent were obtained from Gibco BRL Life Technologies. All fatty acids were purchased from Cayman Chemical, Ann Arbor, MI, U.S.A.

The polyclonal antisera against protein kinase C ζ (PKC ζ), as well as the immunizing peptide, were purchased from Boehringer Mannheim GmbH, Germany. All other chemicals, unless otherwise stated, were of molecular biology grade and purchased from Sigma.

3T3-L1 cell culture

The murine 3T3-L1 cells used in this study were originally obtained from Dr. Howard Greene, Harvard University, Boston, MA, U.S.A. Cells were cultured, maintained, and differentiated as previously described [27]. The cells were maintained for 10 days post-differentiation and then treated with the indicated agents for various times prior to RNA isolation. Concentrations of TNF used in the study were based on previous studies [9,10,13]. The concentrations of C₆-ceramide added were determined by preliminary experiments over a concentration range from 0.1 μM to 10 μM (based on an estimated intracellular concentration of 0.1 μM to 0.5 μM after TNF treatment). Concentrations at the upper end of this range were lethal to the cells.

SM assay

Cells were labelled with [³H]choline chloride (specific radioactivity of 1 mCi/ml) at 0.5 $\mu\text{Ci/ml}$ for 6 days after initiation of differentiation and then the cells were grown in normal media for the remaining 3 days. On day 9 the cells were treated with 5 nM TNF and the lipids were extracted and labelled. SM was quantified by the bacterial SMase method as described by Jayadev et al. [26].

RNA isolation and Northern blot analyses

Total RNA was isolated by extraction with guanidine isothiocyanate and centrifugation through 5.7 M caesium chloride [30] or by the TRIzol method (Gibco BRL Life Technologies). Northern blot analyses were performed as described previously [9].

Nuclei isolation and transcription run-on assays

Nuclei isolated from appropriately treated 3T3-L1 adipocytes were used in run-on transcription assays as previously described [31].

DNA probes

The following DNA probes were used: GLUT1, a 2.7 kb *Eco*R1 fragment encoding the murine 3T3-L1 homologue of the HepG2/brain glucose transporter [32]; GLUT4, a 2.8 kb *Eco*R1 fragment encoding the 3T3-L1 homologue of the adipose/muscle (insulin-responsive) glucose transporter [32]; and β -actin, a 1.9 kb *Hind*III fragment obtained from Dr. D. W. Cleveland [33]. Genomic 3T3-L1 DNA was isolated as described by Gross-Bellard et al. [34] and digested with *Eco*RI prior to use in run-on assays.

Preparation of nuclear extract

After the indicated treatments for various times, the cells were pelleted in PBS for 10 min at 1000 g. The pellet was then resuspended in 3 ml of M3 lysis buffer (250 mM sucrose, 25 mM Tris, pH 7.8, 1.1 mM MgCl₂, 0.2% Triton X-100, 0.5 mM PMSF, 10 mM EDTA, 10 mg/ml leupeptin and 1 mg/ml aprotinin) and centrifuged at 12000g for 15 min. The nuclei were extracted using 250 μl of cold M4 extraction buffer [20 mM Hepes, pH 7.8, 0.4 M KCl, 2 mM dithiothreitol (DTT), 20% (v/v) glycerol, 0.5 mM PMSF, 10 mM EDTA, 10 mg/ml leu-

peptin and 1 mg/ml aprotinin) for 30 min and then the nuclear debris and DNA were pelleted at 17500 *g* for 15 min. Nuclear extracts were assayed for protein concentrations by the method of Bradford [35].

