Tumour necrosis factor-α-induced glucose-stimulated insulin secretion inhibition in INS-1 cells is ascribed to a reduction of the glucose-stimulated Ca\(^{2+}\) influx

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Abstract

The present study was undertaken to determine how tumour necrosis factor-α (TNF-α) elicits the inhibition of glucose-stimulated insulin secretion (GSIS) in rat insulinoma cells (INS-1 β-cells). TNF-α pretreatment did not change the expression levels of insulin, PDX-1, glucose transporter 2, glucokinase, K\(_{\text{ATP}}\) channels, Ca\(^{2+}\) channels, and exocytotic molecules and, furthermore, did not reduce the glucose-stimulated ATP level. On the other hand, TNF-α reduced the glucose-stimulated influx of Ca\(^{2+}\). The TNF-α treatment was thought to activate c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and NF-κB inflammatory signals, since TNF-α increased phospho-JNK and phospho-p38 and reduced IkB levels. Inhibitors of these signaling pathways prevented the TNF-α-induced reduction of the Ca\(^{2+}\) influx and GSIS. Overexpression of MEKK3, a possible mediator from the TNF-α receptor to the JNK/p38 and NK-κB signaling cascade, increased the levels of phospho-JNK, phospho-p38, and NF-κB, and reduced the glucose-stimulated Ca\(^{2+}\) influx and GSIS. The reduction of the Ca\(^{2+}\) influx and GSIS in MEKK3-overexpressing INS-1 cells was also prevented by inhibitors of JNK, p38, and NF-κB. These data demonstrate that TNF-α inhibits GSIS by reducing the glucose-stimulated Ca\(^{2+}\) influx, possibly through the activation of JNK and p38 MAPK and NF-κB inflammatory signals. Thus, our findings suggest that the activation of stress and inflammatory signals can contribute to the inhibition of GSIS in the development of diabetes.


Introduction

Secretion of insulin from pancreatic β-cells is an essential prerequisite to maintaining an appropriate level of blood glucose. For appropriate glucose-stimulated insulin secretion (GSIS) to occur, all steps, from glucose sensing to insulin granule exocytosis in β-cells, should be optimally processed (Rorsman et al. 2000). Glucose is first transported into β-cells and metabolized, and the ATP/ADP level is then increased. Elevation of ATP/ADP closes K\(_{\text{ATP}}\) channels and elicits membrane depolarization. The change of membrane potential triggers the opening of voltage-dependent calcium channels (VDCC) and increases the flux of Ca\(^{2+}\). The elevated [Ca\(^{2+}\)], elicits insulin exocytosis through fusion between insulin granules and the plasma membrane. Thus, the Ca\(^{2+}\)-requiring exocytotic pathway is K\(_{\text{ATP}}\) channel dependent and plays a major role in the first phase of insulin secretion (Henquin et al. 2003, Seino & Miki 2003). Conversely, K\(_{\text{ATP}}\)-independent amplifying pathways are also required for the secretion of sufficient amounts of insulin in response to continuing exposure to glucose (Henquin et al. 2003). The amplification signals mainly operate at the level of granule mobilization from cytosol to the plasma membrane and, thus, determine the second phase of insulin secretion (Troitza et al. 2002). Although the identities of amplifying signals have not been examined completely, intermediates generated from the TCA cycle and lipogenic pathway have been thought to contribute to the amplification of Ca\(^{2+}\)-mediated insulin exocytosis (Troitza et al. 2002, Wiederkehr & Wollheim 2006). Protein kinase A (PKA) and protein kinase C (PKC), which are activated by cAMP and diacylglycerol respectively, and Ca\(^{2+}\)-activated calcium/calmodulin kinase II are known to be typical amplification signals (Nesher et al. 2002).

The inhibition of GSIS as a functional defect of pancreatic β-cells was reported to be a characteristic of the pre-diabetic stage in autoimmune type 1 diabetic subjects (Conget et al. 1993, Sherry et al. 2005), but this inhibition could be restored in in vitro cultures (Strandell et al. 1990, Lupi et al. 2004).
This finding suggests that chronic exposure of β-cells in inflamed islets to high glucose or inflammatory cytokines may reduce GSIS. Failure of insulin secretion is also observed in patients with type 2 diabetes (Polonsky et al. 1988). During progression to type 2 diabetes, selective loss of GSIS was observed as an initial defect of diabetes, whereas secretion in response to other secretagogues such as sulfonylureas, arginine, and KCl was preserved (Porte 1991). Many studies have suggested that functional impairment of β-cells in type 2 diabetes results from chronic exposure of β-cells to high glucose and free fatty acids (Grill & Bjorklund 2000, McGarry 2002, Kjorholt et al. 2005). A proinflammatory cytokine, tumour necrosis factor-α (TNF-α), which is produced from activated macrophages or adipocytes, was also suggested to be a stimulus that plays a role in impaired secretion of insulin, which frequently occurs in type 2 diabetes (Donath et al. 2003, Scheen 2003). Specifically, in vitro studies demonstrated that TNF-α alone could induce the inhibition of GSIS in various β-cells (Campbell et al. 1988, Zhang & Kim 1995, Dunger et al. 1996, Park et al. 1999, Tsiotra et al. 2001).

