Biochimica et Biophysica Acta 1790 (2009) 8–15

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Prolonged high glucose suppresses phorbol 12-myristate 13-acetate and ionomycin-induced interleukin-2 mRNA expression in Jurkat cells

Koji Higai *, Masatoshi Tsukada, Yumiko Moriya, Yutaro Azuma, Kojiro Matsumoto

Department of Clinical Chemistry, School of Pharmaceutical Sciences, Toho University Miyama 2-2-1, Funabashi, Chiba 247-8510, Japan

ARTICLE INFO

Article history:
Received 21 May 2008
Received in revised form 29 September 2008
Accepted 3 October 2008
Available online 17 October 2008

Keywords:
Interleukin-2
Nuclear factor of activated T cells
Inducible cAMP early repressor
High concentration of glucose

ABSTRACT

Background: The disturbance of immunological responses is a complication of diabetes mellitus.
Methods and Results: We cultured Jurkat cells in 11.1 (normal) and 22.2 mmol/l (high) glucose for 12 weeks and stimulated them with 10 nmol/l phorbol 12-myristate 13-acetate (PMA) and 500 nmol/l ionomycin. RT-PCR revealed that induced interleukin (IL)-2 mRNA expression levels were suppressed in high glucose cultures compared to those in normal glucose. Promoter activities of IL-2, nuclear factor of activated T cells (NFAT), and activator protein-1 (AP-1), after 6 h stimulation with PMA and ionomycin, gradually decreased in high glucose cultures to approximately 20% of those in normal glucose at 12 weeks. The prolonged culture in high glucose increased inducible cAMP early repressor (ICER) II mRNA and protein levels, and overexpression of ICER II dose-dependently suppressed promoter activities of IL-2, NFAT, and AP-1. Moreover, ICER II mRNA expression was transiently induced by stimulation with PMA and ionomycin in normal glucose cultures; however, with high glucose, the induction disappeared.
Conclusion: These results indicate that ICER II protein accumulates during prolonged culture in high glucose and suppresses IL-2 mRNA expression in Jurkat cells.

1. Introduction

Infections and inadequacies in the innate and acquired immune systems are complications of diabetes mellitus [1,2]; however, the mechanisms are still under investigation. Elevated resting values of tumor necrosis factor (TNF)-α [3], interleukin (IL)-6 [4], and IL-8 [5] have been found in diabetic patients compared to nondiabetic controls. On the other hand, after stimulation of peripheral blood mononuclear cells (PBMCs) and monocytes, IL-1 secretion in PBMCs [6] and IL-1 and IL-6 production in monocytes [7], in response to lipopolysaccharide (LPS), were reduced in diabetic patients, while there was no difference in the TNF-α response between the groups. Decreased IL-2 production from mitogen-stimulated mononuclear cells has been reported in patients with type-1 diabetes [8]. IL-2 production is decreased in patients with type-1 but not type-2 diabetes [9]. After mitogen stimulation, TNF-α levels were increase and the percentage of IL-2 receptor positive cells decrease in PBMCs in type-2 diabetes; however, the production of IL-1β, IL-2, and interferon (INF)-γ is not significantly different between diabetic and healthy subjects [10].

In acquired immunity, T cells mediate a complex network of cellular and humoral factors. IL-2 is a potent T cell growth factor and can induce T cell expansion *in vitro*. IL-2 has been used clinically to enhance T cell immunity, and blocking antibodies to the IL-2 receptor are used to suppress T cell response. Nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) are the two major transcription factors in proliferating T lymphocytes implicated as promoters of gene transcription, for genes such as IL-2, IL-4 [11,12], granulocyte-macrophage colony-stimulating factor (GM-CSF) [13], and TNF-α [14]. NFAT proteins form strong cooperative complexes on DNA with the unrelated transcription factor AP-1, composed of Fos/Jun dimers. The strong cooperative binding of NFAT and AP-1 on specific DNA composite sites forms significantly more stable and higher affinity complexes than binding of the individual proteins alone, for example in the expression of IL-2, GM-CSF, IL-3, IL-4, INF-γ, TNF-α, macrophage inflammatory protein (MIP)-1α, and Fas ligand mRNAs [15,16]. Ternary NFAT and Fos/Jun complexes serve as signal integrators for two diametrically different signaling pathways: the calcium and calcinurin pathway that promotes dephosphorylation, nuclear translocation, and activation of NFAT; and the phorbol ester-responsive protein kinase C (PKC) and Ras pathway that promotes the synthesis, phosphorylation, and activation of Fos and Jun family proteins [17].

In this paper, we studied whether the production of immuno-modulating cytokine IL-2 is disturbed in diabetes. Jurkat cells were cultured in a high concentration of glucose and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Prolonged culture in high glucose markedly suppressed transcriptional

* Corresponding author. Tel.: +81 474 72 1481; fax: +81 474 1485.
E-mail address: koji@phar.toho-u.ac.jp (K. Higai).

0304-4165/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2008.10.002

expression of IL-2 mRNA and induced mRNA and protein expression levels of inducible cAMP early repressor (ICER) in Jurkat cells.

2. Experimental procedures

2.1. Cells

Jurkat cells, a human acute T lymphoblastic leukemia cell line, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lanexa, KS), 2.5% sodium bicarbonate, and 1.0% glutamate (Wako Pure Chemical Co., Osaka, Japan) at 37 °C under a humidified atmosphere containing 5% CO_2. RPMI medium originally contains 11.1 mmol/l glucose (referred to hereafter as "normal"). Jurkat cells were also cultured in media containing 22.2 mmol/l glucose (referred to hereafter as "high") and 11.1 mmol/l glucose + 11.1 mmol/l mannitol for 0 to 12 weeks. The media were exchanged every 2 or 3 days.

2.2. RT-PCR for IL-2, GM-CSF, c-Jun, and NFAT

Jurkat cells (1×10^6 cells/ml) were stimulated with 10 nmol/l PMA (Sigma-Aldrich, St Louis, MO) and 500 nmol/l ionomycin (Sigma-Aldrich) for 0 to 24 h at 37 °C. Total cellular RNA was extracted using TRIzol reagent (Invitrogen Co., Carlsbad, CA). First-strand cDNA (20 μl) was prepared from total RNA (5 μg) using ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan) and oligo $(dT)_{20}$ primers followed by DNase treatment according to the manufacture's instructions. cDNA (5 μl) was amplified by PCR on a PC-812 Thermal cycler (Astec Co., Fukuoka, Japan) using KOD Dash (Toyobo) and specific sense and antisense primer sets: 5′-ATGTACAGGATGCAACTCCTGTCTT-3′ and 5′-GTCAGTGTTGAGATGATGCTTTAC-3′ for IL-2 (*NM_000586*), 5′-GAGCA-TGTGAATGCCATCCAGGAG-3′ and 5′-CTCCTGGACTGGCTCCCAGCAGTCA-3′ for GM-CSF (*NM_000758*), 5′-CACCGCCAGGTCGGCAGTATAGTC-3′ and 5′-TTCTTCTCTTGCGTGGCTCT-3′ for c-Jun (*NM_002228*), 5′-ATGA-CCCCACTCTGATCTGC-3′ and 5′-GCCTCTGACCTTGACTGACC-3′ for NFAT (*NM_172390*), and 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTA-GACGGCAGGTCAGGTCCACC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*M_33197*). PCR conditions for IL-2, GM-CSF, c-Jun, and NFAT were 95 °C for 2 min, followed by 25 cycles of 98 °C for 20 s, 60 °C for 5 s, and 72 °C for 30 s, and finally 72 °C for 5 min. PCR for GAPDH was 95 °C for 2 min, followed by 20 cycles of 98 °C for 20 s, 68 °C for 5 s, 72 °C for 30 s, and finally 72 °C for 5 min. PCR products were separated on a 1.5% agarose gel stained with 0.05% ethidium bromide.

