Involvement of c-Jun N-terminal Kinase in Oxidative Stress-mediated Suppression of Insulin Gene Expression *

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Running title: Oxidative Stress and JNK Pathway in Pancreatic β-Cells
ABSTRACT

Oxidative stress, which is found in pancreatic β-cells in the diabetic state, suppresses insulin gene transcription and secretion, but the signaling pathways involved in the β-cell dysfunction induced by oxidative stress remain unknown. In this study, subjecting rat islets to oxidative stress activates c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK), and protein kinase C (PKC), preceding the decrease of insulin gene expression. Adenovirus-mediated overexpression of dominant-negative type (DN) JNK, but not the p38 MAPK inhibitor SB203580 nor the PKC inhibitor GF109203X, protected insulin gene expression and secretion from oxidative stress. Moreover, wild type (WT) JNK overexpression suppressed both insulin gene expression and secretion. These results were correlated with changes in the binding of the important transcription factor PDX-1 to the insulin promoter; adenoviral overexpression of DN-JNK preserved PDX-1 DNA binding activity in the face of oxidative stress, while WT-JNK overexpression decreased PDX-1 DNA binding activity. Furthermore, to examine whether suppression of the JNK pathway can protect β-cells from the toxic effects of hyperglycemia, rat islets were infected with DN-JNK expressing adenovirus or control adenovirus and transplanted under renal capsules of streptozotocin-induced diabetic nude mice. In mice receiving DN-JNK overexpressing islets, insulin gene expression in islet grafts was preserved and hyperglycemia was ameliorated compared with control mice. In conclusion, activation of JNK is involved in the reduction of insulin gene expression by oxidative stress and suppression of the JNK pathway protects β-cells from oxidative stress.
INTRODUCTION

Some of the β-cell failure that is fundamental to diabetes appears to be due to the adverse effects of chronic hyperglycemia on β-cells, a process called glucose toxicity, which leads to suppression of insulin gene transcription and glucose-stimulated insulin secretion (1-7). The reduction of expression or DNA binding activity of the pancreatic and duodenal homeobox factor-1 (PDX-1) (also known as IDX-1/STF-1/IPF1) (8-12) is often observed simultaneously with suppression of insulin gene transcription (3, 4, 7). PDX-1, a member of the homeodomain family of transcription factors, plays an important role in pancreas development (13-16) and differentiation (17-20) and in maintaining normal β-cell function by regulating the expression of multiple genes, including insulin, GLUT2 and glucokinase (21-24).

It has been postulated that oxidative stress (25) which is found in the diabetic state (26-28) is involved in the progression of β-cell deterioration (29-35) as well as in the development of diabetic complications (27, 36-38). In fact, in the presence of diabetes, reactive oxygen species (ROS) are produced in islets (26) as well as in various tissues (27, 28) through several processes such as the non-enzymatic glycosylation reaction (37, 39, 40), the electron transport chain in mitochondria (27), and the hexosamine pathway (35). Furthermore, it has been reported that levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker for oxidative stress, are increased in the blood of type 2 diabetic patients (41, 42). Since expression levels of the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase, are relatively low in islets as compared with other tissues (43), β-cells can be expected to be vulnerable to oxidative stress. Indeed, studies have shown that oxidative stress exerts deleterious effects upon β-cells in the diabetic state, suppressing insulin gene transcription and glucose-stimulated insulin secretion, and even producing apoptosis (29-35). Expression and DNA binding activity of PDX-1 are also suppressed by oxidative stress (30, 32, 33, 35). Furthermore, some toxic effects of hyperglycemia on β-cells in rodent models are reduced by antioxidant treatment (32-34). Thus, it is likely that oxidative stress mediates some of the toxic effects of hyperglycemia.
Several signal transduction pathways including c-Jun N-terminal kinase (JNK) (also known as stress-activated protein kinase (SAPK)) (44, 45), p38 mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) are known to be activated by oxidative stress (25, 46) or high glucose (46-48) in several cell types. However, it is not known which of these kinases is activated in pancreatic islets and involved in oxidative stress-mediated suppression of insulin gene transcription. In this study, we show that activation of JNK is involved in reduction of insulin gene expression by oxidative stress and that suppression of the JNK pathway can protect β-cells from oxidative stress.
MATERIALS AND METHODS

Isolation and culture of rat pancreatic islets.
Islets were isolated from pancreases of 200-250 g male Sprague-Dawley (SD) rats (Taconic Farms, Germantown, NY) with collagenase digestion. The common bile duct was cannulated and injected with 6 ml of cold M199 medium containing 1.5 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN). The islets were separated on a Histopaque 1077 (Sigma, St. Louis, MO) density gradient. The washed islets were hand-picked individually under a dissecting microscope to ensure a pure islet preparation and cultured in RPMI 1640 medium (11 mM glucose, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO2 at 37 °C. All animal procedures were approved by the Animal Care Committee of the Joslin Diabetes Center.