TNF-α is a pleiotropic cytokine that elicits a wide spectrum of physiological effects, including cell proliferation, differentiation, and apoptosis (Locksley et al. 2001). TNF-α-induced responses are mediated through a TNF-α receptor (Locksley et al. 2001). Binding of TNF-α to the TNF-α receptor leads to the recruitment of the TNF receptor-associated death domain (TRADD) and subsequent recruitment of the FAS-associated death domain (FADD), TNF-associated factor 2 (TRAF2), and receptor-interacting protein (RIP). While the association of FADD with TRADD triggers an apoptotic signal, the binding of TRAF2 and RIP to TRADD activates c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and IκB kinase (Wajant et al. 2003). MEKK3 was reported to be an essential mediator for TNF-α-induced activation of NF-κB through interaction with RIP (Yang et al. 2001) or for JNK and p38 activation through interaction with TRAF 6 and TRAF 7 (Huang et al. 2004, Xu et al. 2004).

This study was initiated to determine how TNF-α elicits the impaired secretion of insulin in INS-1 rat β-cells. First, we determined whether TNF-α had a pleiotropic inhibitory effect on insulin secretion stimulated by various insulin secretagogues. Secondly, we investigated which of the steps, from glucose sensing to insulin granule exocytosis, was involved in TNF-α-induced inhibition of GSIS. We specifically focused on the reducing effect of TNF-α on the glucose-stimulated influx of Ca2+. Thirdly, to determine which TNF-α-mediated signals were involved in TNF-α-induced inhibition, the effects of different pharmacological inhibitors of JNK, p38, NF-κB, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS) on TNF-α-induced GSIS inhibition were investigated. Lastly, the involvement of JNK/p38 MAPK and NF-κB inflammatory signals in GSIS inhibition was studied using an MEKK3 expression system.

Materials and Methods

Materials

Recombinant murine TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Specialty chemicals, i.e. glucose, leucine, glutamine, pyruvate, tolbutamide, glimepiride, repaglinide, efaroxan, phenotamine, KCl, Bay K8633, phorbol 12-myristate 13-acetate (PMA), forskolin, glucagon-like peptide 1 (GLP-1), Na42+-methyl-L-arginine acetate, N-acetyl-L-cysteine (NAC), Fluo-3/AM, and doxycycline, were purchased from Sigma–Aldrich. Inhibitors such as SP600125, SB203580, and SN50 were obtained from Merck Bioscience. Chemicals were dissolved in either appropriate medium solution or dimethyl sulfoxide and were then used for treatment at the required working dilution. Anti-Kir 6.2, anti-sulfonylurea receptor 1 (SUR1), anti-NF-κB p50, anti-lamin B, anti-Na+_/K+ ATPase, anti-actin, and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Cav 1.3 (α1D) antibody was purchased from Alomone Labs (Jerusalem, Israel). Anti-phospho-JNK, anti-JNK, anti-phospho-p38 MAPK, anti-p38, and anti-IκB-α antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-MEKK3 antibody was purchased from Upstate (Charlottesville, VA, USA).

Cells and culture

INS-1 rat insulinoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technology Inc., Gaithersburg, MD, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technology Inc).

Methyl thiazol tetrazolium (MTT) cell viability assay

Cell viabilities were determined using an MTT assay. In brief, the cells were incubated in the presence of MTT (0.5 mg/ml) solution for 2 h at 37 0C. Medium supernatants were discarded and acidic isopropanol (0.04 M HCl) was added. After further incubation for 30 min, absorbency was measured at 570 nm using a microplate reader (BioRad).

Semi-quantitative RT-PCR

Expression levels of mRNAs were compared using the semi-quantitative RT-PCR technique. Semi-quantitative RT-PCR was carried out with Takara RNA PCR kit Ver 3.0 (Takara, Shiga, Japan). In brief, INS-1 cell cDNAs were synthesized with avian myeloblastosis virus (AMV) reverse transcriptase and random 9-mers, and were then subjected to PCR amplification with primer sets of different genes. The nucleotide sequences of primer sets and reaction conditions used are listed in Table 1. The amplified DNA was analyzed by agarose gel electrophoresis. The relative quantities of amplified DNA was compared on the basis of amplified cyclophilin DNA.
**Real-time quantitative PCR**

Real-time quantitative PCR was performed in triplicate using the ABI Prism 7900 sequence detection system (PE Applied Biosystems). The fluorescence emission from each sample was collected by a charge-coupled device camera, and the quantitative data were then analyzed using Sequence Detection System software (SDS version 2.0, PE Applied Biosystems). Reaction mixtures contained 10 pmol/mL of each primer and 2X SYBR PCR master mix (PE Applied Biosystems), which includes the HotStarTaq DNA polymerase, dNTP mix, SYBR Green I fluorescent dye, and ROX dye as a passive reference. Serial dilutions (1/2, 1/4, 1/8, and 1/16) of cDNA were used to generate relative standard curves. Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, different annealing temperatures (Table 2) for 30 s, and 72 °C for 30 s. In order to exclude the presence of non-specific products, a melting curve analysis of products was performed routinely by high-resolution data collection during an incremental temperature increase from 60 to 95 °C, with a ramp rate of 0.2 °C/s. Real-time PCR cycle numbers were converted to gene amounts on the basis of the equation (ABI Prism 7900 sequence detection system).