2.3. Luciferase reporter gene assay for NFAT, AP-1, and IL-2 promoters

Genomic DNA in Jurkat cells (1×10^6 cells/ml) was extracted with the Wizard SV Genomic DNA preparation system (Promega Co., Madison, WI). IL-2 promoter construct (IL-2p) containing nt −400 to +44 from transcription initiation site was cloned by PCR from genomic DNA using the KOD plus system (Toyobo) and specific primers, 5′-CTTGCTCT-TGTCCACCACAA-3′ (sense) and 5′-GCAAGACAGGAGTTGCATCC-3′ (antisense). PCR products were 5′-phosphorylated using T4 Polynucleotide Kinase (Toyobo) and then ligated into pGL4.11 vector that had been digested with *EcoRV* (Toyobo) and treated with alkaline phosphatase from *E. coli* (Toyobo). The 5′ regions were then sequenced using a 3730×l DNA analyzer (Applied Biosystems, Foster, CA).

IL-2p, NFAT, or AP-1-Luc reporter plasmid (0.25 μg each) were transfected into Jurkat cells (1×10^5 cells) together with TK-Luc (0.1 μg) using DMRIE-C (Invitrogen). 16 h after transfection, Jurkat cells were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 16 h, and luciferase activities were determined by a 20/20n luminometer (Promega) using a Dual Luciferase Assay Kit (Promega). Luciferase activities of reporter plasmids were normalized to those of TK-Luc transfected control cells.

2.4. Western blot for ICER

Jurkat cells (1×10^6 cells/ml) were sonicated for 3s three times on ice. The lysate was centrifuged at 15,000 rpm for 15 min and the supernatant was separated on 10% SDS-PAGE. The proteins were electrophoretically transferred to a Hybond-P PVDF membrane (Amersham Bioscience, Piscataway, NJ). The membrane was blocked with 3% BSA/PBS and washed 3 times with 0.1% Tween 20 in PBS (PBS-T). The membrane was then incubated with goat anti-ICER (Santa Cruz) (IgG, 1:2000) or mouse anti-β-actin (Santa Cruz) antibodies (IgG, 1:1,000) in PBS-T for 1 h at room temperature. After washing 3 times with PBS-T, the membrane was incubated with peroxidase (POD)-conjugated rabbit anti-goat IgG antibody (1:10,000) (Rockland Immunochemicals, Gilbertsville, PA) or POD-conjugated goat anti-mouse IgG+M antibody (1:10,000) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS-T for 1 h at room temperature. The membrane was then washed 3 times with PBS-T and the proteins were incubated with ECL plus (Amersham Bioscience) for 5 min at room temperature and analyzed with a Bioimager Strom™ 830 (Amersham Bioscience). ICER protein levels were normalized to those of β-actin. Protein contents were determined using the advanced protein assay reagent (Cytoskeleton, Denver, CO) with BSA as a standard.

2.5. Construction of ICER II expression plasmids pIRES/ICER II and promoter assay

Human ICER II full-length cDNAs were PCR-amplified from the cDNA of Jurkat cells stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 24 h using the forward and reverse primer set, 5′-CACCATGGCTGTAACTGGAGATGACAC-3′ and 5′-CTAG-TAATCTGTTTTGGAGAA-3′. PCR products were cloned into the pIRES/puro3 vector (Promega) using Ligation High. Recombinant plasmids were transformed into DH5α competent cells and positive clones were confirmed using a Model 3730xl DNA analyzer.

The relevant plasmids were transiently transfected into Jurkat cells using DMRIE-C. IL-2p, NFAT, or AP-1-Luc reporter plasmids (0.25 μg), 0.1 μg of pRL-CMV, and 0 to 250 ng of pIRES/ICER II expression vector (or 250 to 0 ng of IRES/empty) were transfected into Jurkat cells (1×10^5 cells / well) in a 96-well plate. 24 h after transfection, the cells were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 6 h, and firefly and *Renilla* luciferase activities were determined as described above.

2.6. mRNA levels of IL-2 and ICER

Jurkat cells (1×10^6 cells/ml) were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 24 h and RT-PCR for ICER mRNA isoforms was performed as described above using the following primer sets: 5′-CACCATGGCTGTAACTGGAGATGACAC-3′ (sense) and 5′-TTACTCTACTTTATGGCAAT-3′ (antisense) for ICER I and Iγ (*NM_182717,20*), and 5′-CACCATGGCTGTAACTGGAGATGACAC-3′ (sense) and 5′-CTAGTAATCTGTTTTGGAGAA-3′ (antisense) for ICER II and IIγ (*NM_182718,9*). PCR conditions for ICER I, Iγ, II, and IIγ were as follows: 95 °C for 2 min, followed by 30 cycles of 98 °C for 20 s, 55 °C for 5 s, and 72 °C for 30 s For GAPDH, 20 cycles of 98 °C for 20 s, 68 °C for 5 s and 72 °C for 20 s were performed.

IL-2 and ICER II mRNA levels in Jurkat cells were also determined by real-time PCR using SYBR-Green PCR Master Mix (Toyobo) and the ABI Prism 7000 detection system (Applied Biosystems) according to the manufacturer's instructions. PCR conditions for amplification of IL-2, ICER II, and GAPDH were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63 °C for 60 s, and 72 °C for 60 s The

levels of IL-2 and ICER II transcripts were determined for each sample and normalized to GAPDH levels. Specific oligonucleotide primers used were 5′-CAGCCTCAAGATCATCAGCA-3′ (sense) and 5′-ACAGTCTTCTGGGTGG-CAGT-3′ (antisense) for GAPDH, 5′-CTGAACTTGCCCCAAGTCACA-3′ (sense) and 5′-CAGTAGGAGCTCGGATCTGGTAA-3′ (antisense) for ICER II, and 5′-AAGAATCCCAAACTCACCAGGAT-3′ (sense) and 5′-TCTAGACACT-GAAGATGTTTCAGTT-3′ (antisense) for IL-2. Primer sequences were designed using Primer Express software version 2.0.0 (Applied Biosystems).

2.7. Statistical analysis

All experimental data were represented as the mean±SD and analyzed by Student's *t*-test, and statistical significance was defined as a *P* value less than 5%.