Autophosphorylation of PKC ζ

Day 10 adipocytes (60-mm-diam. dishes, 1×10^7 cells) were incubated in phosphate-free medium for 2 h. Then 50 $\mu\text{Ci/ml}$ [^{32}P]P $_i$ was added for an additional 4 h after which time the cells were exposed to TNF (5 nM) or C $_6$ -ceramide (3 μM) for the appropriate times. The cells were washed once in ice-cold PBS and lysed in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM PMSF, 100 $\mu\text{g/ml}$ leupeptin, 0.5% Triton X-100, 5 mM EDTA, 20 mM *p*-nitrophenyl phosphate, 1 mM NaF and 1 mM sodium orthovanadate. Immunoprecipitation was achieved using a polyclonal antiserum against PKC ζ (Boehringer Mannheim GmbH, Germany) as per Boehringer's instructions. PKC ζ was separated by SDS/PAGE and autophosphorylation analysed on autoradiograms of Kodak X-OMAT films. Specificity of the immunoprecipitation was demonstrated by competition studies using the antigen peptide provided by Boehringer Mannheim, which consists of amino acids 577–592.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using the classic NF- κB response element corresponding to the NF- κB site in the HIV-LTR [36] (5'-AATTTCCCTGGAAAGTCCCCAGCGAAAGTCCCT-TGT-3'). Briefly, ~ 100 ng (60000 c.p.m.) of a [^{32}P]-labelled double-stranded DNA oligomer was incubated with 5 μg of nuclear extract for 15 min at room temperature in a total volume of 20 μl of reaction buffer [25 mM Tris, pH 7.8, 0.5 mM EDTA, 88 mM KCl, 1 mM DTT, 150 μg of poly(dI.dC)-poly(dI.dC), 0.05% Triton X-100 and 12.5 $\mu\text{g/ml}$ salmon sperm DNA]. Reaction mixtures were subjected to electrophoresis on 5% non-denaturing polyacrylamide gels at 4 $^\circ\text{C}$, at 40 A (200 V), for ~ 2 h. The gels were dried and exposed to X-ray film for 12 h at -80 $^\circ\text{C}$.

RESULTS

TNF induces SM turnover in 3T3-L1 adipocytes

TNF has been demonstrated to induce SM hydrolysis in HL-60 cells [37] and NIH-3T3 fibroblasts [38] in a time- and dose-dependent manner with the generation of phosphocholine and ceramide. The effects of TNF on activation of SMase in 3T3-L1 adipocytes were determined by measuring SM hydrolysis. Treatment of the adipocytes with 5 nM TNF resulted in maximal SM turnover of approx. 55% of basal, which was observed as early as 40 min after TNF addition (Figure 1A). Coincident with these results, previous studies from our laboratory have demonstrated that free ceramide levels rise to $\sim 74\%$ above basal within 45 min of TNF treatment (P. H. Pekala and Y. A. Hannun, unpublished work), consistent with TNF activation of a SMase.

The effects of exogenous AA on SM turnover in the adipocytes were also determined since AA is a potential mediator of TNF and has also been shown to activate SMase in HL-60 cells [26]. However, in contrast to TNF, AA does not significantly induce SM hydrolysis, as seen in Figure 1(A). AA treatment resulted in a slight decrease in SM levels (approx. 10–15%) after 2 h, suggesting that AA does not activate SMase in the 3T3-L1 adipocytes. In addition, two other fatty acids, oleic acid (18:1) and ETYA, were tested for their ability to activate SMase (Figure 1B). The results demonstrated that, like AA, neither oleic acid nor ETYA induced SM turnover in the cells.