**Measurement of cellular ATP**

Cellular ATP content was determined using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). To measure the glucose-stimulated increase of ATP in INS-1 cells, cells were seeded in 24-well plates at a density of 1.5 × 10^5 cells per well and were cultured for 2 days. After treatment with TNF-α for 16 h, the cells were washed with KRB buffer (0.2 mM glucose, 24 mM NaHCO_3, 1.2 mM MgCl_2, 1 mM HEPES, 129 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2, and 0.5% BSA). The cells were stimulated by 16.7 mM glucose for 5 min. After the supernatant was discarded, the cells were disrupted by sonication (10 s, four times) in the presence of 0.2 ml of 5% trichloroacetic acid. Cell pellets were removed by centrifugation (12 000 g, 10 min), and the trichloroacetic acid was extracted from the supernatant by repetitive extraction with ether. After neutralization by the addition of the same amount of HEPES buffer (20 mM HEPES and 3 mM MgCl_2 (pH 7.4)), the sample solution was mixed with the same volume of CellTiter-Glo reagent. Luminescence was measured with a luminometer (Turner Design Instrument, Sunnyvale, CA, USA). The quantity of ATP was determined using a standard ATP curve.

**Measurement of [Ca^{2+}]_i**

[Ca^{2+}]_i levels were determined by the measurement of Fluoro-3/AM fluorescence using a confocal laser scanning microscope (Zeiss, Jena, Germany). Dispersed INS-1 cells were plated onto glass bottom culture dishes (MatTek Co., Ashland, MA, USA) at 37 °C in 10% FBS-RPMI 1640 culture medium for 24 h. The cells were then loaded with Fluoro-3/AM (5 μM) for 30 min at 37 °C in Hank’s balanced salt solution (HBSS; 145 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl_2, 1 mM CaCl_2, 10 mM glucose, and 20 mM HEPES (pH 7.4)). After stimulation with glucose (16.7 mM) or KCl (30 mM), the fluorescent images were captured (excitation: 488 nm and emission: 505 nm) and subsequently digitalized. The calcium intensities were calculated (DVC-1310, DVC Co., Austin, TX, USA) and presented as percent changes compared with the basal level (F/Fo).

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**Table 1** Primer sequence and the reaction conditions for semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Annealing Tm (°C)</th>
<th>Cycle</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>(F) ccaaggcttgctcaacagcac (R) cagttgcagttgctctccagttg</td>
<td>60</td>
<td>21</td>
<td>267</td>
</tr>
<tr>
<td>PDX-1</td>
<td>(F) cctaatccctctgcgtcagttg (R) ctcctccggttctgctgtagc</td>
<td>59</td>
<td>26</td>
<td>349</td>
</tr>
<tr>
<td>GLUT2</td>
<td>(F) cagttgcagttgctctccagttg (R) agcgctgctgctgctgctgct</td>
<td>55</td>
<td>28</td>
<td>183</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>(F) tccttcgctctcactgc (R) tccttcgctctcactgc</td>
<td>61</td>
<td>32</td>
<td>299</td>
</tr>
<tr>
<td>iNOS</td>
<td>(F) gcgaaatgacacatcag (R) acaacctgggtggtggaagc</td>
<td>55</td>
<td>33</td>
<td>356</td>
</tr>
<tr>
<td>Kir 6.2</td>
<td>(F) ccagttacctttaggccagtct (R) ccagttacctttaggccagtct</td>
<td>64</td>
<td>30</td>
<td>954</td>
</tr>
<tr>
<td>SUR1</td>
<td>(F) fccagttacctttaggccagtct (R) fccagttacctttaggccagtct</td>
<td>64</td>
<td>30</td>
<td>1048</td>
</tr>
<tr>
<td>α1D</td>
<td>(F) gcaggaaacttgaagagccag (R) ctgactcagatatggctgctgag</td>
<td>64</td>
<td>30</td>
<td>868</td>
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<tr>
<td>β3</td>
<td>(F) cccttcgctctcactgc (R) cccttcgctctcactgc</td>
<td>60</td>
<td>30</td>
<td>415</td>
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<tr>
<td>Cyclophilin</td>
<td>(F) ccacagccagcag (R) gaaattagagttgtcaccag</td>
<td>60</td>
<td>26</td>
<td>410</td>
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</tbody>
</table>
Quantitation of insulin

To measure the insulin secreted in INS-1 cells, cells were seeded in 48-well plates at a density of $1 \times 10^5$ cells per well and were cultured for 2 days. After treatment with TNF-α for 16 h, the cells were washed with KRB buffer and then incubated with the same KRB buffer for 1 h. Insulin secretion was elicited by incubation for 2 h with appropriate insulin secretagogues. The insulin concentration in supernatant was determined with a Rat Insulin RIA kit (Linco Research, St Charles, MO, USA).

Immunoblotting

Cells were suspended in radio-immuno precipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris–Cl (pH 7.5), protease inhibitor cocktail (Roche Applied Science) and were then incubated on ice for 15 min. Whole proteins were extracted by differential centrifugation $(10000 \times g, 10 \text{ min})$. Protein concentrations were determined using protein assay kits (BioRad). An equal volume of 2X SDS sample buffer (125 mM Tris–Cl (pH 6.8), 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 20% glycerol) was added to cell lysates. Equivalent amounts of protein (30 μg) were loaded onto 10–15% polyacrylamide gels, electrophoresed, and then transferred to polyvinylidine fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After these membranes had been blocked 5% skim milk for 1 h, target antigens were reacted with primary antibodies. After washing with PBS, secondary antibodies, i.e., horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG antibodies, were then bound. The immunoreactive bands were then detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Extraction of nuclear and membrane proteins