3. Results

3.1. IL-2, GM-CSF, c-Jun, and NFAT mRNA expression after stimulation with PMA and ionomycin

To investigate whether IL-2 production is disturbed by high glucose concentrations, Jurkat cells were cultured in normal RPMI medium (11.1 mmol/l glucose), RPMI medium containing high glucose (22.2 mmol/l) and containing mannitol (11.1 mmol/l glucose+11.1 mmol/l mannitol) for 12 weeks at 37 °C (the difference of proliferation speed was not seen between 11.1 mmol/L, 22.2 mmol/L glucose- and 11.1 mmol/l glucose+11.1 mmol/l mannitol -treatment in Jurkat cells) and stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 24 h. Expression levels of IL-2, GM-CSF, c-Jun, and NFAT mRNAs were determined by RT-PCR (Fig. 1). Both IL-2 and GM-CSF mRNA expression levels in cells cultured in normal glucose and glucose+mannitol were markedly enhanced by stimulation with PMA and ionomycin; however, PMA and ionomycin only slightly enhanced IL-2 and GM-CSF mRNA expression in high glucose cultures. The mRNA expression patterns of transcription factors c-Jun and NFAT in high glucose cultures were similar to those in normal glucose, with a transient enhancement of c-Jun mRNA expression at 1 h and a constant expression of NFAT mRNA. Since NFAT and AP-1 regulate transcription of IL-2 and GM-CSF, these results indicate that high glucose suppresses the promoter activities, rather than the expression levels, of NFAT and AP-1. Furthermore, PMA+ionomycin-induced IL-2 mRNA expression suppressed by high glucose was not caused by osmolarity in Jurkat cells.

3.2. Prolonged culture in high glucose suppresses promoter activities of IL-2p, NFAT, and AP-1

IL-2 mRNA expression is regulated by a TATAA box (at −50 nt upstream of the transcription initiation site) and cooperatively enhanced by four NFAT and AP-1 binding sites [12]. To test for suppression of IL-2 mRNA expression by prolonged culture in high glucose, we constructed a luciferase reporter vector containing an IL-2 promoter region (−400 to +44 nt) (IL-2p-Luc), and transfected NFAT, AP-1, or IL-2p-Luc into Jurkat cells cultured in 11.1 (normal) and 22.2 mmol/l (high) glucose for 12 weeks. 16 h after transfection, cells were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 24 h, and luciferase activities for NFAT, AP-1, and IL-2p were determined by dual luciferase assay (Fig. 2).

In normal glucose cultures, the NFAT, AP-1, and IL-2p-Luc promoter activities were enhanced to reach maximum activities at 8 h stimulation and declined thereafter. As expected, in high glucose cultures, all of the promoter activities were extremely low even after 8 h stimulation. These results indicate that prolonged culture in high glucose suppresses promoter activities of NFAT, AP-1, and IL-2p.

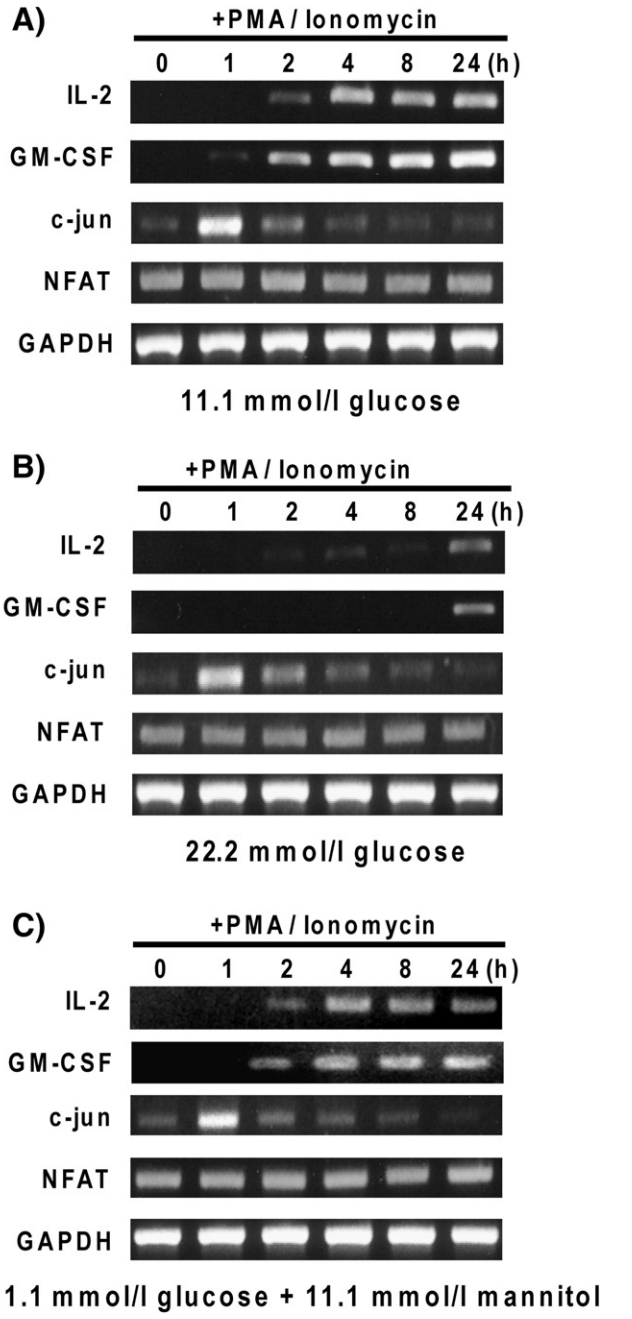

Fig. 1. IL-2, GM-CSF, c-Jun and NFAT mRNA expression induced by stimulation with PMA and ionomycin. Jurkat cells cultured in (A) 11.1 or (B) 22.2 mmol/l glucose (C) 11.1 mmol/l glucose+11.1 mmol/l mannitol for 12 weeks were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 24 h. IL-2, GM-CSF, c-Jun, and NFAT mRNA expression levels were determined by RT-PCR.

3.3. Promoter activities of NFAT, AP-1, and IL-2p during high glucose culture

To investigate the effects of the duration of high glucose cultures on IL-2 mRNA expression, we cultured Jurkat cells in 11.1 (normal) and 22.2 mmol/l (high) glucose for 0 to 12 weeks at 37 °C and determined the promoter activities of NFAT, AP-1, and IL-2p-Luc induced by 6 h stimulation with PMA and ionomycin as described above (Fig. 3A). In high glucose cultures, the promoter activities of NFAT, AP-1, and IL-2p were suppressed time-dependently to approximately 20% of those in normal glucose cultures by 12 weeks.

After culturing in high glucose for 12 weeks, cells were further incubated in normal glucose for 0 to 5 weeks, and the promoter activities of NFAT, AP-1, and IL-2p were measured (Fig. 3B). NFAT and IL-2p