Preparation of recombinant adenoviruses expressing wild type (WT) and dominant-negative type (DN) JNK.
Recombinant adenoviruses expressing WT- and DN-JNK (44, 45) were prepared using the AdEasy system (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) (49). It should be noted that this DN-JNK is a kinase-dead mutant (the ATP-binding site is mutated); this DN-JNK can be phosphorylated but cannot phosphorylate c-Jun. In brief, the encoding region of WT- and DN-JNK (K55R) was cloned into the XbaI-EcoRV site of a shuttle vector pAdTrack-CMV. To produce homologous recombination, 1.0 µg of linearized plasmid containing WT- or DN-JNK and 0.1 µg of the adenoviral backbone plasmid, pAdEasy-1 were introduced into electrocompetent E. coli BJ5183 cells with electroporation (2,500 V, 200 Ohms). Then the resultant plasmids were re-transformed into E. coli XL-Gold Ultracompetent Cells (Strategene, La Jolla, CA). The plasmids were linearized with PacI and then transfected into the adenovirus packaging cell line 293 using LipofectAMINE (Invitrogen, Carlsbad, CA). Ten days after transfection, cell lysate was obtained from the 293 cells. The cell lysate was added to 293
cells again and when most of the cells were killed by the adenovirus infection and detached, the cell lysate was obtained again (this process was repeated three times). Control adenovirus expressing green fluorescent protein (Ad-GFP) was prepared in the same manner. To determine viral titers, confluent 293 cells were infected with a 1:10,000 dilution of the final lysate containing Ad-WT-JNK or Ad-DN-JNK (with GFP). After 18 h incubation, the effective titer was determined by the following formula: $10^7 \times \text{the average number of GFP-positive cells per field (X100 magnification)}$, which was considered equivalent to plaque forming units (PFU)/ml. This number was considered to be proportional to the number of infective particles in the original lysates. Isolated rat islets (~300 islets) were infected with Ad-WT-JNK, Ad-DN-JNK, or Ad-GFP, using a 1 h exposure to the adenovirus ($1 \times 10^8$ plaque forming units (PFU)/ml). One hour after infection the islets were cultured in 3 ml of RPMI medium in 6 cm bacteriologic Petri dishes.

**RNA extraction and cDNA synthesis.**

Total RNA was extracted from islets using Trizol (Invitrogen). After quantification by spectrophotometry, 500 ng of RNA was heated at 85 °C for 3 min and then reverse-transcribed into cDNA in a 25 µl solution containing 200 units of Superscript II RNase H− reverse transcriptase (Invitrogen), 50 ng random hexamers (Invitrogen), 160 µM dNTP, and 10 mM dithiothreitol. The reactions took place for 10 min at 25 °C, 60 min at 42 °C, and 10 min at 95 °C. The final cDNA reaction products were then diluted with 50 µl H2O to a concentration corresponding to 20 ng of starting RNA (20 ng RNA equivalents) per 3 µl.

**Semiquantitative radioactive PCR.**

Polymerization reactions were performed with a Perkin-Elmer 9700 Thermocycler using a 50 µl reaction volume containing 3 µl of cDNA (20 ng RNA equivalents), 5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT), 1.5 mM MgCl₂, 160 µM cold dNTPs, 2.5 µCi of [α-32P]dCTP, and 10 pmol of appropriate oligonucleotide primers. The oligonucleotide primers were as follows: insulin: (forward) TCT TCT ACA CAC CCA TGT CCC, (reverse) GGT GCA GCA CTG ATC CAC;
cyclophilin: (forward) AAC CCC ACC GTG TTC TTC, (reverse) TGC CTT CTT TCA CCT TCC C; α-tubulin: CTC GCA TCC ACT TCC CTC, (reverse) ATG CCC TCA CCC ACG TAC; glucagon: (forward) ACC TAG ACT CCC GCC GTG, (reverse) ATG TCT GCG CCC AAG TTC; JNK: (forward) ATC CAG CAG AAG CAA GCG, (reverse) GCC AGA CCG AAG TCA AGA ATC. The primers for 18S ribosomal RNA (rRNA) was purchased from AMBION (Austin, TX). The thermal cycle profile employed a 10 min denaturing step at 94 ºC followed by the number of amplification cycles (1 min of denaturation at 94 ºC, 1 min of annealing at 55 ºC, and 1 min of extension at 72 ºC) and an extension step of 10 min at 72 ºC. The amplimers were separated on a 6% polyacrylamide gel. The amount of [α-32P]dCTP incorporated into each amplimer was measured with a PhosphorImager and quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The steps taken to validate the measurements of mRNA levels by semiquantitative-radioactive multiplex PCR have been reported previously (7).

Gel-mobility shift assay.

Cells were treated with 1 ml of hypotonic buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃P₂O₇, 1 mM Na₃VO₄, 1 mM DTT); then 50 µl of high salt buffer (420 mM NaCl and 20% glycerol in hypotonic buffer) was added to the pellet, followed by 1 h of incubation at 4ºC. The supernatants were used as nuclear extracts. Two micrograms of nuclear extract were incubated with 2 µg of poly (dl-dC), 10 mM HEPES pH 7.8, 0.1 mM EDTA, 75 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol, 3% Ficoll at room temperature. The binding reaction was initiated by adding [32P]-labeled double-stranded oligonucleotide probes. A double-stranded oligonucleotide reproducing the rat insulin gene II PDX-1 binding region and surrounding sequences (ACG TCC TCT TAA GAC TCT AAT TAC CCT ACG T) (Sigma Genosys, Woodlands, TX) was used as a binding probe. The sequences of the mutated type competitor were as follows: ACG TCC TCT TAA GAC TC[G] CCG TAC CCT ACG T. In some of the binding assays, anti-PDX-1 antiserum (24) was added to the reaction mixture 1 h before
addition of the DNA probes. After the binding reactions, samples were analyzed by separation on a 6% polyacrylamide gel in 1 x TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA), followed by autoradiography.

**Cell viability assays.**

Isolated rat islets (~200 islets) were exposed to 0-250 µM H₂O₂ and incubated for 15 min with fluorescein diacetate (10 µg/ml) (Sigma, St. Louis, MO) and propidium iodide (0.5 µg/ml) (Molecular Probes, Eugene, OR) in 3 ml phosphate-buffered saline (PBS). Fluorescein diacetate enters cells and fluoresces green in viable cells; propidium iodide enters only dead or dying cells, binds to nucleic acids and fluoresces red. The islets were then washed in PBS and images acquired with a Magnafire camera on an Olympus fluorescent microscope.

