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Role of JNK activation in pancreatic β-cell death by streptozotocin

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c-Jun N-terminal kinase (JNK) is activated by cellular stress and plays critical roles in diverse types of cell death. However, role of JNK in β-cell injury is obscure. We investigated the role for JNK in streptozotocin (STZ)-induced β-cell death. STZ induced JNK activation in insulinoma or islet cells. JNK inhibitors attenuated insulinoma or islet cell death by STZ. STZ-induced JNK activation was decreased by PARP inhibitors, suggesting that JNK activation is downstream of PARP-1. Phosphatase inhibitors induced activation of JNK and abrogated the suppression of STZ-induced JNK activation by PARP inhibitors, suggesting that the inhibition of phosphatases is involved in the activation of JNK by STZ. STZ induced production of reactive oxygen species (ROS) as potential inhibitors of phosphatases, which was suppressed by PARP inhibitors. PARP-1 siRNA attenuated insulinoma cell death and JNK activation after STZ treatment, which was reversed by MKP (MAP kinase phosphatase)-1 siRNA. These results suggest that JNK is activated by STZ downstream of PARP-1 through inactivation of phosphatases such as MKP, which plays important roles in STZ-induced β-cell death.

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1. Introduction

Streptozotocin (STZ) is a well-known diabetogenic agent and has been widely used to study the mechanism of pancreatic β-cell death and diabetes. STZ is a glucose analogue that is transported into pancreatic β-cells via GLUT-2 transporter system (Schnedl et al., 1994). STZ, as an alkylating agent similar to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or other nitrosoureas, induces strand breaks and methylation of genomic DNA, leading to activation of poly(ADP-ribose) polymerase-1 (PARP-1) (LeDoux et al., 1988). While PARP-1 activation in response to DNA breaks is a mechanism to repair damaged DNA and to maintain genomic DNA stability (de Murcia et al., 1997; Wang et al., 1997), excessive PARP-1 activation, e.g., that after STZ treatment, could be deleterious to the cells by depleting NAD^+ through alternating formation and degradation of poly(ADP-ribose) (PAR) (Hoorens and Pipeleers, 1999; Okamoto and Takasawa, 2002). Indeed, *PARP-1*-null mice are resistant to the development of diabetes after STZ treatment (Burkart et al., 1999b; Pieper et al., 1999).

However, it is still controversial whether NAD^+ depletion by PARP-1 activation is the sole mechanism of cell death by STZ (Chiarugi, 2002). Furthermore, intracellular signaling downstream of PARP-1 activation and the biochemical mechanism by which nuclear damage by STZ is translated to the entire series of cell death events in cytoplasm and mitochondria are not clearly elucidated.

Mitogen-activated protein (MAP) kinase is a typical element of cellular responses against extracellular stimuli. The c-Jun N-terminal kinase (JNK) is a group of MAP kinases that is activated by cytokines and cellular stress. While activation of MAP kinases including JNK is essentially an adaptive process to environmental changes, excessive or prolonged activation of JNK leads to cell death, which may represent a mechanism to remove extensively damaged cells and to avoid futile expenditure of cellular energy. Thus, JNK activation plays an important role in a variety of cell death types including apoptosis and necrosis (Kamata et al., 2005; Tournier et al., 2000).

As a cellular response against cell stress, JNK activation is likely to occur in DNA damage and cell death by STZ. Indeed, activation of JNK has been reported to occur when PARP-1 is activated by an alkylating agent, MNNG (Xu et al., 2006). While these results suggest that JNK activation could be involved in STZ-induced β-cell death, this possibility has not been systematically studied. We studied whether JNK that has been reported to play a role in the

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pathogenesis of both natural type 1 and type 2 diabetes (Hirosumi et al., 2002; Jaeschke et al., 2005) is involved in the STZ-induced β-cell death.

2. Materials and methods

2.1. Cell cultures

NIT-1, insulinoma cells established from SV40 T-transgenic NOD/Lt mice, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l glutamine at 37 °C in humidified 5% CO_2/95% air.