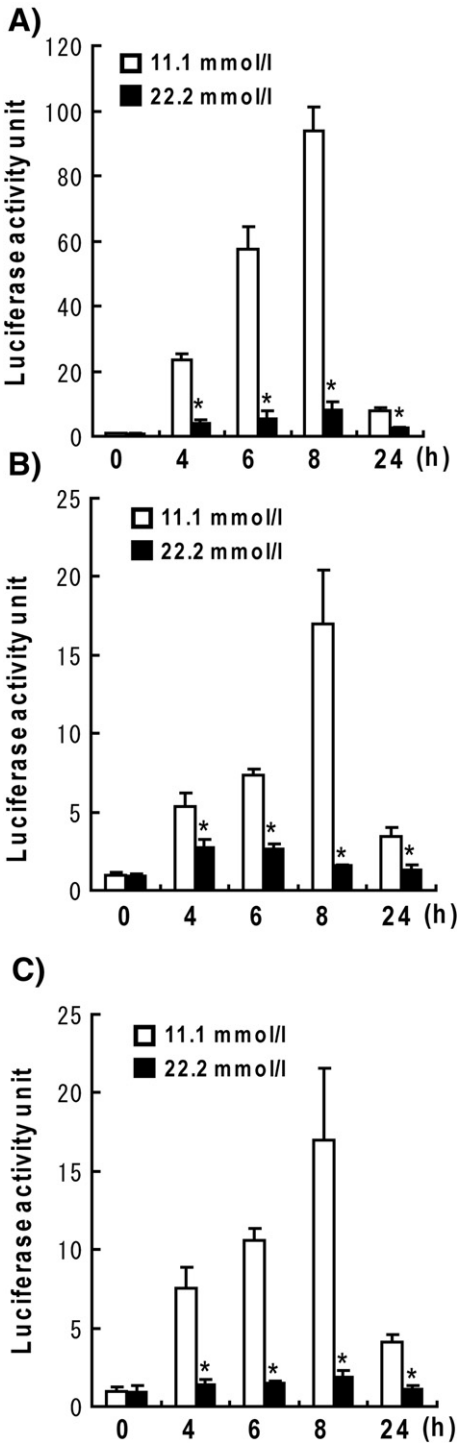

Fig. 2. NFAT, AP-1, and IL-2p promoter activities induced by stimulation with PMA and ionomycin after 12 weeks of culture in high glucose. Jurkat cells were incubated in 11.1 (open bars) and 22.2 (closed bars) mmol/l glucose. Reporter vectors of (A) NFAT, (B) AP-1, or (C) IL-2p-Luc (0.25 μg each) were co-transfected with control vector TK-Luc (0.1 μg) into Jurkat cells. 16 h after transfection, cells were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin. Luciferase activities were measured 0 to 24 h post-stimulation and were normalized to TK-Luc. Results were obtained by 3 independent experiments and are represented as mean±SD. Asterisks indicate significant differences ($P<0.05$) compared with 11.1 mmol/l glucose cultures at the same time points.

activities almost recovered to levels measured in normal glucose cultures after 5 weeks; AP-1 activity however still remained low even after 5 weeks. These results indicate that suppression of IL-2 gene transcription enhanced by stimulation with PMA and ionomycin is chronically induced, but not regulated, by direct signaling transduction of high glucose.

3.4. Prolonged culture in high glucose enhances ICER mRNA and protein expression

ICER is well known to compete with NFAT and AP-1 binding sites and suppress their promoter activities. To determine if prolonged

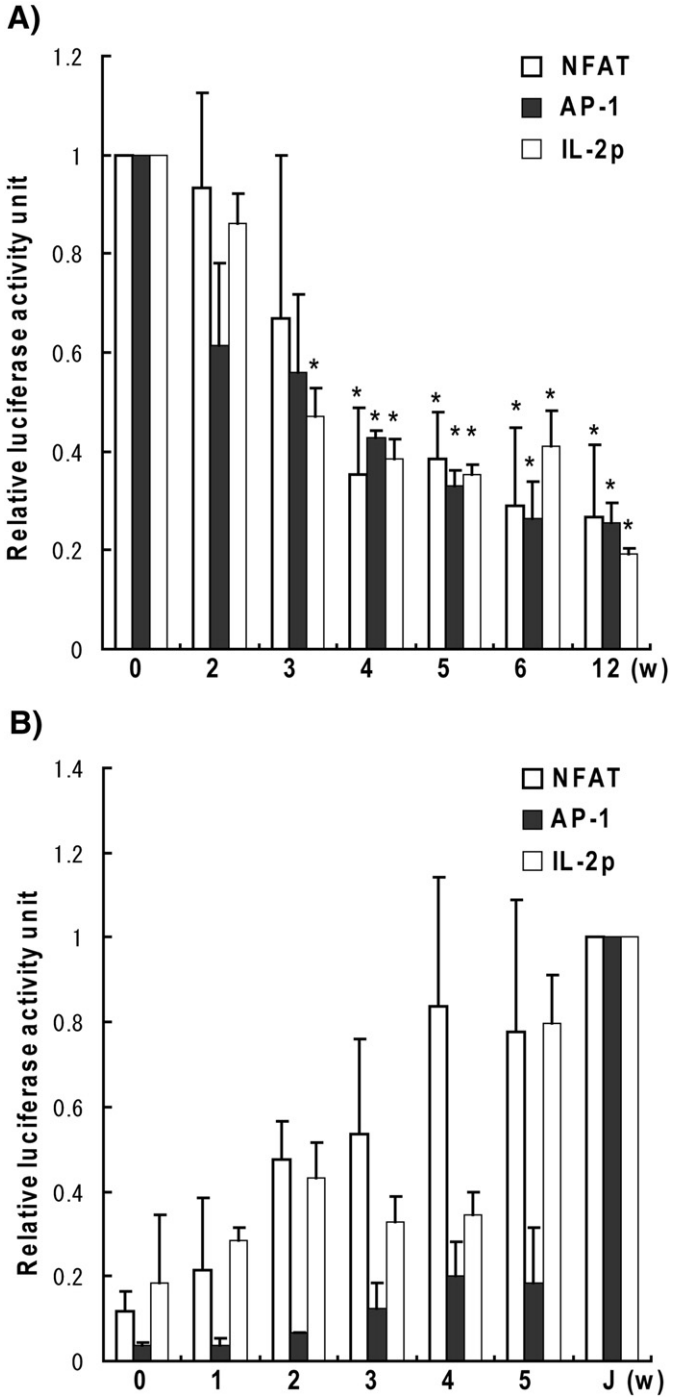

Fig. 3. Duration of high glucose culture and promoter activities of NFAT, AP-1, and IL-2p. (A) Jurkat cells were cultured in 11.1 and 22.2 mmol/l glucose for 0 to 12 weeks and transfected with NFAT (open bars), AP-1 (closed bars), or IL-2p-Luc (gray bars) and TK-Luc. 16 h after transfection, cells were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin. Luciferase activities were determined after 6 h stimulation and normalized to those of 11.1 mmol/l glucose cultures. Asterisks indicate significant differences ($P<0.05$) compared with 11.1 mmol/l cultures (0 week). (B) After being cultured in 22.2 mmol/l glucose for 12 weeks, cells were further cultured in 11.1 mmol/l glucose for 0 to 5 weeks. The results were obtained by 3 independent experiments and are represented as mean±SD. J: Jurkat cells cultured in 11.1 mmol/l glucose.

culture in high glucose induces ICER II mRNA and subsequent protein expression levels, we measured ICER mRNA and protein levels in 0 to 12 week normal and high glucose cultures. As shown in Fig. 4A, RT-PCR revealed that Jurkat cells prominently expressed ICER II and IIγ, but not ICER I and Iγ. ICER II and IIγ mRNA expression levels were further measured by real-time PCR, revealing that their expression levels were time-dependently enhanced and doubled by 4 weeks of culture in high glucose compared to normal glucose (Fig. 4B). ICER protein expression levels as determined by Western blot analysis also time-dependently increased in high glucose cultures, doubling by 4 weeks compared to normal glucose (Fig. 4CD). These results indicate that prolonged culture in high glucose enhances ICER II mRNA expression and accumulates ICER protein, which inhibits the promoter activities of NFAT and AP-1, thus suppressing IL-2 mRNA expression.