**Western blotting.**

Cells were treated with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40); then supernatants were collected. Ten micrograms of cell extracts were fractionated by 10% SDS-PAGE, and transferred to a polyvinylidene fluoride membranes (Immun-Blot™ PVDF Membrane, Bio-RAD, Hercules, CA) using transfer buffer containing 20% methanol, 25 mM Tris base and 192 mM glycine (300 mA, 2 h). After blocking the membranes at room temperature for 1 h in 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 with 5% nonfat dry milk, the membranes were incubated at 4 °C overnight in TBS buffer (50 mM Tris-HCl, 150 mM NaCl) containing a 1:1000 dilution of rabbit antibody for JNK, phosphorylated JNK, p38 MAPK, phosphorylated p38 MAPK, c-Jun, or phosphorylated c-Jun (Santa Cruz, CA), and washed three times in TBS buffer with 0.1% Tween-20 (TBS-T). The membranes were then incubated for 1 h at room temperature in TBS containing a 1:1,000 dilution of anti-rabbit IgG antibody (Bio-RAD) coupled to horseradish peroxidase, followed by washing with TBS-T. Immunoreactive bands
were made visible by incubation with LumiGLO (Cell Signaling, Beverly, MA) and exposed to X-ray film (Kodak, New Haven, CT).

**Assay for PKC activity.**

Total PKC enzyme activity was determined using SignaTECT protein kinase C assay system (Promega, Madison, WI) using the conditions recommended by the manufacturer. In brief, cell lysates from islets were incubated with $[^\gamma-32P]ATP$ and PKC biotinylated protein peptide substrate buffer at 30 °C for 5 min, and then spotted on SAM biotin capture membrane (Promega). After washing with 2 M NaCl and 2 M NaCl in 1% H$_3$PO$_4$, PKC activity was examined with scintillation counter.

**Evaluation of glucose-stimulated insulin secretion.**

Glucose-stimulated insulin secretion was determined by static incubation. Isolated rat islets (50 islets) were pre-incubated for 30 min in 2 ml of HEPES-balanced Krebs-Ringer bicarbonate buffer and then incubated for 60 min in the same buffer supplemented with 0.5% BSA and either 2.8 mM or 16.7 mM glucose in 6-well bacteriologic Petri dishes. The insulin secreted into the medium was determined with a radioimmunoassay (RIA) kit (Linco Research, St Charles, MO), using rat insulin as the standard.

**Islet transplantation.**

Male immune-deficient athymic Swiss nude mice (8 weeks old) (Taconic Farms) were made diabetic by intraperitoneal injection of streptozotocin (STZ) (250 mg/kg) (Sigma), freshly dissolved in citrate buffer (pH 4.5), and used as graft recipients. Islets isolated from SD rats were infected with Ad-DN-JNK or Ad-GFP (1X10$^8$ PFU/ml), using a 1 h exposure to the adenovirus. One hour after infection the islets were cultured for 2 days in 3 ml of RPMI medium in 6 cm bacteriologic Petri dishes, and then 500 islets were transplanted under kidney capsules of Swiss nude mice. Aliquots of islets were sedimented in a PE-50 polyethylene tube (Becton Dickson, Sparks, MD) attached to a 1 ml of Hamilton syringe (Fisher), which
allowed the injection of an islet pellet under the kidney capsule (50). Then the islet pellets were transplanted beneath the left kidney capsule under methoxyflurane anesthesia (Metofane, Mallinckrodt Veterinary Inc., Mundelin, IL). After transplantation, nonfasting blood glucose levels and body weights were measured weekly. Blood glucose was measured with a portable glucose meter (Precision QID, Medisense Inc., St. Charles, MA) after tail snipping. For measurement of plasma insulin levels 4 weeks after transplantation, nonfasting blood samples were collected into heparinized capillary tubes and plasma insulin was determined with a radioimmunoassay kit (Linco Research), using rat insulin as the standard. Also 4 weeks after transplantation, islet grafts were excised under methoxyflurane anesthesia and lysed in RNA isolation solution Trizol (Invitrogen) for analysis of gene expression by RT-PCR as described above.

**Data analysis.**

All results are presented as mean ± S.E. of at least three independent experiments. Statistical analysis was performed using the unpaired Student's $t$ test.
RESULTS

Suppression of insulin gene expression by oxidative stress in rat pancreatic islets.

As shown in Fig. 1A, when pancreatic islets isolated from SD rats were exposed to 0-50 μM H2O2 for 48 h, insulin mRNA levels were reduced in a dose-dependent manner, whereas levels of cyclophilin, α-tubulin, glucagon, and rRNA were not affected, suggesting that insulin gene expression is particularly vulnerable to oxidative stress. Also, DNA binding activity of PDX-1, an important transcription factor for insulin gene, was reduced by oxidative stress in association with decrease of insulin mRNA level (Fig. 1B). The specificity of the band for PDX-1 was confirmed by its ablation by PDX-1 antibody, but not by pre-immune serum (lanes 5 and 6), and by wild type competitor, but not by mutated type competitor (lanes 7 and 8). Since it is known that oxidative stress triggers apoptosis in several cell types, after subjecting rat islets to 0-250 μM H2O2 for 48 h, cell viability was assessed with two different reagents, fluorescein diacetate (upper panel) and propidium iodide (lower panel). Fluorescein diacetate enters cells and fluoresces green in viable cells; propidium iodide enters only dead or dying cells, binds to nucleic acids and fluoresces red. As shown in Fig. 1C, there was no difference in cell viability after exposure to vehicle alone (left panel) and 50 μM H2O2 (middle panel), although we cannot exclude the possibility that this low concentration of H2O2 has some less obvious effect on cell viability. In contrast, when islets were exposed to a higher concentration of H2O2 (250 μM), cell viability was markedly decreased (right panel). These results suggest that suppressive effects of oxidative stress on insulin gene expression and PDX-1 DNA binding activity (Fig. 1A and B) can occur separately from its effects upon cell viability.