2.2. MTT assays

Cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay as described (Suk et al., 2001b). In some experiments, cells were pretreated with various inhibitors such as SP600125 (Calbiochem, La Jolla, CA, USA), 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; Alexis Biochemicals, Grünberg, Germany), nicotinamide (Sigma), okadaic acid (Sigma) or vanadate (Sigma) before STZ treatment.

2.3. Hoechst/PI staining

To distinguish apoptotic cells from necrotic cells, double staining with 5 μg/l Hoechst 33342 and 2.5 μg/l PI (Molecular Probes, Eugene, OR, USA) was done. Cells with intact blue nuclei, condensed/fragmented blue nuclei, and intact pink nuclei were considered as viable, apoptotic, and necrotic cells, respectively (Suk et al., 2001a).

2.4. RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After reverse transcription using SuperScript II (Invitrogen), cDNA was subjected to PCR amplification employing specific primer sets (*JNK1* forward, 5′-CAGAGGTCATTCTCGGCATG-3′; reverse, 5′-GGAGAGCTTCATCTACGGAG-3′; *JNK2* forward, 5′-GCATTCAGCTGGTATCATTC-3′; reverse, 5′-GATGGTGTTCCTAGCTGTTC-3′; *JNK3* forward, 5′-GGTGATTCAGATGGAGCTGG-3′; reverse, 5′-CCACGTAGTTT-CTGACTGTG-3′; *PARP-1* forward, 5′-CGGAGAGGCTTTATCGAGTG-3′; reverse, 5′-TACCCAGCTGTGGCTTCTCT-3′; *MKP-1* forward, 5′-AGGACAACCACAAGGCAGAC-3′; reverse, 5′-GGGATGGAAACAGGGAAGTT-3′).

2.5. ROS measurement

Intracellular reactive oxygen species (ROS) content was measured using fluorescent probes as described (Park et al., 2004). In short, cells plated in 6-well plates were incubated with 1 μmol/l dihydroethidium (HE; Molecular Probes) or 1 μmol/l 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) at 37 °C for 20 min without light exposure. Then, the cells were harvested and analyzed by FACS analysis (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. Western blot

After lysis of cells in a buffer (10 mmol/l Tris–HCl, pH 7.5, 100 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, 1 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride and protease inhibitor cocktail), protein expression was examined by immunoblotting using specific antibodies to phospho-JNK (Cell Signaling Technology, Beverly, MA, USA), total JNK (Santa Cruz, Santa Cruz, CA, USA), PAR (Calbiochem), PARP-1 (Cell Signaling Technology) and β-actin (Abcam, Cambridge, MA, USA) as described (Suk et al., 2001b).

2.7. siRNA experiment

siRNA for murine *PARP-1* (5′-GACACAGAAUCCUAGCCGA-3′), *MKP-1* (5′-CUAGAAACUUCAUGCUUGA-3′) or an irrelevant green fluorescence protein (GFP) (Bioneer, Daejeon, Korea) was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The expression of PARP-1 and MKP-1 was evaluated by RT-PCR and Western blotting as in Sections 2.4 and 2.6.

2.8. Isolation of pancreatic islets

Pancreatic islets were isolated from C57BL/6 mice using the previously described method of collagenase digestion and Ficoll density gradient centrifugation (Suk et al., 2001b). Then, trypsinized single islet cells were incubated in RPMI 1640 medium-10% FCS.

2.9. Statistical analysis

The results were presented as means ± SEM. Student's *t*-test was employed to compare the differences between two groups. When more than two groups were compared, statistic significance was determined by one-way ANOVA with Newman–Keuls *post hoc* test. For all statistical analyses, *P* values less than 0.05 were regarded as significant.

3. Results

3.1. JNK expression in insulinoma cell lines and primary islet cells

We first studied whether various isoforms of JNK are expressed in pancreatic islet cells because JNK isoforms have different expression pattern and distinct role in response to cell stress and in various modes of cell death. RT-PCR analysis showed that JNK1, 2 and 3 were all expressed in NIT-1 insulinoma cells and primary islet cells (Fig. 1a).