3.5. Promoter activities of NFAT, AP-1, and IL-2p in Jurkat cells transfected with ICER II expression vector

To clarify whether ectopically transfected ICER II can transcriptionally suppress IL-2 mRNA expression, we co-transfected an ICER II expression vector and luciferase reporter vectors NFAT, AP-1, or IL-2p-Luc together with TK-Luc into Jurkat cells. 16 h after transfection, cultures with normal levels of glucose were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 6 h, and promoter activities were determined by dual luciferase assay and compared to those in empty vector pIRES/empty-transfected Jurkat cells cultured with normal glucose. Transfection of ICER II expression vector suppressed transfection dose-dependently for all three promoter activities (Fig.5). These results indicate that ICER II specifically suppresses the promoter activities of NFAT, AP-1, and IL-2p.

3.6. Prolonged culture in high glucose suppresses PMA and ionomycin-induced ICER II and IIγ mRNA expression

To clarify whether ICER II and IIγ mRNA expression induced by stimulation with PMA and ionomycin is suppressed in prolonged high glucose culture, we cultured Jurkat cells in high and normal levels of glucose for 12 weeks and determined IL-2 and ICER II and IIγ mRNA expression levels after 0 to 24 h stimulation with 10 nmol/l PMA and 500 nmol/l ionomycin (Fig. 6). IL-2 (Fig. 6A) and ICER (Fig. 6B) mRNA levels in normal glucose cultures were markedly enhanced by stimulation with PMA and ionomycin. On the other hand, in high glucose cultures, basal ICER II and IIγ mRNA levels were high, but were not enhanced by stimulation with PMA and ionomycin. The results reveal that prolonged high glucose

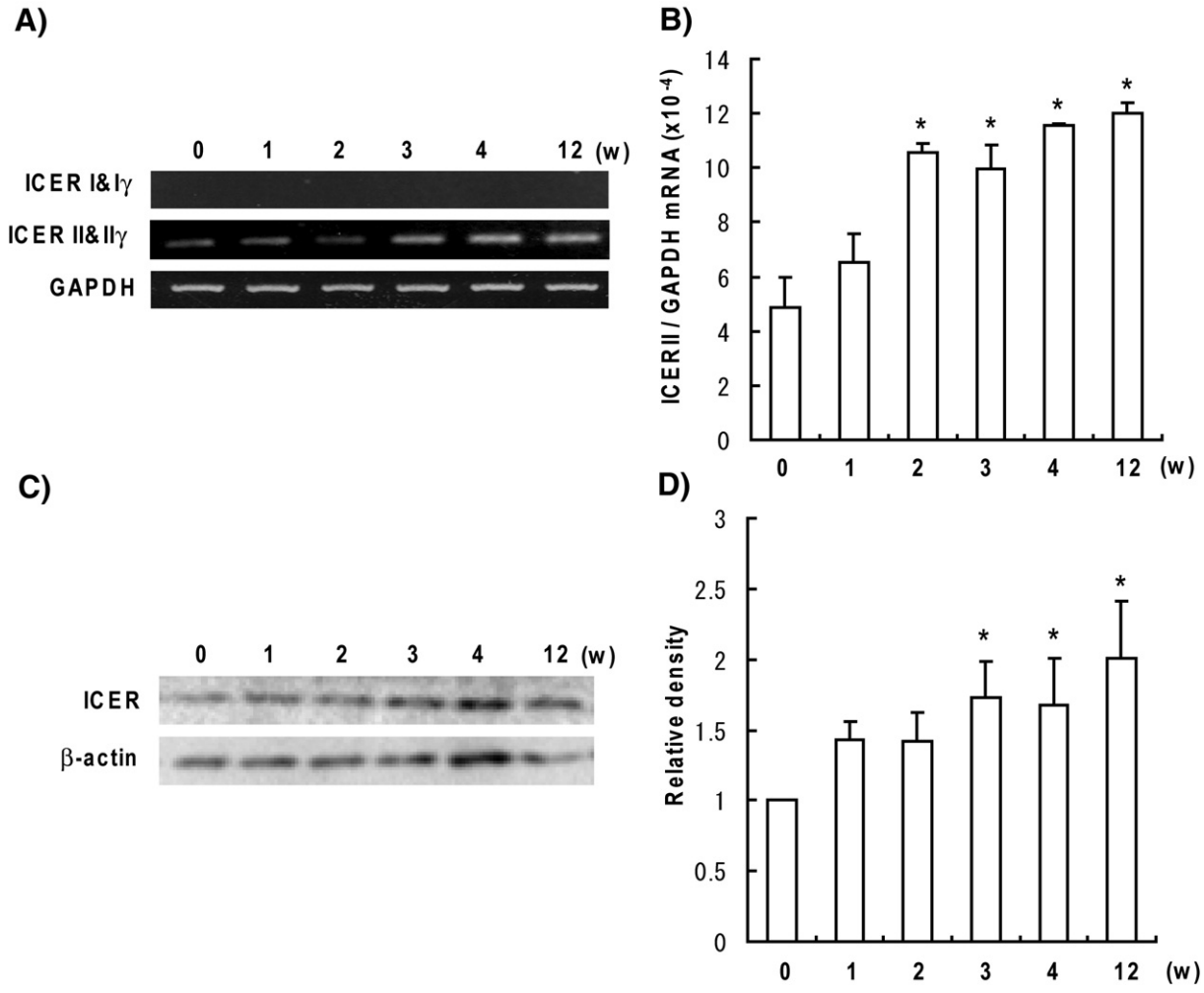

Fig. 4. ICER II mRNA and protein expression in Jurkat cells cultured in high glucose. Jurkat cells were cultured in 11.1 and 22.2 mmol/l glucose for 12 weeks. (A and B) ICER II and IIγ mRNA expression levels determined by RT- and real-time PCR. (C) ICER protein in the cells determined by Western blotting. Proteins were separated by 15% SDS-PAGE and were transferred to a membrane. ICER and β-actin protein levels were determined using anti-ICER and anti-β-actin and POD-conjugated secondary antibodies. (D) Densitometry analysis of ICER protein. Each value is shown relative to that of Jurkat cells cultured in normal glucose. Results were obtained by 3 independent experiments and represent mean±SD normalized to β-actin. Asterisks indicate significant differences compared with normal glucose cultures ($P<0.05$).