Activation of the pathways JNK, p38 MAPK, and PKC by oxidative stress in rat pancreatic islets.

Although several pathways such as JNK, p38 MAPK, and PKC are known to be activated by oxidative stress (25, 46) or high glucose (46-48) in several cell types, it is not known which kinase is activated in
pancreatic islets. Therefore, we examined signaling pathways that could be involved in suppression of insulin gene expression by oxidative stress. As shown in Fig. 2A and B, expression levels of the phosphorylated forms of JNK and p38 MAPK were markedly increased in isolated islets treated with H2O2, while expression levels of total JNK and p38MAPK were not affected by H2O2 treatment. Total PKC enzyme activity was also increased by treatment with H2O2 in isolated islets (Fig. 2C), although the increase of PKC activity was not so remarkable as those of phosphorylated forms of JNK and p38 MAPK. Also, we examined time course of insulin gene suppression by oxidative stress. As shown in Fig. 2D, when isolated islets were exposed to 50 μM H2O2 for 0-48 h, insulin mRNA levels were reduced in a time-dependent manner. Thus, activation of several pathways (JNK, p38MAPK, and PKC) by oxidative stress (Fig. 2A, B, and C) preceded suppression of insulin gene expression (Fig. 2D). Additionally, as shown in Fig. 2E and F, phosphorylation of p38MAPK and activation of PKC enzyme activity by H2O2 were completely blocked by the p38MAPK inhibitor SB203580 (10 μM) or the PKC inhibitor GF109203X (5 μM), respectively, indicating that these reagents are acting as inhibitors in islets.

**Effects of adenovirus-mediated overexpression of wild type (WT) and dominant-negative type (DN) JNK on insulin gene expression.**

To learn about the pathways involved in oxidative stress-mediated suppression of insulin gene expression, we tested the effects of candidate kinases. To evaluate the possible implications of JNK in β-cell dysfunction, we prepared WT-JNK and DN-JNK-expressing adenoviruses (Ad-WT-JNK and Ad-DN-JNK), and control adenovirus (Ad-GFP). Fig. 3A depicts representative islets 72 h after exposure to Ad-WT-JNK. As seen by GFP in this light micrograph of whole floating islets, many cells are infected after exposure to Ad-WT-JNK, which also contains GFP. To confirm that infected WT- and DN-JNK adenoviruses can function appropriately in islets, whole cell extracts were isolated after infection with these adenoviruses and used for Western blotting. As shown in Fig. 3B, infection with Ad-WT-JNK or Ad-DN-JNK increased both total and phosphorylated JNK expression in islets as compared with islets.
infected with Ad-GFP. Moreover, phosphorylated JNK expression levels were further increased by H2O2 in both WT-JNK and DN-JNK overexpressing islets. Also, as shown in Fig. 3C, total and phosphorylated c-Jun expression levels were moderately increased by H2O2. In contrast to JNK phosphorylation, c-Jun phosphorylation was clearly induced by H2O2 only in WT-JNK overexpressing islets, but not in DN-JNK overexpressing islets. Considering that this DN-JNK is a kinase-dead mutant which can be phosphorylated but cannot phosphorylate c-Jun, these results are expected and thus suggest that these infected WT- and DN-JNK adenoviruses function appropriately in islets. Effects of the JNK pathway upon insulin gene transcription were examined by using these adenoviruses. As shown is shown in Fig. 3C, the decrease of insulin mRNA levels by H2O2 was prevented by infection with Ad-DN-JNK, while insulin mRNA levels were decreased in the WT-JNK overexpressing islets compared to that in Ad-GFP-infected islets. Also, as shown in Fig. 3D and E, the decrease of insulin mRNA levels by H2O2 was prevented by the antioxidant N-acetyl-L-cysteine (NAC), but no effect was seen with the p38MAPK inhibitor SB203580 or the PKC inhibitor GF109203X. These results suggest that the JNK activation is likely to be involved in decrease of insulin gene expression by oxidative stress.

Involvement of the JNK pathway in reduction of insulin gene expression by glucosamine.

Glucose metabolism through the hexosamine pathway has been implicated in many of the adverse effects of chronic hyperglycemia (51-54). Increased glucose flux through this pathway results in the generation of an amino sugar, glucosamine-6-phosphate, which is formed from fructose-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT) and leads to increase of O-linked glycosylation of many proteins (55, 56). Since it is known that glucosamine induces oxidative stress and is possibly involved in β-cell dysfunction in diabetes (35), we tested whether JNK is involved in β-cell dysfunction produced by glucosamine. As shown in Fig. 4A, expression level of phosphorylated form of JNK was increased in islets treated with glucosamine, while expression level of total JNK was not affected by glucosamine treatment. Also, as shown in Fig. 4B, insulin mRNA level was reduced by glucosamine treatment, and the suppressive
effect of glucosamine on insulin gene expression was reduced by the antioxidant NAC (Fig. 4B). These results imply that glucosamine decreases insulin mRNA level mainly through the induction of oxidative stress, although we cannot exclude the possibility that some other effect of glucosamine also contributes to suppression of insulin gene expression. Furthermore, as shown in Fig. 4C, the decrease of insulin gene expression by glucosamine was prevented by infection with Ad-DN-JNK. Thus, these results strengthen the hypothesis that DN-JNK protects insulin gene expression from oxidative stress.

Effects of adenoviral overexpression of WT- and DN-JNK on glucose-stimulated insulin secretion.

To see if JNK is involved in the reduction of insulin secretion, static incubations were performed after exposure to Ad-WT-JNK or Ad-DN-JNK. As shown in Fig. 5, after exposure to H2O2, glucose-stimulated insulin secretion was decreased. The decrease of insulin secretion was prevented by infection with Ad-DN-JNK, while insulin secretion was decreased in the WT-JNK overexpressing islets compared to that in Ad-GFP-infected islets. These results were correlated with change of insulin mRNA levels in islets (Fig. 3C). Thus, we assume that preservation of insulin secretion from oxidative stress is at least in part due to protection of insulin mRNA level by DN-JNK. Taken together, these results indicate that overexpression of DN-JNK can protect both insulin gene expression and secretion from oxidative stress.