Cells $(1 \times 10^6)$ were suspended in 1 ml RSB buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris–Cl (pH 7.4), 0.5% NP-40, protease inhibitor cocktail, and 0.2 mM PMSF) and were then incubated on ice for 5 min. After centrifugation at 500 g for 5 min, the pellets were re-suspended with 1 ml RSB buffer. Production of nuclei was evaluated by microscopic observation. To obtain intact nuclei, repetitive extraction was carried out with RSB buffer. After the nuclear pellets were suspended in RIPA buffer, nuclear protein was extracted by differential centrifugation $(10000 \times g, 10 \text{ min})$. To obtain membrane proteins, cells were first suspended in a lysis buffer (150 mM NaCl, 50 mM Tris–Cl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail), disrupted by sonication (10 s, five times), and centrifuged at 700 g for 15 min. After the pellets containing nuclei and undisrupted cells were discarded, the supernatant was centrifuged at 12000 g for 15 min. The pellets that contained plasma membranes were suspended in RIPA buffer and used for immunoblotting.

Construction of MEKK3-expressing INS-1 cells (TRE-MEKK3) using the Tet-On system

INS-1 β-cell cDNAs were synthesized with AMV reverse transcriptase (Takara) using random 9-mers and were then subjected to PCR amplification using mouse MEKK3
primes (5'-gaagtcgacatggatcaagagcattagactc-3’ and 5'-gtcttagctgtagcactagctggtcga-3’). After the amplified DNA was cut with SalI and XbaI, the MEKK3 fragments were subcloned to the pTRE2 vector (Clontech) in order to construct pTRE-MEKK3. The mouse MEKK3 nucleotide sequences were confirmed using the ABI BigDye sequencing kit (Applied Biosystems, Foster City, CA, USA). The stable INS-1 cells containing pTK-Hyg (Clontech) were transfected with the pTRE-MEKK3 DNAs, and the stable INS-1 cells (TRE-MEKK3) that were able to express MEKK3 following treatment with doxycycline were then selected.

**Statistical analysis**

Data are presented as means ± S.D. of at least three independent experiments. Statistically significant differences between groups were determined using Student’s t-test. *P<0.05 were considered to be statistically significant.

**Results**

**TNF-α-induced inhibition of GSIS in INS-1 β-cells**

To determine whether treatment with TNF-α inhibits GSIS in β-cells, INS-1 rat β-cells were treated with different concentrations of TNF-α for 16 h, and the amount of insulin secreted by glucose stimulation (16.7 mM, 2 h) was then measured by insulin RIA. The cytotoxic effect of TNF-α on INS-1 cells was investigated before determining the inhibitory effect of TNF-α on GSIS. Cell viability was little affected by treatment with 20 ng/ml TNF-α for 16 h (Fig. 1A). As shown in Fig. 1B and C, pretreatment with TNF-α significantly reduced GSIS in a concentration- and time-dependent manner, while the same treatment of TNF-α did not affect basal secretion of insulin. Pretreatment with 10 ng/ml TNF-α for 16 h reduced GSIS by one-third of its maximal secretion and was sufficient for obtaining a maximal inhibitory effect. To determine whether the inhibitory effect of TNF-α on GSIS is reversible, GSIS was re-assessed with the INS-1 cells incubated in TNF-α-free media after TNF-α pretreatment (10 ng/ml, 16 h). INS-1 cells that had been incubated again in the absence of TNF-α showed similar GSIS capacity compared with INS-1 cells that had never been treated with TNF-α (Fig. 1C), which demonstrated that the inhibitory effect of TNF-α on insulin secretion was reversible.

**Inhibitory effect of TNF-α on various insulin secretagogue-stimulated insulin secretions in INS-1 cells**

To determine whether the TNF-α-induced inhibitory effect on insulin secretion was pleiotropic, we investigated the effect of TNF-α on insulin secretion stimulated by various insulin secretagogues. Pretreatment with 10 ng/ml TNF-α for 16 h significantly inhibited insulin secretion stimulated by ATP-generating nutrients such as leucine/glutamine and pyruvate. Two-hour stimulation of INS-1 cells with leucine (20 mM)/glutamine (10 mM) showed secretion of a similar amount of insulin compared with that shown with 16.7 mM glucose, but pre-incubation of INS-1 cells with TNF-α (10 ng/ml, 16 h) was found to reduce leucine/glutamine-stimulated insulin secretion (Table 3). Pretreatment with TNF-α also showed a significant inhibitory effect on pyruvate-stimulated insulin secretion, although 10 mM pyruvate had a weaker stimulatory effect on insulin secretion than 16.7 mM glucose. All of the K_{ATP} channel closures, including tolbutamide, glibenpiride, repaglinide, efloxane, and phenotolamine, had stimulatory effects on insulin secretion, but pretreatment with TNF-α inhibited the K_{ATP} channel closure-stimulated secretion of insulin. In addition, TNF-α pretreatment had a significant inhibitory effect on calcium channel opener Bay K8644-stimulated insulin secretion. In contrast, insulin secretion stimulated by a depolarizing agent, KCl, was not inhibited by pre-incubation with TNF-α. On the other hand,
the potentiation effects of PKC- and PKA-mediated amplification signals on insulin secretion were not blocked by pretreatment with TNF-α. Phorbol myristate acetate (PMA) and forskolin as a PKC activator and a PKA activator, respectively, showed potentiation effects on insulin secretion, and these effects were not inhibited by pretreatment with TNF-α. Furthermore, the GLP-1-mediated potentiation effect on GSIS was not impaired by TNF-α treatment either (Table 3).