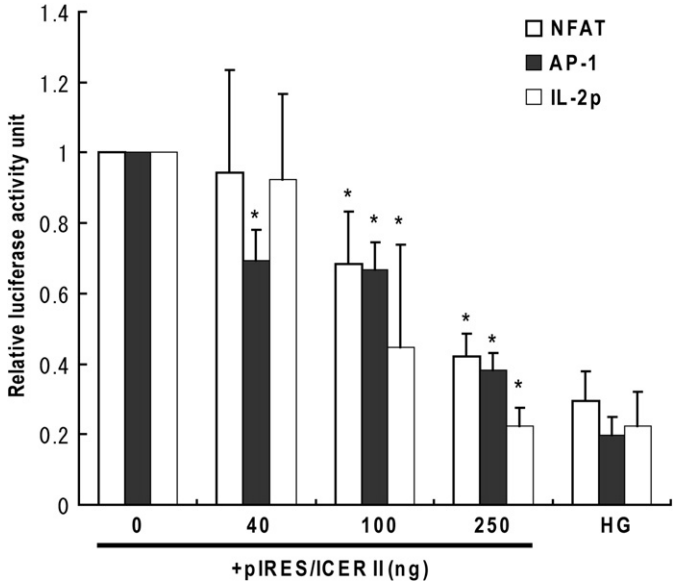

Fig. 5. Promoter activities of NFAT, AP-1, and IL-2p in ICER II expression vector-transfected Jurkat cells. After 16 h co-transfection with ICER II and IIγ expression vector (0, 40, 100, and 250 ng) and reporter genes for NFAT, AP-1, and IL-2p-Luc (0.25 μg each) together with TK-Luc (0.1 μg) into Jurkat cells cultured in 11.1 mmol/l glucose, cells were stimulated with PMA and ionomycin for 4 h, NFAT (open bars), AP-1 (closed bars), or IL-2p-Luc (gray bars). Promoter activities were determined by dual luciferase assay. Activities were normalized to TK-Luc and compared to those in empty vector-transfected cells. Results were obtained by 3 independent experiments and represent mean ± SD. Asterisks indicate significant differences compared with normal glucose cultures ($P<0.05$). (HG): 12 week high glucose cultures.

culture suppresses PMA and ionomycin-induced ICER mRNA expression and auto-repression.

4. Discussion

In this study we investigated the mechanism of the immune response disturbance in diabetes mellitus. T cells produce and secrete various cytokines by activation through the interaction of antigens with T cell receptors and play a central role in acquired immunity. We measured IL-2 mRNA expression induced by stimulation with PMA and ionomycin in Jurkat cells cultured in high concentrations of glucose (22.2 mmol/l) compared to that of RPMI media with normal concentrations (11.1 mmol/l) and with osmolarity control (11.1 mmol/l glucose + 11.1 mmol/l mannitol). The results indicate that prolonged culture (12 weeks) in high glucose suppresses IL-2 mRNA expression by suppression of the promoter activities of NFAT, AP-1, and IL-2p.

Furthermore, prolonged high glucose culture induced an increase in ICER II (a strong repressor of NFAT and AP-1) mRNA and protein expression levels, and negated ICER gene expression induced by stimulation with PMA and ionomycin.

Transcription of IL-2 and GM-CSF genes is mainly regulated by NFAT and AP-1. Various factors modulate translocation and activation of these factors. Inhibition of AMP-activated protein kinase (AMPK) suppresses IL-2 mRNA expression induced by stimulation with PMA and ionomycin through suppression of transcriptional activation of NFAT and AP-1, but not NF-κB in Jurkat cells [18]. The combined increase in protein kinase A (PKA) and decrease in PKCθ activities leads to enhanced inhibition of nuclear NFAT translocation; however, this crosstalk affects neither the NF-κB, AP-1, nor the cAMP responsive elements (CRE) binding protein (CREB) pathway [19].

High glucose is known to enhance the hexosamine biosynthesis pathway, and the addition of O-linked N-acetylglucosamine (O-GlcNAc) to serine or threonine residues of nuclear and cytoplasmic proteins is ubiquitous and reversible. Protein O-GlcNAcylation is accelerated by accumulated UDP-GlcNAc and protein O-GlcNAc transferase (OGT), and O-GlcNAc is removed by O-GlcNAc selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase) [20]. Activation of the hexosamine biosynthesis pathway by the addition of glucosamine suppresses NFAT trafficking to the nucleus to reduce IL-2 production in Jurkat cells in response to phytohemagglutinin (PHA) [21]. The calcium and calcinurin signaling pathway dephosphorylates serine residues in the amino terminus of NFAT, resulting in nuclear import of NFAT, whereas PKA and glycogen synthase kinase (GSK)-3 phosphorylate NFAT for export from the nucleus [22].

In a previous study, we reported that human hepatocellular HuH-7 cells cultured in high glucose (33 mmol/l) or treated with GlcNAcase inhibitor O-(2-acetoamide-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) for 24 h had enhanced nuclear transport of GlcNAcylated CREB, suppressed CRE, and enhanced AP-1 promoter activity [23]. In this study, prolonged high glucose culture

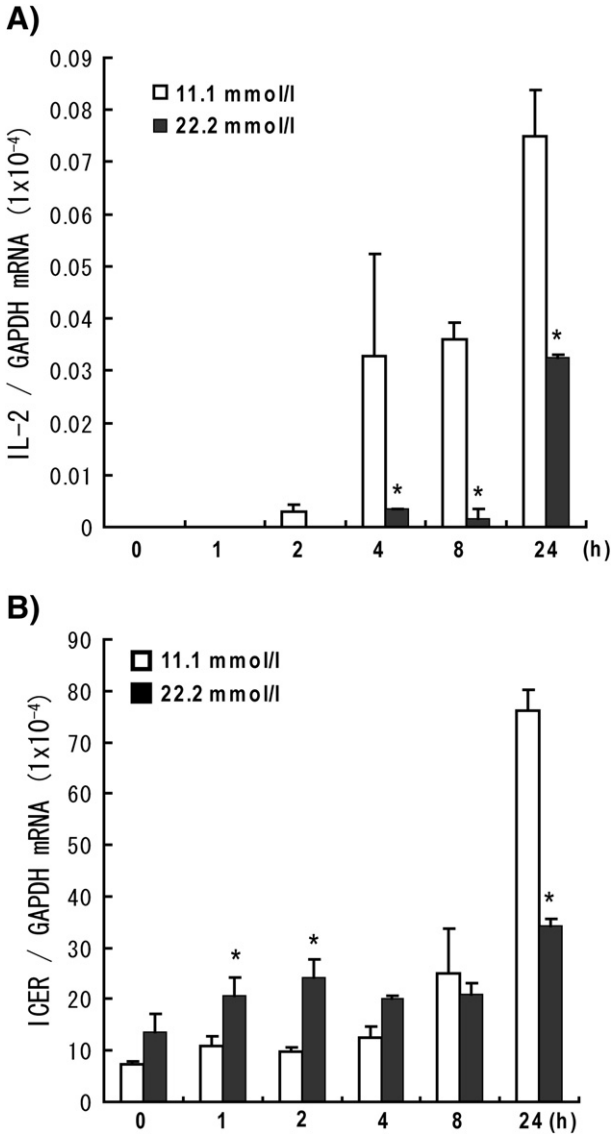

Fig. 6. IL-2 and ICER II mRNA expression after stimulation with PMA and ionomycin. Jurkat cells cultured in 11.1 (open bars) and 22.2 (closed bars) mmol/l glucose for 12 weeks were stimulated with 10 nmol/l PMA and 500 nmol/l ionomycin for 0 to 24 h. IL-2 (A) and ICER II (B) mRNA expression levels were determined by real-time PCR using SYBR-Green and specific primers, and normalized to GAPDH mRNA levels. Data from 3 independent experiments are included at each time point; error bars represent SD of each data set. Asterisks (*) indicate significant difference ($P<0.05$) compared with normal glucose cultures.

also suppressed CRE promoter activity (data not shown) but suppressed AP-1 promoter activity. Contradictory results for the AP-1 promoter activity may have been caused by the duration of high glucose culture and cell types used. This is currently under investigation.