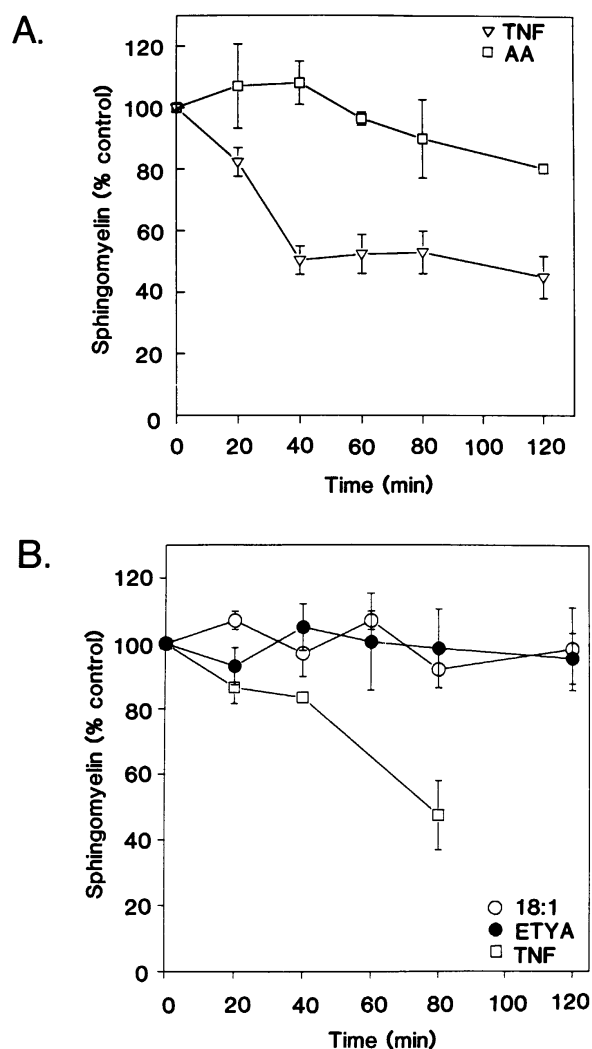


Figure 1 Time course of TNF-stimulated SM hydrolysis

(A) [^3H]Choline-labelled adipocytes were treated at time 0 with 5 nM TNF or 100 μM AA. At the indicated times cells were harvested, lipids were extracted, and SM was quantified as described in the Experimental section. The data are plotted as the mean percentages of SM remaining \pm S.D. and are representative of results from three separate experiments. (B) As described in (A) with the exception that the cells were treated at time 0 with 5 nM TNF or 100 μM oleic acid (18:1) or 100 μM ETYA. As indicated above the data are plotted as the mean percentages of SM remaining \pm S.D. and are representative of results from three separate experiments.

C $_6$ -ceramide suppresses GLUT4 mRNA levels

TNF activation of SMase and generation of ceramide has been suggested as an effector mechanism mediating biological activities of TNF [16]. Ceramide has been implicated as a mediator of the effects of TNF such as programmed cell death [39] and monocyte differentiation in HL-60 cells [22]. To establish a role for ceramide in the TNF-induced GLUT4 mRNA suppression, fully differentiated 3T3-L1 adipocytes were treated with the membrane-permeable C $_6$ -ceramide for 12 h at 1 and 3 μM as shown in Figure 2. The levels of GLUT4 mRNA were determined via Northern blot analysis using cDNA probes corresponding to either GLUT4 or β -actin (results not shown), and quantification of the hybridization pattern of each mRNA species was determined by phosphorimager analysis. As a result of C $_6$ -ceramide