3.2. JNK activation by STZ

We next investigated whether JNK is activated in insulinoma cells by STZ treatment. Immunoblotting showed that JNK was strongly activated in insulinoma cells after 10 mmol/l STZ treatment for 30–120 min (Fig. 1b). Persistent activation of JNK over 90 min suggested that the JNK activation by STZ was a prolonged one that could exert cell death (Tang et al., 2001). We next studied the role for JNK activation in insulinoma cell death by STZ because JNK activation is involved in diverse types of cell death and JNK inhibitors are able to suppress cell death in such models. SP600125, a JNK inhibitor that has been used in a wide variety of cell death models, significantly inhibited insulinoma cell death by STZ treatment for 24 h in a dose-dependent manner, suggesting that JNK activation contributes to the death of insulinoma cells by STZ (Fig. 1c). Type of STZ-induced cell death suppressed by JNK inhibitors was mostly necrosis because fluorescent microscopy after Hoechst/PI staining showed that, while 86% of total insulinoma cells underwent necrosis characterized by PI uptake without nuclear condensation/fragmentation after treatment with 10 mmol/l STZ, percentage of such cells was significantly decreased to 27% in the presence of SP600125 ($P < 0.01$) (Fig. 1d). Only a few apoptotic cells characterized by nuclear condensation/fragmentation without PI uptake were observed after 10 mmol/l STZ treatment of NIT-1 insulinoma cells (Fig. 1d), while apoptotic cells were well observed after treatment with lower dose of STZ (data not shown).

3.3. JNK activation downstream of PARP-1 activation

We next studied the relationship between PARP-1 activation that plays a crucial role in STZ-induced β-cell injury and JNK activation. Treatment with PARP inhibitors such as DPQ or nicotinamide, inhibited STZ-induced insulinoma cell death as previously reported (Burkart et al., 1999a) (Fig. 2a). Here, we examined whether JNK activation observed after STZ treatment of insulinoma cells could be modulated by PARP inhibitors. DPQ inhibited STZ-induced JNK activation in doses that suppressed PARP-1 activation and PAR production (Fig. 2b), suggesting that JNK activation is downstream of PARP-1 activation. Nicotinamide, another PARP inhibitor, also inhibited STZ-induced JNK activation in NIT-1 insulinoma cells (Fig. 2b). On the contrary, JNK inhibitor did not inhibit PARP-1 activation by STZ in doses that inhibited JNK activation (Fig. 2c), supporting that JNK is not upstream but downstream of PARP-1 activation.

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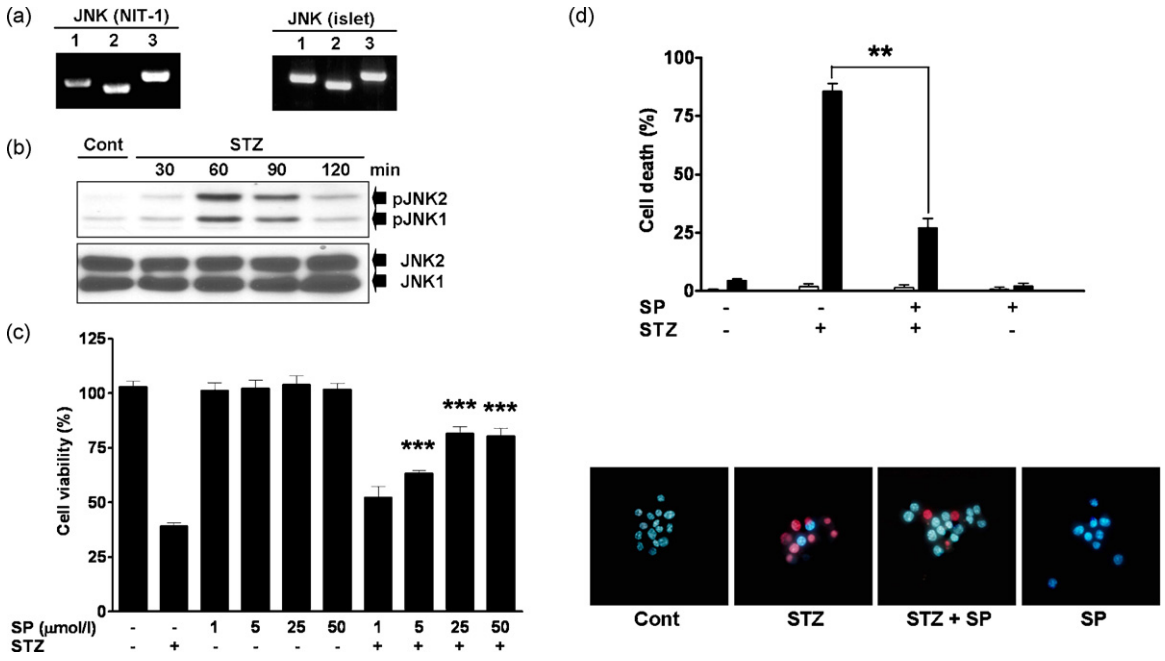