Effects of adenoviral overexpression of WT- and DN-JNK on PDX-1 DNA binding activity.

Since gene expression and DNA binding activity of PDX-1 are reduced by oxidative stress provoked in the diabetic state, in association with suppression of insulin gene expression (32, 33), we examined the effects of the JNK pathway on PDX-1 DNA binding activity with the gel-shift assay. As shown in Fig. 6A and B, oxidative stress-mediated suppression of PDX-1 DNA binding activity was reduced by DN-JNK overexpression or the antioxidant NAC, but not by the p38 MAPK inhibitor SB203580 or the PKC inhibitor GF109203X. Furthermore, WT-JNK overexpression reduced PDX-1 DNA binding activity.
These results further implicate JNK in oxidative stress-mediated suppression of PDX-1 DNA binding activity and of insulin gene expression.

**Effects of transplantation of DN-JNK overexpressing islets on insulin gene expression and glucose tolerance in STZ-induced diabetic mice.**

Since it is currently not possible to culture isolated islets for a long enough period to fully bring out the effects of glucose toxicity, we performed islet transplantation into diabetic mice to examine whether DN-JNK can protect β-cells from the toxic effects of hyperglycemia and to explore the potential therapeutic application for islet transplantation. Isolated rat islets were infected with Ad-DN-JNK or Ad-GFP and cultured for 2 days; then 500 islets were transplanted under kidney capsules of STZ-induced diabetic Swiss nude mice. Whereas 500 islets cultured overnight can usually normalize glucose levels in diabetic mice, we anticipated that the viral infection and longer time in culture would produce some alteration of islet cells, thus making these 500 islets a more marginal number. Blood glucose levels were not sufficiently decreased by transplantation of islets infected with Ad-GFP, which was probably due to toxic effects of hyperglycemia upon a marginal islet number, but were markedly decreased by Ad-DN-JNK (Fig. 7A). There was no clear difference in weight gain between these two groups (Fig. 7B). Plasma insulin levels examined 4 weeks after transplantation showed a tendency to be preserved by infection with Ad-DN-JNK, although statistical significance was not reached (Fig. 7C). To examine how long the overexpressed JNK lasts in the transplanted islets, we measured JNK mRNA levels before and 4 weeks after transplantation of Ad-DN-JNK-infected islets. As shown in Fig. 7D, in Ad-GFP-infected islets JNK expression levels were relatively low both before and 4 weeks after transplantation (lanes 1 and 3). After infection of Ad-DN-JNK (before transplantation), however, JNK expression was markedly increased (lane 2); 4 weeks after transplantation JNK expression in the grafts was decreased by 32%, but still clearly detected (lane 4). Furthermore, insulin mRNA levels in either islet pellets or islet grafts were examined before and 4 weeks after transplantation. As shown in Fig. 7E, there was no difference in insulin mRNA
levels between Ad-GFP and Ad-DN-JNK group before transplantation (2 days after exposure of isolated islets to each adenovirus). Four weeks after transplantation of islets infected with Ad-GFP, insulin mRNA levels in islet grafts were clearly decreased compared with those before transplantation, but relatively preserved by DN-JNK overexpression (Fig. 7E). These results suggest DN-JNK can protect β-cells from some of the toxic effects of hyperglycemia during this transplant period.
DISCUSSION

It has been postulated that oxidative stress provoked under diabetic conditions is involved in β-cell dysfunction characterized by decreases in insulin gene transcription and glucose-stimulated insulin secretion (29-35). For example, reactive oxygen species (ROS) are produced in β-cells in the diabetic state (26), and antioxidant treatment can provide some protection against this dysfunction and improve glucose tolerance (32-34). Thus, it is likely that oxidative stress mediates some of the toxic effects of hyperglycemia upon β-cells. In this study, we examined the mechanism for oxidative stress-mediated suppression of insulin gene expression. We have shown that in isolated rat islets several pathways (JNK, p38 MAPK, and PKC) are activated by oxidative stress, preceding suppression of insulin gene expression (Figs. 1 and 2). Furthermore, oxidative stress-mediated suppression of insulin gene expression and secretion was prevented by overexpression of DN-JNK (Figs. 3, 4 and 5), suggesting that JNK is involved in oxidative stress-mediated suppression of insulin gene expression and secretion found in diabetes.

Suppression of insulin gene expression by JNK overexpression was accompanied by a decrease of PDX-1 DNA binding activity (Fig. 6), which may explain at least part of the suppression of insulin gene expression. PDX-1 (8-12) plays an important role in pancreas development (13-16) and differentiation (17-20) and in the regulation of cell-specific expression of insulin and various other genes essential for β-cell function (21-24). Moreover, mutations in PDX-1 are known to cause some cases of maturity-onset diabetes of the young (MODY) (57). Thus, it is likely that JNK-mediated suppression of PDX-1 DNA binding activity accounts for some of the suppression of insulin gene transcription and of β-cell function, which fits with reports that PDX-1 expression DNA binding activity is decreased in association with reduction of insulin gene transcription after chronic exposure to a high glucose concentration (3, 4, 6, 7, 32, 33). Thus, we postulate that activation of JNK pathway leads to decreased PDX-1 activity and subsequent suppression of insulin gene transcription in the diabetic state. Although not examined in this study, there are several possible mechanisms for suppression of PDX-1 DNA binding activity by JNK.
pathway. One possibility is that activation of the JNK pathway influences nuclear accumulation of PDX-1, as has been shown for NFAT4, a member of the REL domain family transcription factors that are important mediators of immune response (58). Another is that JNK activation changes the phosphorylation state of PDX-1 (59), which could influence the PDX-1 DNA binding activity. As is discussed later, we do not propose that suppression of insulin gene expression by oxidative stress is exclusively mediated through lowering of PDX-1; c-Jun, c-Myc and other factors are likely to make contributions.