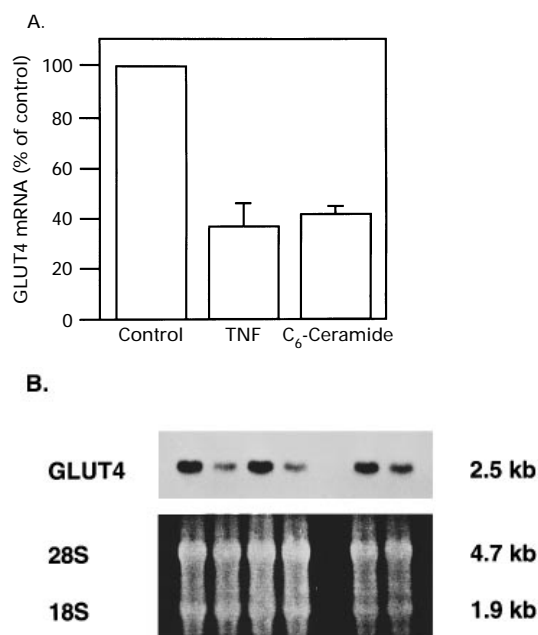


Figure 2 Effect of C₆-ceramide on GLUT4 mRNA expression

Fully differentiated 3T3-L1 cells were treated with 5 nM TNF and 1–3 μ M C₆-ceramide for 12 h. Total cellular RNA was isolated and 20 μ g/lane was subjected to electrophoresis and Northern blot analysis. **(A)** Graphical representation of three independent experiments. In each case the levels of GLUT4 mRNA were normalized to β -actin or the 18 S ribosomal RNA band. Data are plotted as the mean percentages of mRNA remaining \pm S.D. ($n = 3$). **(B)** Representative Northern blot analysis. Lanes 1 and 3 are controls (no treatment), lanes 2 and 4 are TNF-treated, and lanes 5 and 6 are 1 and 3 μ M C₆-ceramide-treated respectively. Lanes are numbered left to right.

treatment, GLUT4 mRNA content decreased by 60%, an effect similar in magnitude to that seen with TNF treatment (~75% decrease in GLUT4 mRNA) (Figure 2), suggesting ceramide may be a possible mediator of the effects of TNF on GLUT4 gene expression. Finally we note that addition of phosphocholine

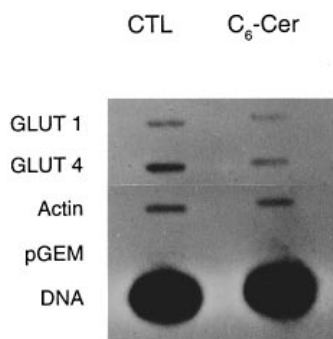


Figure 3 Regulation of GLUT4 gene transcription by ceramide

Nuclei were isolated from 3T3-L1 adipocytes, both control (CTL) and 3 μ M C₆-ceramide treated (12 h) cells, and subjected to run-on transcription analysis as we have previously described [31]. The results demonstrate the hybridization of *in vitro* transcribed RNA to the indicated cDNAs (2 μ g each) or 3T3-L1 genomic DNA (0.1 μ g), which were immobilized on nylon membranes. Vector DNA (pGEM plasmid, Promega; 2 μ g/lane) was present as a control. Following autoradiography, results were quantified using Imagequant software, Molecular Dynamics (Sunnyville, CA, U.S.A.). The results shown are representative of an experiment performed three times with similar results.

to the cultures to a final concentration of 50 μ M had no effect on GLUT4 mRNA accumulation (results not shown).

C₆-ceramide decreases GLUT4 gene transcription

Since C₆-ceramide can mimic TNF's effects on GLUT4 mRNA, the question became at what level mechanistically did C₆-ceramide suppress GLUT4 message levels, as TNF decreases both GLUT4 gene transcription and mRNA stability [13]. To determine whether the ceramide-induced suppression of GLUT4 mRNA content arose at the level of mRNA synthesis, transcription of the GLUT4 gene was measured in nuclei obtained from both control and 12 h C₆-ceramide-treated adipocytes (Figure 3). Nascent ³²P-labelled GLUT4 and GLUT1 run-on transcripts were quantified using Imagequant software. Exposure of the 3T3-L1 adipocytes to C₆-ceramide markedly suppressed run-on transcription of the GLUT4 and GLUT1 genes, which decreased to ~35% and 30% respectively of their initial rates. The effect of ceramide on the transcription of the two transporter genes is identical to that which we have previously reported for TNF [9,13]. Subsequent investigations revealed that, in contrast to TNF, C₆-ceramide had no effect on the stability of GLUT4 mRNA (results not shown). These results are consistent with a reduction in GLUT4 gene transcription as being responsible for the observed effects of C₆-ceramide on GLUT4 mRNA content shown in Figure 2.