Fig. 1. JNK activation in STZ-induced insulinoma cell death. (a) Total RNA was extracted from NIT-1 insulinoma cells (left) or primary islet cells (right). RT-PCR was done using primer sets specific for each JNK isotype. (b) NIT-1 cells were treated with STZ (10 mmol/l) or sodium citrate (Cont) for the indicated time period. Whole cell lysates were then subjected to immunoblot analysis using antibodies against phospho-JNK and total JNK. (c) NIT-1 cells were pretreated for 1 h with the indicated concentrations of a JNK inhibitor, SP600125 (SP) and then exposed to STZ (10 mmol/l). MTT assay was carried out 24 h later. The data in this figure are presented as the mean ± SEM of three independent experiments. (***$P < 0.001$ compared to NIT-1 cells treated with STZ alone) (d) Insulinoma cells were treated with STZ (10 mmol/l) for 24 h in the presence or absence of SP, and apoptotic or necrotic cells were counted after Hoechst/PI double staining on a fluorescent microscope (400×) (*left bars*, apoptosis; *right bars*, necrosis). The data in this figure are presented as the mean ± SEM of three independent experiments. (**$P < 0.01$ compared to NIT-1 cells treated with STZ alone).

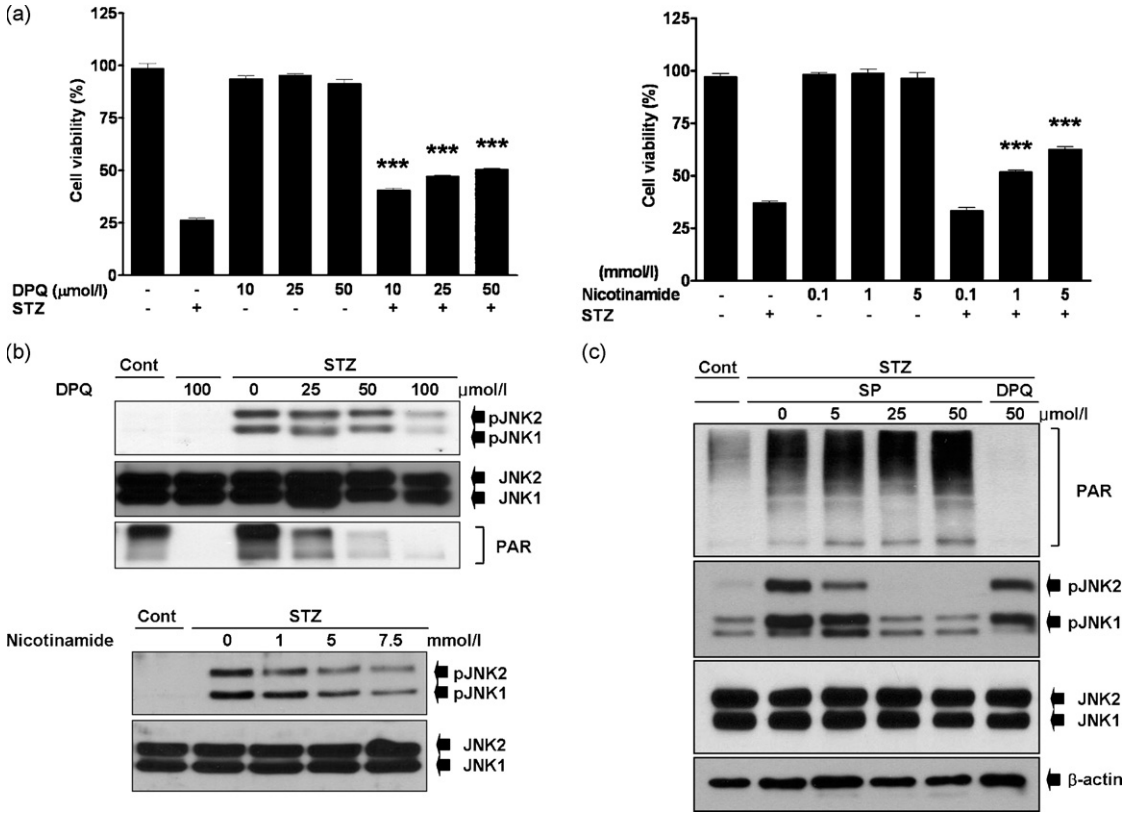

Fig. 2. Effect of PARP inhibitors on JNK activation by STZ. (a) NIT-1 cells were pretreated for 1 h with the indicated concentrations of PARP inhibitor, DPQ or nicotinamide, and then exposed to STZ (10 mmol/l). MTT assay was carried out 24 h later. The data in this figure are presented as the mean ± SEM of three independent experiments. (***$P < 0.001$ compared to NIT-1 cells treated with STZ alone) (b) NIT-1 cells were pretreated for 1 h with the indicated concentrations of DPQ or nicotinamide, and then treated with STZ (10 mmol/l) for 1 h. Total cell lysates were subjected to immunoblotting using anti-phospho-JNK, -total JNK or -PAR antibody. (c) NIT-1 cells were pretreated for 1 h with the indicated concentrations of the JNK inhibitor, SP600125, and then treated with STZ (10 mmol/l) for 1 h. Whole cell lysates were subjected to immunoblotting as in (b).

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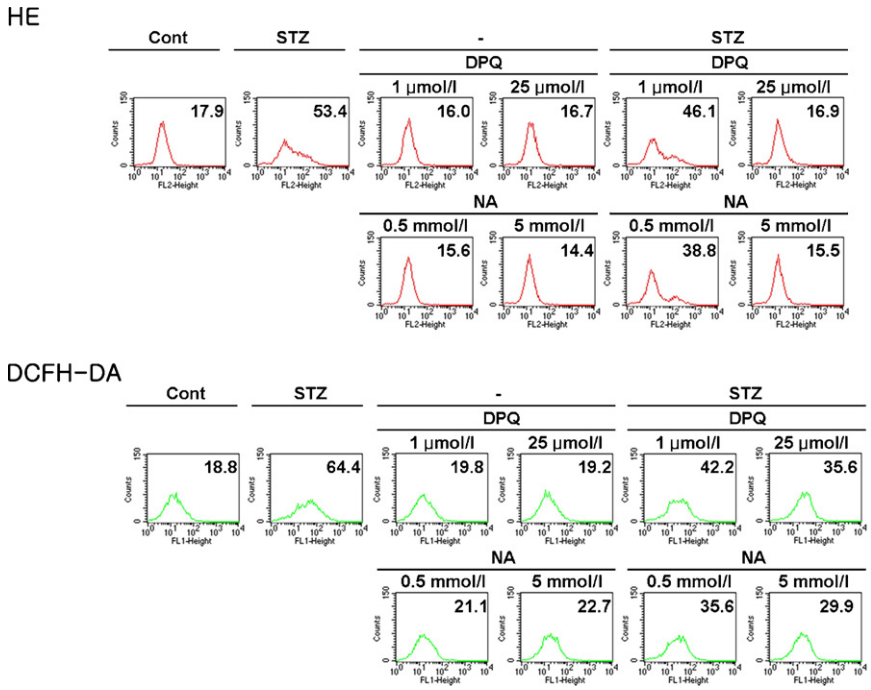