To examine the potential therapeutic application for islet transplantation, we transplanted DN-JNK overexpressing islets under kidney capsules of STZ diabetic nude mice and found that DN-JNK provides some protection for β-cells, thus ameliorating hyperglycemia (Fig. 7). Under hyperglycemic conditions oxidative stress is provoked in β-cells (26) and some other cell types (27, 28) through processes such as the non-enzymatic glycosylation reaction (36, 37, 39, 40), the electron transport chain in mitochondria (27), and the hexosamine pathway (35). Moreover, the JNK pathway has been found to be activated by oxidative stress (25, 46) or high glucose (46-48) in several cell types. Because we used immune-deficient nude mice as recipients, we assume DN-JNK protected islet grafts from oxidative stress induced by chronic hyperglycemia.

Since it has been reported that expression of c-Jun, a b-Zip transcription factor which is known to be phosphorylated by JNK, is upregulated by oxidative stress in several cell types (60, 61) and suppresses insulin gene transcription (62, 63) by affecting the transactivation potential of the E2A gene products (64), it is possible that c-Jun expression itself is involved in suppression of insulin gene expression by oxidative stress. Actually, as shown in Fig. 3B, total c-Jun expression levels were moderately increased by H2O2, which should, at least in part, account for suppression of insulin gene expression by oxidative stress. As shown in Fig. 3C and D, however, JNK overexpression suppressed insulin gene expression without affecting c-Jun expression levels and DN-JNK protected insulin gene expression from oxidative stress without apparently affecting c-Jun expression levels. Thus, we propose that activation of the JNK pathway is also, at least in part, involved in suppression of insulin gene expression by oxidative stress in addition to
c-Jun expression. However, we also suspect that c-Jun, even at low levels, is contributing to this effect. Additionally, c-Myc, a bHLH-LZ transcription factor, is also upregulated by oxidative stress (60, 61) or high glucose *per se* (65, 66) and suppresses insulin gene expression by inhibiting NeuroD-mediated transcriptional activation (67). We assume, however, that some of the suppressive effects of JNK on the insulin gene transcription are independent of c-Jun or c-Myc, because JNK activation reduces insulin gene transcription in association with suppression of PDX-1 DNA binding activity (Fig. 6) while both c-Jun and c-Myc suppress the insulin gene transcription through mechanisms other than PDX-1 (64, 67).

Although JNK activation by oxidative stress preceded suppression of insulin gene expression (Fig. 2A and D), there was some difference in timing between activation of JNK and suppression of insulin gene expression. We think this is due to stability of insulin mRNA (about 24-48 h) (68); even if insulin gene transcription is completely suppressed, insulin mRNA levels should not fall very quickly. Also, although WT-JNK suppressed insulin gene expression and that DN-JNK protected some of the reduction of insulin gene expression from oxidative stress (Fig. 3D), the extent of insulin gene expression was not completely correlated with phosphorylated c-Jun levels which is regulated by JNK pathway. While insulin gene expression was clearly decreased by H2O2 treatment alone or Ad-WT-JNK overexpression alone, expression levels of total and phosphorylated c-Jun were only slightly induced by these treatments (Fig. 3C and D). We think this may be due to very low expression levels of c-Jun in islets; c-Jun expression in islets is quite low and thus H2O2 treatment alone or Ad-WT-JNK overexpression alone was not enough to induce clear bands of phosphorylated c-Jun. Also, although phosphorylated c-Jun expression was markedly induced by H2O2 in the presence of WT-JNK, the extent of decrease in insulin gene expression was similar to that after H2O2 treatment alone or Ad-WT-JNK overexpression alone. This may be also due to stability of insulin mRNA (half life is 24-48 h) (68); even if insulin gene transcription is strongly suppressed, insulin mRNA levels should not be markedly decreased in 48 h. Thus, we assume that since H2O2 treatment alone or Ad-WT-JNK alone exerts maximal suppressive effects on insulin gene
transcription, the combination of H2O2 and WT-JNK overexpression would not be expected to show additional effects (Fig. 3C).

It has been reported that β-cell destruction by cytokines such as interleukin-1β (IL-1β) (69-73) can be prevented by inhibition of the JNK pathway (74-77), implying that JNK plays a role in autoimmune β-cell destruction. It should be noted, however, since in this study we used immune-deficient nude mice as recipients, it is not likely that DN-JNK protected islet grafts from oxidative stress induced by immune reaction, although we cannot totally exclude the possibility that DN-JNK exerted some beneficial effects on less obvious oxidative injury induced during the transplantation operation. It has been reported that under hyperglycemic conditions production of reactive oxygen species (ROS) is increased in β-cells (26) and some other cell types (27, 28) and that levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG), a marker for oxidative stress, are increased in the blood of type 2 diabetic patients (41, 42). Also, as shown in this study, JNK is activated by oxidative stress in islets, and the JNK activation reduces the PDX-1 DNA binding activity and insulin gene transcription. Thus, we assume that JNK is involved in deterioration of β-cell function in both type 2 diabetes and the early stages of type 1 diabetes.