C₆-ceramide stimulates the autophosphorylation of PKC ζ and activation of NF- κ B in the 3T3-L1 adipocytes

We next investigated which signalling intermediates were activated by ceramide in the adipocytes. Recently, ceramide has been shown to bind to and regulate the kinase activity of PKC ζ in response to TNF treatment in U937 cells [40]. Activation of the PKC is accompanied by intramolecular autophosphorylation, which reflects the activity state of the kinase [41]. Based on these observations the phosphorylation state of PKC ζ was determined in the adipocytes treated with either C₆-ceramide or TNF. Exposure of ³²P-labelled cells to 3 μ M C₆-ceramide resulted in a rapid and transient increase in the phosphorylation of PKC ζ (Figure 4A). When the cells were exposed to 5 nM TNF, similar results were observed; however, as shown in Figure 4(B), both the magnitude of autophosphorylation as well as the time course were altered. These results are consistent with the hypothesis that PKC ζ participates in TNF-induced signalling pathways after activation by ceramide.

Previous data from our laboratory have demonstrated that 1 h of TNF treatment results in activation of NF- κ B in the adipocytes [42]. We therefore evaluated the ability of C₆-ceramide to serve as a second messenger for TNF in the activation of NF- κ B. For these studies, 3T3-L1 adipocytes were treated with TNF for 1 and 2 h, AA for 1.5 h, or C₆-ceramide for 1.5 h, and then nuclear proteins were extracted. NF- κ B activation was determined by DNA gel shift analysis as shown in Figure 5. The data clearly demonstrate that C₆-ceramide, as well as AA, is capable of activating NF- κ B in the adipocytes (Figure 5), although the response was not as robust as that seen after TNF treatment. The gel shift assay detected a specific DNA–protein complex formed in response to TNF, AA or C₆-ceramide that was competed by a 100-fold excess of unlabelled DNA probe but not by a similar non-specific probe (results not shown) and thus demonstrated a specific activation of NF- κ B.

These data represent the first demonstration of ceramide activation of both PKC ζ and NF- κ B in terminally differentiated 3T3-L1 adipocytes. While we have not established a link between their activation and GLUT4 gene expression they provide the

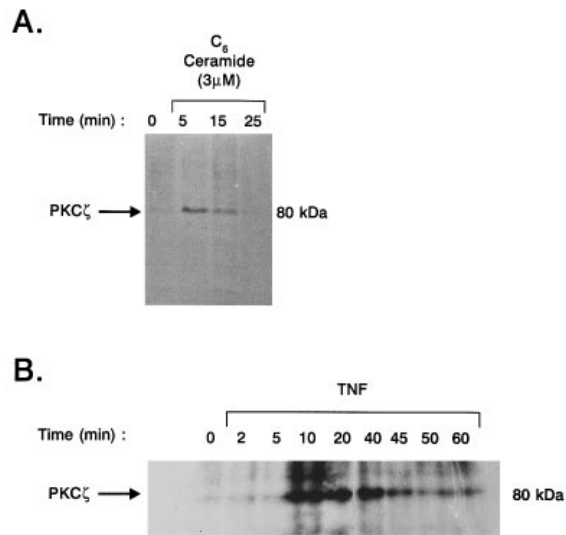


Figure 4 SDS/PAGE analysis of PKC ζ immunoprecipitations from C₆-ceramide- and TNF-treated 3T3-L1 adipocytes

3T3-L1 adipocytes were labelled for 4 h with [³²P]P_i (100 μ Ci/ml) and incubated for the indicated times with 3 μ M C₆-ceramide (A) or 5 nM TNF (B). The cells were lysed and PKC ζ was immunoprecipitated. Immunoprecipitates of 7.5×10^6 cells/lane were separated on SDS/PAGE and the gel was dried down and autoradiographed for 7 days. Identical results were observed in three separate experiments. Specificity of the immunoprecipitation was demonstrated by competition studies using the cognate antigen (amino acids 557–592) provided by Boehringer Mannheim (results not shown).