Prolonged culture in high glucose in this study did not affect c-Jun and NFAT mRNA expression levels in response to stimulation with PMA and ionomycin (Fig. 1). Suppression of the promoter activities of NFAT, AP-1, and IL-2p in high glucose cultures was induced chronically and recovered gradually in normal glucose cultures (Figs. 2 and 3). Furthermore, stimulation with PMA and ionomycin did not cause a difference between NFAT translocation into the nucleus, nor binding of NFAT and AP-1 to their specific DNA probes, in normal and high glucose cultures (Data not shown).

We also estimated ICER mRNA and protein levels in high glucose cultures. ICER is a powerful transcriptional repressor and plays an important role in the regulation of the cyclic AMP-dependent transcriptional response. In the well-characterized type 2 diabetes Goto–Hakozaki rat model, ICER levels are increased, and the expression of insulin mRNA, whose gene transcription is modulated by CREs, is decreased [24]. Up-regulation of ICER by either free fatty acid or high glucose deteriorates β-cell functions in type 2 diabetes by suppressing insulin gene transcription [25]. ICER induced by incubation of the rat pancreatic β-cell line INS-1E in high glucose diminishes the expression of the Rab GTPases Rab3a and Rab27a, and their effectors Granuphilin and Noc2, by binding to CRE located on the promoters of these genes and inhibiting insulin exocytosis [26]. Transgenic mice with β-cell directed expression of ICER acquire early severe diabetes [27]. However, the mechanism by which ICER is induced by incubation in high glucose remains unclear.

In the immune system, ICER serves as a repressor of T cell proliferation and effector functions acting through the inhibition of carcineulin-mediated IL-2 expression [28,29]. ICER is transcribed via an alternative internal promoter in the CRE modulator (CREM) gene and consists of four different isoforms generated by alternative splicing of its transcript: ICER I, Iγ, II, and IIγ [30]. ICER binds to a variety of CREs and suppresses transcriptional activities of CREB, CREM, activating transcription factors (ATFs), and Fos and Jun families. It lacks the transactivation domain of CREM required for recruitment of CREB binding protein (CBP)/p300 but retains the DNA binding domains.

Among the four isoforms, ICER II and IIγ are predominantly expressed in Jurkat cells. We measured ICER II and IIγ mRNA expression by real-time PCR and ICER protein expression by Western blotting, which indicated that prolonged culture in high glucose enhances ICER II and IIγ mRNA and leads to the accumulation of ICER protein (Fig. 4). Furthermore, ectopically transfected ICER II and IIγ expression vectors suppressed the promoter activities of NFAT, AP-1, and IL-2p dose-dependently (Fig. 5).

ICER activity is primarily determined by its intracellular concentration rather than by post-transcriptional modifications such as phosphorylation. Intracellular levels of ICER are controlled by the ubiquitin–proteasome pathway for protein breakdown [31]. Activation of the mitogen-activated protein kinase (MAPK) pathway increases ICER phosphorylation followed by degradation by the ubiquitin–proteasome pathway, and cAMP stabilizes ICER protein levels by inhibiting the MAPK cascade [32]. Protein O-GlcNAcylation modifies signal transduction, transcriptional activities, and proteasome-dependent degradation of proteins [33]. Modification of proteins with O-GlcNAc inhibits ubiquitination. The ubiquitin-dependent proteasome is also O-GlcNAcylated, and the activities of the proteasome are inhibited by O-GlcNAcylation [34]. We measured proteasome activities using three kinds of substrates; however, the suppression of proteasome activity in high glucose cultured Jurkat cells was not significant compared to that of normal glucose cultures (data not shown).

Expression of ICER mRNA was transiently enhanced by stimulation of PMA and ionomycin in Jurkat cells cultured in normal glucose; however, in high glucose cultures, PMA and ionomycin-induced enhancement of ICER mRNA expression disappeared (Fig. 6). Prolonged high glucose disturbed ICER gene transcription and the autoregulatory loop.

Transcription of the ICER gene is regulated by four CRE-binding domains and repressed by an autoregulatory loop [35]. Once expressed, ICER competes with CREB for binding to CRE-like DNA binding motifs, including ICER's own cAMP-autoregulatory elements (CAREs) in the P2 promoter, eventually leading to its own down-regulation. Treatment with forskolin, an activator of adenylate cyclase, induces transcription of the ICER gene through two CRE-like elements, CARE 3 and 4 [36], while calcium inhibits the responsiveness of the ICER promoter to cAMP-activated CREB [37]. Multiple NFAT and AP-1 binding sites have been reported in IL-2 and GM-CSF promoter regions, and CRE-binding sites have been found in the P2 promoter region of ICER. Homologies and binding affinities of these ICER binding sites in NFAT and AP-1 in IL-2 and GM-CSF promoter regions and CRE in ICER promoter regions will influence the repressive and transcriptional activities of these genes. Further investigation is necessary to clarify the mechanism by which ICER mRNA levels are enhanced and ICER protein accumulates during prolonged high glucose culture.

In conclusion, prolonged culture in high glucose enhances ICER II mRNA expression, and accumulated ICER protein suppresses IL-2 mRNA expression in Jurkat cells.

Acknowledgements

This research was partially supported by the “Open Research Center” Project.

References

[1] N. Joshi, G.M. Caputo, M.R. Weitekamp, A.W. Karchmer, Infections in patients with diabetes mellitus, N. Engl. J. Med. 341 (1999) 1906–1912.
[2] A.Y. Peleg, T. Weererethna, J.S. McCarthy, T.M.E. Davis, Common infections in diabetes: pathogenesis, management and relationship to glycaenic control, Diabetes Metab. Res. Rev. 23 (2007) 3–13 (Review).
[3] J. Myśliwska, K. Zorena, A. Bakowska, A. Skuratowicz-Kubica, A. Myśliwski, Significance of tumor necrosis factor α in patients with long-standing type-I diabetes mellitus, Horm. Metab. Res. 30 (1998) 158–161.
[4] J.C. Pickup, M.A. Crook, Is type II diabetes mellitus a disease of the innate immune system? Diabetologia 41 (1998) 1241–1248.
[5] D. Zozuliñska, A. Majchrzak, M. Sobieska, K. Wiktorowicz, B. Wierusz-Wysocka, Serum interleukin-8 level is increased in diabetic patients, Diabetologia 42 (1999) 117–118.
[6] A.D. Mooradian, R.L. Reed, K.E. Meredith, P. Scuderi, Serum levels of tumor necrosis factor and IL-1α and IL-1β in diabetic patients, Diabetes Care 14 (1991) 63–65.
[7] Y. Ohno, N. Aoki, A. Nishimura, In vitro production of interleukin-1, interleukin-6, and tumor necrosis factor-α in insulin-dependent diabetes mellitus, J. Clin. Endocrinol. Metab. 77 (1993) 1072–1077.
[8] U. Shah, L. Karch, L. Barker, K.S. Zier, Low interleukin 2 synthesis by type 1 diabetes is regulated at pretranslational level, Clin. Immunol. Immunopathol. 61 (1991) 177–190.
[9] W.A. Kaye, M.N. Adri, J.S. Soeldner, S.L. Rabinowe, A. Kaldany, C.R. Kahn, B. Bistrian, S. Srikanta, O.P. Ganda, G.S. Eisenbarth, Acquired defect in interleukin-2 production in patients with type I diabetes mellitus, N. Engl. J. Med. 315 (1986) 920–924.
[10] F.Y. Chang, M.F. Shaio, Decreased cell-mediated immunity in patients with non-insulin-dependent diabetes mellitus, Diabetes Res. Clin. Pract. 28 (1995) 137–146.
[11] J.P. Northrop, S.N. Ho, L. Chen, D.J. Thomas, L.A. Timmerman, G.P. Nolan, A. Admon, G.R. Crabtree, NF-AT components define a family of transcription factors targeted in T-cell activation, Nature 369 (1994) 497–502 (6480).
[12] M.B. Wisniewska, M. Ameyar-Zazoua, L. Bakiri, B. Kaminska, M. Yaniv, J.B. Weitzman, Dimer composition and promoter context contribute to functional cooperation between AP-1 and NFAT, J. Mol. Biol. 371 (2007) 569–576.
[13] B.V. Johnson, A.G. Bert, G.R. Ryan, A. Condina, P.N. Cockerill, Granulocyte-macrophage colony-stimulating factor enhancer activation requires cooperation between NFAT and AP-1 elements and is associated with extensive nucleosome reorganization, Mol. Cell Biol. 24 (2004) 7914–7930.
[14] J.H. Oum, J. Han, H. Myung, M. Hleb, S. Sharma, J. Park, Molecular mechanism of NFAT family proteins for differential regulation of the IL-2 and TNF-α promoters, Mol. Cells 13 (2002) 77–84.
[15] A. Rao, C. Luo, P.G. Hogan, Transcription factors of the NFAT family: regulation and function, Annu. Rev. Immunol. 15 (1997) 707–747 (Review).