In conclusion, the present results provide new insights into the mechanism through which oxidative stress suppresses insulin gene transcription in pancreatic β-cells, and the finding that this adverse outcome can be prevented by DN-JNK overexpression suggests that the JNK pathway in β-cells could become a new therapeutic target for diabetes.
ACKNOWLEDGMENTS

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FOOTNOTES

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The abbreviations used are: PDX-1, pancreatic and duodenal homeobox factor-1; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; SD rat, Sprague-Dawley rat; WT, wild type; DN, dominant-negative type; GFP, green fluorescent protein; PFU, plaque forming unit; rRNA, ribosomal RNA; STZ, streptozotocin; NAC, N-acetyl-L-cysteine.
REFERENCES


FIGURE LEGENDS

Fig. 1. Suppression of insulin gene expression by oxidative stress in rat pancreatic islets.

(A) After treatment of isolated islets (~100 islets) with 0-50 µM H2O2 for 48 h in RPMI 1640 medium (11 mM glucose), insulin mRNA levels were examined by RT-PCR. Relative ratios of insulin / cyclophilin mRNA levels are expressed as mean ± S.E. in bar graphs with those of untreated islets being arbitrarily set at 100 (n = 3). (B) After treatment with 0-50 µM H2O2 for 48 h, nuclear extracts were obtained from the islets and gel-shift assays were performed. A double-stranded oligonucleotide containing the rat insulin gene PDX-1 binding region was used as a binding probe. In lanes 5 and 6, anti-PDX-1 antiserum and pre-immune serum were employed, and in lanes 7 and 8, wild type and mutated type competitor (X100) were employed. The arrow indicates the supershifted band. Similar results were obtained in three independent experiments. (C) Isolated islets (~200 islets) were exposed to 0-250 µM H2O2 for 48 h and stained with fluorescein diacetate (upper panel) and propidium iodide (lower panel). Fluorescein diacetate enters cells and fluoresces green in viable cells; propidium iodide enters only dead or dying cells, binds to nucleic acids and fluoresces red. There was no difference in cell viability between exposure to vehicle alone (left panel) and 50 µM H2O2 (middle panel), whereas viability was markedly decreased (right panel) after treatment with a higher concentration of H2O2 (250 µM) (right panel).

Fig. 2. Activation of the pathways JNK, p38 MAPK, and PKC in rat pancreatic islets.

(A, B) After treatment of isolated islets with 50 µM H2O2 for 0-120 min, whole cell extracts were obtained and Western blot analysis was performed. JNK and p38 MAPK expression levels and their phosphorylated form levels were examined. Relative ratios of the phosphorylated form of JNK / total JNK levels and the phosphorylated form of p38 MAPK / total p38 MAPK levels are expressed as mean ± S.E. in line graphs with those of untreated islets being arbitrarily set at 1 (n = 3). (C) After treatment with 50 µM H2O2 for 0-120 min, cell lysates were obtained and total PKC enzyme activity was examined. Fold
increase of total PKC activity is expressed in line graphs as mean ± S.E. with those of untreated islets being arbitrarily set at 1 (n = 4). (D) After treatment of isolated islets with 50 μM H2O2 for 0-48 h, insulin mRNA levels were examined by RT-PCR. Relative ratios of insulin / cyclophilin mRNA levels are expressed as mean ± S.E. in bar graphs with those of untreated islets being arbitrarily set at 100 (n = 3). (E, F) Islets were exposed to 50 μM H2O2 (or vehicle) for 30 min with and without the p38 MAPK inhibitor SB203580 (10 μM) (E) or the PKC inhibitor GF109203X (5 μM) (F), and then phosphorylated form level of p38 MAPK and total PKC activity were examined. Relative ratios of the phosphorylated form of p38 MAPK / total p38 MAPK levels and fold increase of total PKC activity are expressed in bar graphs as mean ± S.E. with those of untreated islets being arbitrarily set at 1 (n = 3).

Fig. 3. Effects of adenovirus-mediated overexpression of WT- and DN-JNK on insulin gene expression.

(A) Isolated pancreatic islets (~300 islets) were infected with recombinant adenoviruses, Ad-WT-JNK, Ad-DN-JNK, or Ad-GFP (1X 10^8 PFU/ml), and cultured for 72 h in RPMI 1640 medium (11 mM glucose). Panel shows representative islets 72 h after exposure to Ad-WT-JNK. As seen by GFP in this light micrograph of whole floating islets, many cells are infected after exposure to Ad-WT-JNK, which also contains GFP. (B, C) Seventy two hours after infection of Ad-WT-JNK, Ad-DN-JNK, or Ad-GFP, islets were exposed to 50 μM H2O2 (or vehicle) for 30 min. Total protein was obtained and used for Western blotting was performed with the antibody for total and phosphorylated forms of JNK (B) and c-Jun (C). Expression levels of total and phosphorylated forms of JNK and c-Jun are expressed as mean ± S.E. with those of control islets being arbitrarily set at 1 (n = 3). (D) Forty eight hours after exposure to Ad-GFP, Ad-WT-JNK, Ad-DN-JNK, islets were exposed to 50 μM H2O2 (or vehicle) for another 48 h and insulin mRNA levels were examined by RT-PCR. (E) After exposure to 50 μM H2O2 (or vehicle) for 48 h with and without p38 MAPK inhibitor SB203580 (10 μM), PKC inhibitor GF109203X (5 μM), or antioxidant N-acetyl-L-cysteine (NAC) (10 mM), insulin mRNA levels were examined. Relative ratios of
insulin / cyclophilin mRNA levels are expressed as mean ± S.E. in bar graphs with those of islets infected with Ad-GFP (without H₂O₂ treatment) being arbitrarily set at 100 (n = 4). *, p<0.01.

**Fig. 4. Activation of the JNK pathway and suppression of insulin gene expression by glucosamine.**

(A) After treatment of isolated islets with 10 mM glucosamine for 0-120 min, whole cell extracts were obtained and Western blot analyses were performed. Levels of JNK expression level and its phosphorylated form were examined. Relative ratios of the phosphorylated form of JNK / total JNK levels are expressed as mean ± S.E. in line graphs with those of untreated islets being arbitrarily set at 1 (n = 3).