No quantitative difference in insulin, PDX-1, glucose transporter 2, glucokinase, ATP, K<sub>ATP</sub> channels, calcium channels, and exocytotic molecules between TNF-α-untreated and TNF-α-treated INS-1 cells

To determine which step, from glucose sensing to insulin exocytosis, is impaired in TNF-α-incubated INS-1 cells, we compared the expression levels of the β-cell-specific molecules that are involved in this process. The expression levels of insulin and insulin transcription factor PDX-1 were determined by PCR or an immunoblotting technique. As shown in Fig. 2A, semi-quantitative RT-PCR analysis showed that there was little change in insulin and PDX-1 mRNA levels following treatment with TNF-α. The insulin content in cytoplasm was not changed either (Fig. 2B). The expressions of glucose transporter 2 and glucokinase were not decreased by TNF-α (Fig. 2A). Pretreatment with TNF-α did not reduce the expression levels of K<sub>ATP</sub> (Kir 6.2 and SUR1) and VDCC components (α1D and β3) at the mRNA and protein levels (Fig. 2C).

<table>
<thead>
<tr>
<th>Insulin secretagogues</th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0.54±0.08</td>
<td>0.59±0.04</td>
</tr>
<tr>
<td>Glucose (16.7 mM)</td>
<td>1.74±0.06</td>
<td>0.71±0.04</td>
</tr>
<tr>
<td>Leucine (20 mM) + glutamine (10 mM)</td>
<td>1.74±0.15</td>
<td>0.75±0.07</td>
</tr>
<tr>
<td>Pyruvate (10 mM)</td>
<td>1.38±0.14</td>
<td>0.85±0.12</td>
</tr>
<tr>
<td>Tollbutamide (300 µM)</td>
<td>1.17±0.18</td>
<td>0.55±0.16</td>
</tr>
<tr>
<td>Glimepiride (3 µM)</td>
<td>1.30±0.01</td>
<td>0.76±0.17</td>
</tr>
<tr>
<td>Repaglinide (1 µM)</td>
<td>1.22±0.10</td>
<td>0.82±0.04</td>
</tr>
<tr>
<td>Efaroxane (200 µM)</td>
<td>1.04±0.11</td>
<td>0.55±0.08</td>
</tr>
<tr>
<td>Phenolthalein (50 µM)</td>
<td>1.70±0.13</td>
<td>0.86±0.04</td>
</tr>
<tr>
<td>KCl (30 mM)</td>
<td>1.95±0.21</td>
<td>1.94±0.27</td>
</tr>
<tr>
<td>Bay K8644 (1 µM)</td>
<td>1.19±0.10</td>
<td>0.63±0.07</td>
</tr>
<tr>
<td>PMA (300 mM)</td>
<td>2.21±0.24</td>
<td>2.32±0.24</td>
</tr>
<tr>
<td>Forskolin (10 µM)</td>
<td>2.32±0.21</td>
<td>2.40±0.22</td>
</tr>
<tr>
<td>Glucose (16.7 mM) + GLP-1 (100 nM)</td>
<td>3.25±0.34</td>
<td>1.62±0.17</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01 versus TNF-α-untreated cells.

Involvement of JNK and p38 MAPK and NF-κB inflammatory signals in TNF-α-induced GSIS inhibition

To determine which of the signals induced by TNF-α may be involved in TNF-α-induced inhibition of GSIS, the INS-1 cells were treated with TNF-α in the presence of different pharmacological inhibitors of JNK, p38, NF-κB, nitric oxide (NO), and ROS, and GSIS was then investigated. The highest concentration of inhibitors having a maximal blocking effect without a cytotoxic effect was first determined, and the blocking effect was then investigated with lower concentrations. As shown in Table 4, SP600125, SB203080, and SN50, as inhibitors of JNK, p38, and NF-κB respectively showed significant protective effects on TNF-α-induced GSIS inhibition. The protective effects of these inhibitors were concentration dependent. A mixture of these inhibitors showed an additive protective effect on TNF-α-induced inhibition of GSIS. Interestingly, the inhibitors themselves had a weakly augmented effect on basal secretion of insulin in TNF-α-treated INS-1 cells. Among the inhibitors, SP600125 had the strongest protective effect on the TNF-α-induced inhibition of

Table 3 Inhibition of secretagogue-induced insulin secretion by tumour necrosis factor-α pretreatment. INS-1 cells were incubated in the absence and presence of TNF-α (10 ng/ml) for 16 h, and insulin secretion was then stimulated with various insulin secretagogues for 2 h. The insulin in medium was quantitated by insulin RIA.


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GSIS. NMMA (iNOS inhibitor) and N-acetyl cysteine (NAC; ROS scavengers) did not have any protective effect against TNF-α-induced GSIS inhibition. To determine whether TNF-α increases JNK, p38, and NF-kB signals in INS-1 cells and whether their pharmacological inhibitors reduce the TNF-α-induced signal increase, the levels of phospho-JNK, phospho-p38, and IκB-α were investigated by immunoblot analysis. As shown in Fig. 4A, the levels of phospho-JNK and phospho-p38 began to increase at 6 min, and were maintained for 30 min after TNF-α treatment, while the IκB-α level was significantly decreased at 12 min and 30 min after TNF-α treatment.