Fig. 3. Effect of PARP inhibitors on ROS production by STZ. After pretreatment with the indicated concentration of DPQ or nicotinamide (NA) for 1 h, NIT-1 cells were treated with STZ (10 mmol/l) for 3 h. Then, FACS analysis after staining with HE and DCFH-DA was done to measure intracellular superoxide and hydrogen peroxide levels, respectively. Numbers in the boxes indicate the percentage of stained cells.

3.4. *Role of ROS in JNK activation by STZ*

Next, we investigated the mechanism of JNK activation by STZ downstream of PARP-1. We studied whether ROS are produced because PARP-1 activation has been reported to mediate ROS production (Gille et al., 2002) and ROS are able to activate JNK by inhibiting phosphatases (Owens and Keyse, 2007). FACS analysis after HE and DCFH-DA staining that detect predominantly superox-

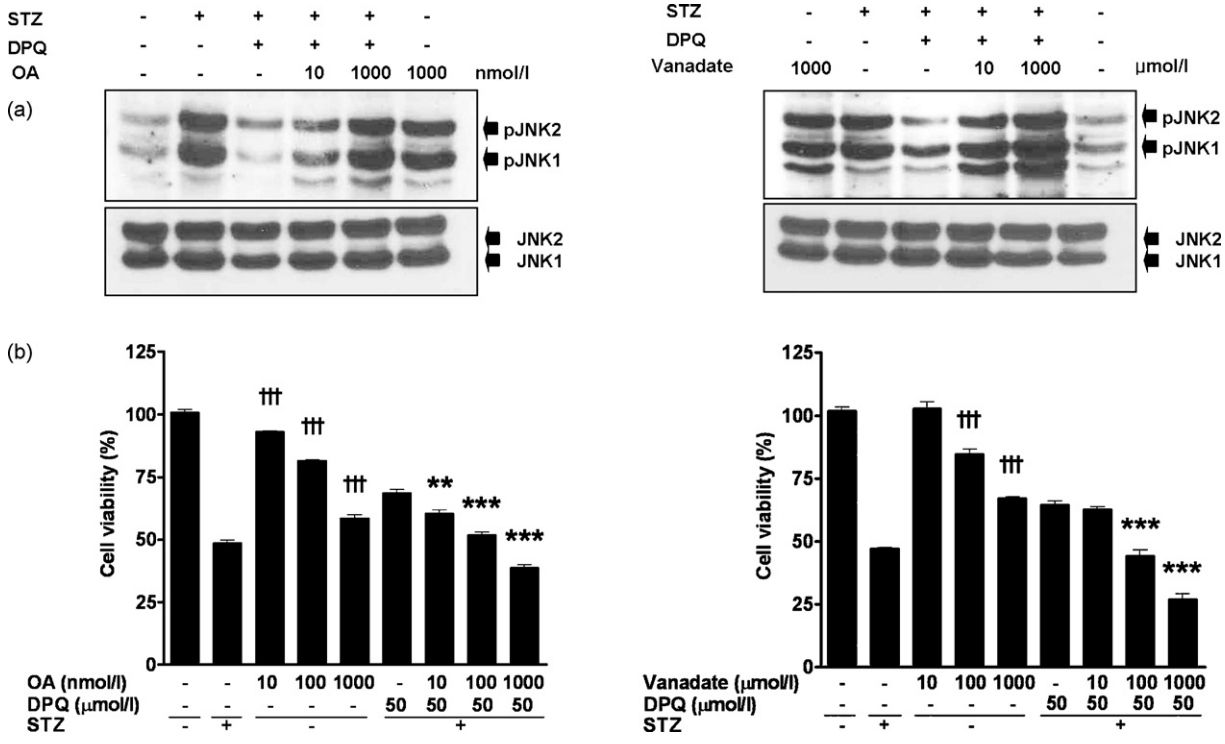

Fig. 4. Effect of phosphatase inhibitors on the suppression of STZ-induced JNK activation and cell death by PAPR inhibitors. (a) After pretreatment of NIT-1 cells with DPQ (50 µmol/l) in the presence or absence of phosphatase inhibitors (okadaic acid (OA) or vanadate) for 1 h, cells were treated with STZ (10 mmol/l). After 1 h of incubation, total cell lysates were subjected to immunoblotting using antibodies against phospho-JNK and total JNK. (b) After pretreatment of NIT-1 cells with DPQ in the presence or absence of phosphatase inhibitors (OA or vanadate) for 1 h, cells were treated with STZ (10 mmol/l). After 24 h of incubation, MTT was done. The data in this figure are presented as the mean ± SEM of three independent experiments. (†††$P < 0.001$ compared to untreated cells; **$P < 0.01$ and ***$P < 0.001$ compared to NIT-1 cells treated with STZ in the presence of DPQ).

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ide anion and hydrogen peroxide, respectively (Kim and Lee, 2005), showed that ROS are produced after STZ treatment of insulinoma cells (Fig. 3). ROS produced by STZ were suppressed by DPQ or nicotinamide (Fig. 3), consistent with a previous paper showing the role for PARP-1 in ROS production during cellular injury (Virag et al., 1998). Because previous papers showed the role of phosphatase inhibition by ROS in JNK activation (Kamata et al., 2005; Owens and Keyse, 2007), we studied whether phosphatases inhibitors could affect the activation of JNK and viability of insulinoma cells. Phosphatase inhibitors such as okadaic acid or