(B) Islets were exposed to 10 mM glucosamine (or vehicle) for 48 h with or without N-acetyl-L-cysteine (NAC) (10 mM) and insulin mRNA levels were examined by RT-PCR. (C) Forty eight hours after exposure to Ad-GFP or Ad-DN-JNK, islets were exposed to 10 mM glucosamine (or vehicle) for another 48 h and insulin mRNA levels were examined by RT-PCR. Relative ratios of insulin / cyclophilin mRNA levels are expressed as mean ± S.E. in bar graphs with those of control islets being arbitrarily set at 100 (n = 3). *, p<0.01.

**Fig. 5. Effects of adenoviral overexpression of WT- and DN-JNK on glucose-stimulated insulin secretion.**

Forty eight hours after infection with Ad-WT-JNK, Ad-DN-JNK, or Ad-GFP, islets were exposed to 50 μM H₂O₂ (or vehicle) for another 48 h and then glucose-stimulated insulin secretion was examined over 60 min using static incubation with batches of 50 islets, comparing 2.8 mM and 16.7 mM glucose. Amounts of secreted insulin (pg/min/islet) are expressed as mean ± S.E. in bar graphs (n = 3). *, p<0.01.

**Fig. 6. Effects of adenoviral overexpression of WT- and DN-JNK on PDX-1 DNA binding activity.**
(A) Forty eight hours after exposure to Ad-WT-JNK, Ad-DN-JNK, or Ad-GFP, islets were exposed to 50 μM H2O2 (or vehicle) for another 48 h and then nuclear extracts were obtained and gel-shift assays were performed. (B) After treatment with 50 μM H2O2 (or vehicle) and SB203580 (10 μM), GF109203X (5 μM), and NAC (10 mM) for 48 h, gel-shift assays were performed. A double-stranded oligonucleotide containing the rat insulin gene PDX-1 binding region was used as a binding probe. Similar results were obtained in three independent experiments.

Fig. 7. Effects of transplantation of DN-JNK overexpressing islets on insulin gene expression and glucose tolerance in STZ-induced diabetic nude mice.

(A, B) Isolated rat islets were infected with Ad-DN-JNK or Ad-GFP and cultured for 2 days; then 500 islets were transplantated under the kidney capsules of STZ-induced diabetic Swiss nude mice. After transplantation, nonfasting blood glucose levels and body weights were measured weekly. Data are expressed as mean ± S.E. in line graphs (n = 6). *, p<0.05. (C) Four weeks after transplantation, nonfasting plasma insulin levels were determined. Data are expressed as mean ± S.E. in bar graphs (n = 6). (D, E) Four weeks after transplantation, islet grafts were retrieved and mRNA levels of JNK (D) and insulin (E) in the grafts were examined by RT-PCR. Relative ratios of JNK / cyclophilin mRNA levels and insulin / cyclophilin mRNA levels are expressed as mean ± S.E. in bar graphs with those of islets infected with Ad-GFP (before transplantation) being arbitrarily set at 100 (n = 3). *, p<0.05.
Fig. 1

A

H_{2}O_{2}  0  10  25  50 (μM)

insulin

relative ratios of insulin / cyclophilin

B

H_{2}O_{2}  0  10  25  50 (μM)

PDX-1 antibody

PDX-1 antibody

C

H_{2}O_{2}  0  50  250 (μM)

fluorescein diacetate

propidium iodide
Fig. 2

A

H$_2$O$_2$ 0 30 60 120 (min)

JNK-P

JNK

Relative ratios of JNK-P / JNK

B

H$_2$O$_2$ 0 30 60 120 (min)

P38MAPK-P

P38MAPK

Relative ratios of P38MAPK-P / P38MAPK

C

Fold increase of total PKC activity

H$_2$O$_2$ 0 30 60 120 (min)

D

H$_2$O$_2$ 0 12 24 48 (h)

insulin
cyclophilin

Relative ratios of insulin / cyclophilin

E

Relative ratios of p38MAPK-P / p38MAPK

control H$_2$O$_2$ H$_2$O$_2$ + SB203580

F

Fold increase of total PKC activity

control H$_2$O$_2$ H$_2$O$_2$ + GF109203X
Fig. 3

D

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insulin

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cyclophilin

Relative ratios of insulin / cyclophilin

E

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*
Fig. 4

A

glucosamine 0 30 60 120 (min)

JNK-P
JNK

Relative ratios of JNK-P / JNK

B

NAC (-) NAC (+)

glucosamine - + - +

insulin

cyclophilin

Relative ratios of insulin / cyclophilin

C

Ad-JNK

Ad-GFP (DN)

- + - +

Relative ratios of insulin / cyclophilin
Fig. 5

Glucose-stimulated Insulin Secretion (pg/min/islet)

H$_2$O$_2$  
-  +  -  +  -  +

Ad-GFP  Ad-JNK (WT)  Ad-JNK (DN)

* * *

* * *
Fig. 6

A

\[ \text{H}_2\text{O}_2^{-} + \text{Ad-GFP (WT)} + \text{Ad-JNK (DN)} \]

B

\[ \text{H}_2\text{O}_2^{-} + \text{SB203580} + \text{GF109203X} + \text{NAC} \]

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PDX-1
Fig. 7

A

Transplantation

Blood Glucose Level (mg/dl)

0 1 3 7 14 21 28 (days)

Ad-GFP

Ad-DN-JNK

B

Body Weight (g)

0 7 14 21 28 (days)

Ad-DN-JNK

Ad-GFP

C

Plasma Insulin Level (ng/ml)

Ad-GFP

Ad-DN-JNK
Fig. 7

D

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JNK

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insulin

cyclophilin

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Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression
Hideaki Kaneto, Gang Xu, Nobuharu Fujii, Shokei Kim, Susan Bonner-Weir and Gordon C. Weir

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