TNF-α-induced signals were selectively blocked by each pharmacological inhibitor. As shown in Fig. 4B, SP600125 reduced the TNF-α-induced phospho-JNK level, SB203580 reduced the TNF-α-induced phospho-p38 level, and SN50 reduced the NF-kB level. Since TNF-α reduced the glucose-stimulated influx of Ca^{2+}, we investigated whether SP600125, SB203580, or SN50 prevented the TNF-α-induced reduction of the Ca^{2+} influx. As shown in Fig. 4C, all inhibitors caused significant recovery of the TNF-α-induced reduction of the Ca^{2+} influx. More specifically, the JNK inhibitor, SP600125, had the highest preventive activity on the TNF-α-induced reduction of the Ca^{2+} influx.

**Inhibition of GSIS by MEKK3 expression**

Since JNK, p38, and NF-kB signals are thought to be critical for TNF-α-induced GSIS inhibition to occur, and MEKK3 is thought to be a central mediator for the activation of JNK, p38, and NF-kB from the TNF-α receptor, the effects of these signals on GSIS were investigated using an inducible MEKK3 expression system. MEKK3-expressing INS-1 cells (TRE-MEKK3) were constructed using a Tet-On expression system, and the expression of MEKK3 was then induced by doxycycline treatment. As shown in Fig. 5A, MEKK3 expression in TRE-MEKK3 INS-1 cells was gradually increased by doxycycline in a concentration-dependent manner. The phospho-JNK and phospho-p38 levels were increased by doxycycline treatment, while the IκB-α level was decreased. The effect of doxycyclin was concentration dependent. These data suggest that artificial induction of MEKK3 expression could activate JNK and p38 MAPK and NF-kB inflammatory signals. We next investigated whether...
TNF-α augments the MEKK3-mediated signal in these MEKK3-overexpressing cells. As shown in Fig. 5B, the treatment with TNF-α resulted in a greater increase in the phospho-JNK level in doxycycline-treated TRE-MEKK3 cells than in untreated TRE-MEKK3 cells. This finding suggests that TNF-α can activate the JNK signal through the MEKK3 signal. Next, we investigated the glucose-stimulated influx of Ca²⁺ and GSIS in MEKK3-overexpressing INS-1 cells in order to determine whether the MEKK3-mediated activation of MAPK and inflammatory signal is involved in the inhibition of GSIS. Figure 5C and D showed that the glucose-stimulated influx of Ca²⁺ and GSIS were significantly decreased in doxycycline-treated TRE-MEKK3 INS-1 cells in a concentration-dependent manner, which suggests that MEKK3-induced signals were involved in the inhibition of GSIS. To determine whether the blocking of the MAPK and inflammatory signals prevents the inhibition of GSIS in MEKK3-expressing INS-1 cells, the effects of inhibitor mixtures (SP600125, SB203580, and SN50) on MEKK3-mediated reduction of GSIS were investigated in doxycycline-treated TRE-MEKK3 INS-1 cells. Figure 5E shows that inhibitor mixtures prevented the inhibition of GSIS in MEKK3-overexpressing cells, and that the preventive effect was inhibitor dose dependent.

**Discussion**

A non-toxic concentration of TNF-α inhibited GSIS in INS-1 β-cells in a time- and concentration-dependent manner. The inhibition of GSIS seemed to be due to the reduction of the glucose-stimulated calcium influx by TNF-α pretreatment. The influx of calcium was determined by measuring the [Ca²⁺]ᵢ level after stimulation with glucose or KCl. TNF-α-treated and untreated INS-1 cells were loaded with 5 μM Fluo3/AM for 30 min, and the cells were then stimulated with (A) 16.7 mM glucose or (B) 30 mM KCl. Fluorescent signals (emission fluorescence at 505 nm after excitation at 488) were captured and the data were then processed using an imaging system. Relative [Ca²⁺]ᵢ was presented as percent changes in the intensity of fluorescence compared with the basal level (F/F₀). Fifteen independent calcium intensity ratios at the indicated time were combined. Averages and standard errors (mean ± S.E.M.) were calculated using Excel 2000 (Microsoft). **P<0.01 versus [Ca²⁺]ᵢ from TNF-α-untreated INS-1 cells.

Table 4 Effect of different inhibitors on tumour necrosis factor-α-induced glucose-stimulated insulin secretion inhibition. INS-1 cells were treated with different inhibitors for 16 h in the presence of TNF-α (10 ng/ml), and insulin secretion was then stimulated with 16.7 mM glucose for 2 h. The insulin in medium was quantitated by insulin RIA