[16] F. Macián, C. García-Rodríguez, A. Rao, Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun, EMBO J. 19 (2000) 4783–4795.
[17] F. Macián, C. López-Rodríguez, A. Rao, Partners in transcription: NFAT and AP-1, Oncogene 20 (2001) 2476–2489 (Review).
[18] B.S. Jhun, J.Y. Lee, Y.T. Oh, J.H. Lee, W. Choe, H.H. Baik, S.S. Kim, K.S. Yoon, J. Ha, I. Kang, Inhibition of AMP-activated protein kinase suppresses IL-2 expression through down-regulation of NF-AT and AP-1 activation in Jurkat T cells, Biochem. Biophys. Res. Commun. 351 (2006) 986–992.
[19] N. Hermann-Kleiter, N. Thuille, C. Pfeifhofer, T. Gruber, M. Schäfer, C. Zitt, A. Hatzelmann, C. Schudt, M. Leitges, G. Baier, PKCθ and PKA are antagonistic partners in the NF-AT transactivation pathway of primary mouse $CD3^+$ T lymphocytes, Blood. 107 (2006) 4841–4848.
[20] N.E. Zachara, G.W. Hart, Cell signaling, the essential role of O-GlcNAc, Biochim. Biophys. Acta 1761 (2006) 599–617 (Review).
[21] J.B. Huang, A.J. Clark, H.R. Petty, The hexosamine biosynthesis pathway negatively regulates IL-2 production by Jurkat T cells, Cell Immunol. 245 (2007) 1–6.
[22] C.R. Beals, C.M. Sheridan, C.W. Turck, P. Gardner, G.R. Crabtree, Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3, Science 275 (5308) (1997) 1930–1934.
[23] Y. Azuma, K. Miura, K. Higai, K. Matsumoto, Protein O-N-acetylglucosaminylation modulates promoter activities of cyclic AMP response element and activator protein 1 and enhances E-selecting expression on HuH-7 human hepatoma cells, Biol. Pharm. Bull. 30 (2007) 2284–2289.
[24] A. Inada, Y. Yamada, Y. Someya, A. Kubota, K. Yasuda, Y. Ihara, S. Kagimoto, A. Kuroe, K. Tsuda, Y. Seino, Transcriptional repressors are increased in pancreatic islets of type 2 diabetic rats, Biochem. Biophys. Res. Commun. 253 (1998) 712–718.
[25] Y.P. Zhou, K. Marlen, J.F. Palma, A. Schweitzer, L. Reilly, F.M. Gregoire, G.G. Xu, J.E. Blume, J.D. Johnson, Overexpression of repressive cAMP response element modulators in high glucose and fatty acid-treated rat islets. A common mechanism for glucose toxicity and lipotoxicity? J. Biol. Chem. 278 (2003) 51316–51323.
[26] A. Abderrahmani, S. Cheviet, M. Ferdaoussi, T. Coppola, G. Waeber, R. Regazzi, ICER induced by hyperglycemia represses the expression of genes essential for insulin exocytosis, EMBO J. 25 (2006) 977–986.
[27] A. Inada, Y. Hamamoto, Y. Tsuura, J. Miyazaki, S. Toyokuni, Y. Ihara, K. Nagai, Y. Yamada, S. Bonner-Weir, Y. Seino, Overexpression of inducible cyclic AMP early repressor inhibits transactivation of genes and cell proliferation in pancreatic beta cells, Mol. Cell Biol. 24 (2004) 2831–2841.
[28] J. Bodor, A.L. Spetz, J.L. Strominger, J.F. Habener, cAMP inducibility of transcriptional repressor ICER in developing and mature human T lymphocytes, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 3536–3541.
[29] J. Bodor, J.F. Habener, Role of transcriptional repressor ICER in cyclic AMP-mediated attenuation of cytokine gene expression in human thymocytes, J. Biol. Chem. 273 (1998) 9544–9551.
[30] J. Bodor, J. Bodorova, R.E. Gress, Suppression of T cell function: a potential role for transcriptional repressor ICER, J. Leukoc. Biol. 67 (2000) 774–779 (Review).
[31] E.J. Folco, G. Koren, Degradation of the inducible cAMP early repressor (ICER) by the ubiquitin–proteasome pathway, Biochem. J. 328 (1997) 37–43.
[32] G. Yehia, F. Schlotter, R. Razavi, A. Alessandrini, C.A. Molina, Mitogen-activated protein kinase phosphorylates and targets inducible cAMP early repressor to ubiquitin-mediated destruction, J. Biol. Chem. 276 (2001) 35272–35279.
[33] J.E. Kudlow, Post-translational modification by O-GlcNAc: another way to change protein function, J. Cell. Biochem. 98 (2006) 1062–1075.
[34] F. Zhang, K. Su, X. Yang, D.B. Bowe, A.J. Paterson, J.E. Kudlow, O-GlcNAc modification is an endogenous inhibitor of the proteasome, Cell 115 (2003) 715–725.
[35] C.A. Molina, N.S. Foulkes, E. Lalli, P. Sassone-Corsi, Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor, Cell 75 (1993) 875–886.
[36] D. Mao, E.A. Warner, S.A. Gurwitch, D.R. Dowd, Differential regulation and transcriptional control of immediate early gene expression in forskolin-treated WEHI7.2 thymoma cells, Mol. Endocrinol. 12 (1998) 492–503.
[37] D.A. Krueger, D. Mao, E.A. Warner, D.R. Dowd, Functional analysis of the mouse ICER (inducible cAMP early repressor) promoter: evidence for a protein that blocks calcium responsiveness of the CAREs (cAMP autoregulatory elements), Mol. Endocrinol. 13 (1999) 1207–1217.