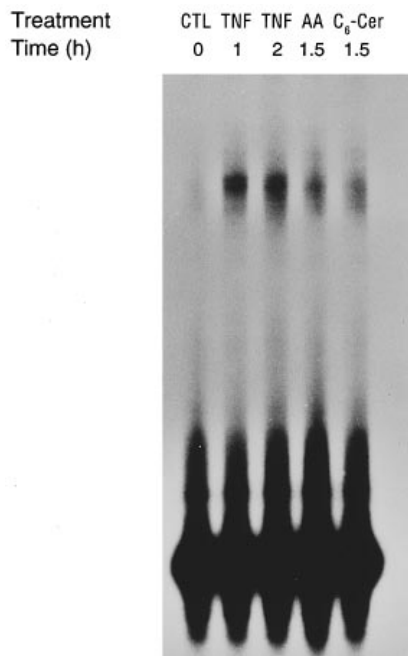


Figure 5 EMSA of nuclear extracts prepared from 3T3-L1 adipocytes

Fully differentiated 3T3-L1 cells were treated with 5 nM TNF for 1 and 2 h, or 100 μ M AA or 3 μ M C₆-ceramide for 1.5 h. Nuclear extracts were then prepared as described in the Experimental section and 10 μ g of protein from each sample was incubated with a radiolabelled double-stranded oligonucleotide containing the NF- κ B response element (5'-AATTCCTGG-AAA-GTCCCAGCGGAAAGTCCCTGT-3'). The binding reactions were then subjected to EMSA analysis. The gel shifts displayed are representative of an experiment performed three times with similar results.

basis for elucidating the components of the ceramide-initiated signal transduction pathway, as well as a testable hypothesis for control of GLUT4 gene expression.

DISCUSSION

In the present study, we have demonstrated that TNF regulates GLUT4 gene expression in the 3T3-L1 adipocytes through a ceramide-dependent pathway of signal transduction. We have found that addition of TNF to the adipocytes resulted in a rapid decrease in SM levels and concomitant increase in ceramide levels. However, unlike the SM cycle described by Jayadev et al. in HL-60 cells [26] (where the PLA₂/AA pathway mediates the effects of TNF on SM hydrolysis) AA does not appear to be involved in the TNF-induced SM turnover in 3T3-L1 cells. This is suggested by the data in Figure 1 where SM levels did not change after treatment with AA, oleic acid or ETYA. The inability of fatty acids to activate the adipocyte SMase suggests that it is not a neutral SMase, as described for the HL-60 cells [26], but an acidic SMase, which has been suggested to mediate the effects of TNF in some cell types. Ceramide, a product formed from the breakdown of membrane SM through the action of SMase, has been demonstrated to act as a second messenger, mediating the effects of TNF on cell growth and differentiation [37,42–44] and, more recently, TNF-induced apoptosis [39]. The current study identifies ceramide as a likely mediator of the TNF-induced decrease in GLUT4 mRNA content by demonstrating that ceramide is capable of mimicking the effects of TNF on GLUT4 gene expression. Like TNF, C₆-ceramide suppressed GLUT4 mRNA accumulation; however, ceramide acted only by decreasing the transcription rate of the gene, without changing the GLUT4 mRNA half-life. Thus, the effect of ceramide is localized to transcription, suggesting that another pathway controls the TNF-induced mRNA destabilization. We also examined the effect of ceramide on GLUT1 transcription and observed that, in a manner identical to TNF [9,13], ceramide decreased the transcription of the GLUT1 gene. These results are consistent with ceramide functioning as the mediator of TNF for the regulation of both transporters. Contrary to our previous studies [9,13], Szalkowski et al. [45] reported the lack of an effect of TNF on GLUT1 expression. However, in that study, the concentrations of TNF and protocol for differentiation, as well as the duration of treatment, were markedly different to those in our studies, suggesting that the discrepancy results from one or more of these differences.