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>TNF-α (10 ng/ml)</th>
<th>Glucose (16.7 mM, 2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>sp600125 (2.5 μM)</td>
<td>–</td>
<td>0.633±0.04</td>
</tr>
<tr>
<td>sp600125 (5 μM)</td>
<td>+</td>
<td>0.620±0.05</td>
</tr>
<tr>
<td>sp600125 (10 μM)</td>
<td>+</td>
<td>0.695±0.07</td>
</tr>
<tr>
<td>SB203080 (5 μM)</td>
<td>+</td>
<td>0.711±0.12</td>
</tr>
<tr>
<td>SB203080 (10 μM)</td>
<td>+</td>
<td>0.729±0.09</td>
</tr>
<tr>
<td>SN50 (2.5 μM)</td>
<td>+</td>
<td>0.618±0.06</td>
</tr>
<tr>
<td>SN50 (5 μM)</td>
<td>+</td>
<td>0.699±0.07</td>
</tr>
<tr>
<td>SN50 (10 μM)</td>
<td>+</td>
<td>0.707±0.10</td>
</tr>
<tr>
<td>sp (2.5 μM) + SB (5 μM) + SN50 (2.5 μM)</td>
<td>+</td>
<td>0.599±0.06</td>
</tr>
<tr>
<td>NMAA (2.5 mM)</td>
<td>+</td>
<td>0.701±0.04</td>
</tr>
<tr>
<td>NMAA (5 mM)</td>
<td>+</td>
<td>0.702±0.09</td>
</tr>
<tr>
<td>NAC (2.5 mM)</td>
<td>+</td>
<td>0.711±0.12</td>
</tr>
<tr>
<td>NAC (5 mM)</td>
<td>+</td>
<td>0.623±0.08</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01 versus TNF-α-treated cells without inhibitor.
The inhibition of GSIS in TNF-α similar to that in untreated INS-1 cells. This finding suggests that TNF-α-induced GSIS inhibition is characterized by the pleiotropic nature of the TNF-α signal transduction. The presence of these signals was analyzed by immunoblotting at 30 min after treatment with TNF-α in the presence of inhibitors. The influx of calcium was determined by measuring the [Ca²⁺]i level after glucose stimulation. *P<0.05 versus NF-κB, **P<0.01 versus phospho-JNK from TNF-α-treated INS-1 cells. Pretreatment with TNF-α did not reduce insulin gene expression and did not affect glucose metabolism from glucose uptake to ATP production. In addition, the expression levels of K_ATP channels, VDCCs, and exocytotic molecules were little changed as well. On the other hand, the levels of phospho-JNK, phospho-p38, and NF-κB were enhanced by TNF-α treatment, and inhibitors of these MAPK and inflammatory signals reduced TNF-α-induced GSIS inhibition. Overexpression of MEKK3, a possible mediator of the TNF-α receptor to JNK and p38 MAPK and NF-κB inflammatory signals, inhibited GSIS and this inhibition was prevented by inhibitors of these signals.

One characteristic of TNF-α-induced GSIS inhibition in INS-1 cells was the reversibility of GSIS inhibition. When TNF-α-treated INS-1 cells were re-incubated in TNF-α-free medium, GSIS was completely restored to a level similar to that in untreated INS-1 cells. This finding suggests that the inhibition of GSIS in TNF-α-treated INS-1 cells is similar to that of islet β-cells isolated from diabetic subjects (Strandell et al. 1990, Lupi et al. 2004, Marchetti et al. 2004). Another characteristic of TNF-α-treated INS-1 cells was the relative pleiotropic nature of the TNF-α-induced inhibition of insulin secretion. The insulin secretion stimulated by most secretagogues was inhibited by TNF-α pretreatment. This finding suggests that functional impairment in TNF-α-treated β-cells is slightly different from that in β-cells that exists in the initial stage of type 2 diabetes, since β-cells in this initial stage of diabetes are still responsive to most secretagogues such as sulfonylureas, although they are unresponsive to glucose (Del Guerra et al. 2005). Thus, TNF-α-treated β-cells appear to be similar to cells that exist in later stages of diabetes, since β-cells from patients at later stages of diabetes are not responsive to insulin secretagogues (Scheen 2004, Weyer et al. 2006). Although our data showed that TNF-α inhibited triggering signals that are stimulated through the enhanced influx of Ca²⁺, TNF-α pretreatment did not inhibit insulin secretion stimulated by a membrane-depolarizing agent like KCl. The reason that TNF-α did not inhibit KCl-stimulated insulin secretion was not clear, but a lack of impairment of KCl-stimulated insulin secretion was frequently observed in β-cells that were not responsive to glucose. Several studies have reported that insulin secretion stimulated by membrane-depolarizing agents such as KCl and arginine was not blunted in secretagogue-induced desensitized β-cells or islet β-cells isolated from diabetic patients (Rustenbeck et al. 2004, Del Guerra et al. 2005, Tsuboi et al. 2005).

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Figure 4 Activation of JNK, p38, and NF-κB signals by TNF-α and the involvement of these signals in the glucose-stimulated calcium influx. (A) Activation of stress (JNK and p38) and inflammatory (NF-κB) signals was analyzed by immunoblotting with anti-phospho-JNK, anti-phospho-p38, and anti-IκB-α antibodies after treatment with TNF-α. P<0.05 versus phospho-JNK from TNF-α-untreated cells. (B) Inactivation of signals was analyzed by immunoblotting at 30 min after treatment with TNF-α in the presence of inhibitors. Nuclear proteins were used for the detection of NFκB. *P<0.05 versus phospho-JNK from TNF-α-treated INS-1 cells. Pretreatment with TNF-α did not inhibit KCl-stimulated insulin secretion. The insulin secretion stimulated by membrane-depolarizing agents such as KCl and arginine was not blunted in secretagogue-induced desensitized β-cells or islet β-cells isolated from diabetic patients (Rustenbeck et al. 2004, Del Guerra et al. 2005, Tsuboi et al. 2005).
and PDX-1 were not decreased either. Little change in the treatment, and also indicated that the expressions of insulin exocytosis, were not quantitatively altered by TNF-α molecules involved in all steps, from glucose sensing to insulin-induced GSIS inhibition. Our data showed that most of the doxycycline-treated INS-1 was analyzed by measuring the [Ca^{2+}]_{i} level. +Auntreated INS-1 cells. (D) Insulin secretion in doxycycline-treated INS-1 cells was analyzed by measuring the quantity of insulin released into the medium after glucose (HG: 16.7 mM) stimulation for 2 h. *P<0.05; **P<0.01 versus glucose-stimulated insulin from Dox-untreated INS-1 cells. (E) Doxycycline-treated INS-1 cells were incubated with inhibitor mixtures (+: 2.5 μM SP600125, 5 μM SB203580, and 2.5 μM SN50; +: 5 μM SP600125, 10 μM SB203580, and 5 μM SN50) of JNK, p38, and NF-κB. Glucose (HG: 16.7 mM, 2 h)-stimulated insulin secretion was analyzed by measuring the amount of insulin released into the medium. +P<0.05 versus glucose-stimulated insulin from Dox-treated INS-1 cells.