With respect to TNF signalling, ceramide has been shown to activate a membrane-associated kinase which is also activated by TNF in intact and cell-free systems and shares the substrate specificity of MAP kinases [46,47]. A ceramide-activated protein phosphatase has also been described in extracts of T9 glioma cells that may be involved in transducing the effects of ceramide [21]. To date, it is not clear whether these kinases and phosphatases are direct or downstream targets for ceramide and what role they may play in mediating the biological effects of ceramide. Previous data from our laboratory demonstrated that the phosphatase inhibitor, okadaic acid, suppressed GLUT4 gene transcription in a similar manner to TNF and ceramide [13]. As the inhibition of a phosphatase is biochemically the same as activating a kinase these data would predict that kinase activation is a logical intermediate in the TNF/ceramide signalling pathway. Recent evidence has demonstrated that ceramide specifically activates PKC ζ , suggesting that it is an immediate target for this lipid second messenger [40]. We have determined that the 3T3-L1 adipocytes predominantly express the atypical isoform, PKC ζ with minor expression of the Ca²⁺-independent isoforms ϵ and θ

[42]. Based on these observations we predicted that ceramide treatment of the cells would stimulate phosphorylation and subsequent activation of PKC ζ and indeed, immunoprecipitations of PKC ζ from [³²P]P_i-labelled cells that were incubated with C₆-ceramide revealed enhanced phosphorylation of the protein within 5 min (Figure 4). Again these observations are consistent with ceramide functioning as the second messenger for TNF, and with the reported direct activation of PKC ζ by ceramide, the data support the placing of this PKC isoform at a primary point in the signal transduction cascade.

The TNF induction or repression of specific genes is carried out by nuclear transcription factors that are at the receiving end of the signalling cascade. TNF is one of the few cytokines known to initiate the translocation of NF- κ B from the cytosol (inactive form) to the nucleus (active form), resulting in the regulation of TNF-responsive genes. Thus, NF- κ B can be viewed as a 'third messenger' in the signal transduction pathway mediating the expression of TNF-responsive genes. The study presented here demonstrates that the SMase-ceramide pathway plays a potential role in the activation of NF- κ B by TNF in the 3T3-L1 adipocytes. Like TNF, addition of ceramide to cells was shown to induce the translocation of NF- κ B from the cytoplasm to the nucleus, identifying a potential mediator for TNF activation of NF- κ B in the adipocytes. The results of numerous other studies have indicated that ceramide produced by SMase functions as a crucial component of the TNF signalling cascade by modulating the levels of NF- κ B [18,24,48,49]. These data are not only diagnostic for the TNF signal transduction pathway in the 3T3-L1 adipocytes, but further confirm that ceramide is a major second messenger of TNF action in these cells. Moreover, based on the studies of Schutze et al. [50] where TNF-induced activation of NF- κ B was shown to be mediated by an acidic SMase, these results would suggest that in the adipocytes an acidic SMase is responsible for the generation of ceramide in response to TNF. It is unclear at this time whether this nuclear transcription factor plays a role in the suppression of the GLUT4 gene. The GLUT4 promoter does contain one NF- κ B-like binding site located at bases -110 to -120; however, DNA gel shift analysis indicated no change in the binding pattern of the transcription factor with TNF or ceramide treatment (results not shown).

In summary, we have demonstrated that ceramide appears to be the functional mediator of TNF in the suppression of GLUT4 gene transcription. In addition, we have established that in terminally differentiated adipocytes, ceramide activates two major signalling intermediates, PKC ζ and NF- κ B. While we have not linked activation of these intermediates to control of GLUT4 gene expression we have established the potential for PKC ζ and NF- κ B to play a role in TNF-regulated events in the fat cell.

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