Earlier reports showed that TNF-α could inhibit GSIS (Campbell et al. 1988, Zhang & Kim 1995, Park et al. 1999, Tsiotra et al. 2001). While the insulin content in cytoplasm was not changed by treatment with TNF-α (Zhang & Kim 1995, Park et al. 1999, Tsiotra et al. 2001), the reduction of glucose utilization via the decreases of GLUT2 or glucokinase was suggested to be a cause for TNF-α-induced inhibition of GSIS (Park et al. 1999). However, our data demonstrate that a defect in glucose metabolism is not a main cause of TNF-α-induced inhibition. Our data showed that most of the molecules involved in all steps, from glucose sensing to insulin exocytosis, were not quantitatively altered by TNF-α treatment, and also indicated that the expressions of insulin and PDX-1 were not decreased either. Little change in the glucose-stimulated ATP level between TNF-α-treated and TNF-α-untreated INS-1 cells demonstrates that TNF-α does not affect glucose metabolism. More specifically, the finding that TNF-α could reduce insulin secretion stimulated by K_{ATP} channel closures and VDCC opener supports the hypothesis that TNF-α may elicit a defect at the levels from the Ca^{2+} influx to insulin exocytosis, rather than at the level of glucose metabolism. However, the finding that components of the Ca^{2+} channel were not quantitatively changed by TNF-α treatment indicates that a qualitative modification of molecules related to the influx of Ca^{2+} or insulin exocytosis may contribute to TNF-α-induced GSIS inhibition. Reports that the glucose-stimulated Ca^{2+} influx was frequently reduced in diabetic islets (Tsujit et al. 1993, Roe et al. 1996, Aschcroft & Rorsman 2004, Tsuboi et al. 2006) support that TNF-α can act as a stimulus for eliciting β-cell impairment in diabetes. However, our suggestion does not completely coincide with the other view that β-cell dysfunction in diabetes is mainly a result of a quantitative change of exocytotic molecules, as well as metabolic
TNF-α rapidly increased phospho-JNK, phospho-p38, and NF-κB levels. All of the inhibitors of these signals showed a preventive effect on TNF-α-induced inhibition of GSIS and reduction of the glucose-stimulated Ca2+ influx. These results suggest that activation of JNK and p38 MAPK and NF-κB inflammatory signals was involved in TNF-α-induced GSIS inhibition through the reduction of the Ca2+ influx. The findings from the experiment with MEKK3-expressing INS-1 cells using the Tet-On system support the role of these signals in TNF-α-induced inhibition of GSIS. Doxycycline-induced MEKK3 overexpression showed persistent activation of JNK, p38, and NF-κB signals in INS-1 cells, and also showed a reduction of the glucose-stimulated Ca2+ influx and GSIS. The reduction was also prevented by treatment with an inhibitor mixture of JNK, p38, and NF-κB. Collectively, JNK and p38 MAPK and NF-κB inflammatory signals were thought be activated by TNF-α, and the activated signals were thought to play a major role in TNF-α-induced GSIS inhibition in INS-1 β-cells.

While JNK was reported to be a critical signal for eliciting β-cell dysfunction, β-cell dysfunction induced by the activation of JNK was reported to result from the suppression of insulin biosynthesis (Kaneto et al. 2006). The p38 MAPK signal was also reported to be dissociated from the exocytotic release of insulin (Burns et al. 1999). These results suggest that JNK or p38 signals may not be directly involved in the inhibition of GSIS. However, our data demonstrate that the activation of JNK or p38 signals might directly affect GSIS through modulation of the Ca2+ influx. On the other hand, the involvement of NF-κB is supported by two previous reports indicating that NF-κB activation plays a role in GSIS inhibition (Tran et al. 2002, Zeender et al. 2004). However, the NF-κB inflammatory signal was also reported to be necessary for GSIS, rather than inhibition of GSIS (Hammar et al. 2005, Norlin et al. 2005). Although the exact mechanism remains to be clarified, it can be proposed that a suitable level of NF-κB activation is necessary for optimal GSIS. It has also been reported that transient and moderate NF-κB activation is essential to the proper function of pancreatic β-cells (Ortiz et al. 2006).

In conclusion, our study demonstrates that TNF-α inhibits GSIS in INS-1 β-cells by reducing the glucose-stimulated influx of Ca2+. Possibly through the activation of JNK and p38 MAPK and NF-κB inflammatory signals, and thus suggest that the activation of these stress and inflammatory signals, possibly stimulated by metabolic or immunologic insults, may elicit β-cell dysfunction through GSIS inhibition in the development of diabetes.

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Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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