vanadate abrogated the suppression of STZ-induced JNK activation by DPQ (Fig. 4a, lanes 3–5 from the left for okadaic acid and vanadate), suggesting that JNK activation by STZ involves phosphatases downstream of PARP-1 activation. Phosphatase inhibitors alone without STZ treatment also induced activation of JNK in insulinoma cells (Fig. 4a, lane 6 from the left for okadaic acid and lane 1 from the left for vanadate), supporting the role of basal phosphatase activity in the modulation of JNK activity. These results suggest that JNK is activated by STZ as a downstream event of PARP-1 activation through inactivation of phosphatases, which plays an important role in the death of insulinoma cells by STZ. Consistent with the prolonged activation of JNK by phosphatase inhibitors, okadaic acid or vanadate induced insulinoma cell death (Fig. 4b, bars 3–5 from the left for okadaic acid and vanadate). They also inhibited suppression of STZ-induced insulinoma cell death by DPQ (Fig. 4b, bars 6–9 from the left for okadaic acid and vanadate), supporting that inhibition of phosphatases after STZ treatment of insulinoma cells contributes to the prolonged JNK activation and ensuing cell death.

3.5. *Effect of PARP-1 and MKP-1 siRNA*

Because these results were obtained using chemical inhibitors that could have nonspecific effects, we validated our results employing siRNA strategy. Transfection of NIT-1 cells with PARP-1 siRNA that was effective in downregulating PARP-1 mRNA expression (Fig. 5a), significantly inhibited NIT-1 cell death by 10 mM STZ compared to control GFP siRNA transfection ($P < 0.001$) (Fig. 5b). PARP-1 siRNA transfection also decreased JNK activation after STZ treatment (Fig. 5c), supporting that JNK activation downstream of PARP-1 is involved in insulinoma cell death by STZ. We also employed siRNA targeting MAP kinase phosphatase (MKP) since MKPs directly dephosphorylate MAP kinase such as JNK or p38 among phosphatases (Kamata et al., 2005; Sakon et al., 2003). We chose MKP-1 that has a critical and nonredundant role in JNK dephosphorylation among MKPs as the target (Wu and Bennett, 2005). Combined transfection of MKP-1 siRNA that suppressed MKP-1 expression (Fig. 5a), abrogated PARP-1 siRNA effect both on the cell death and JNK activation (Fig. 5b and c), suggesting that the effects of chemical inhibitors of phosphatase are most likely due to the inhibition of MKP rather than their nonspecific effects.

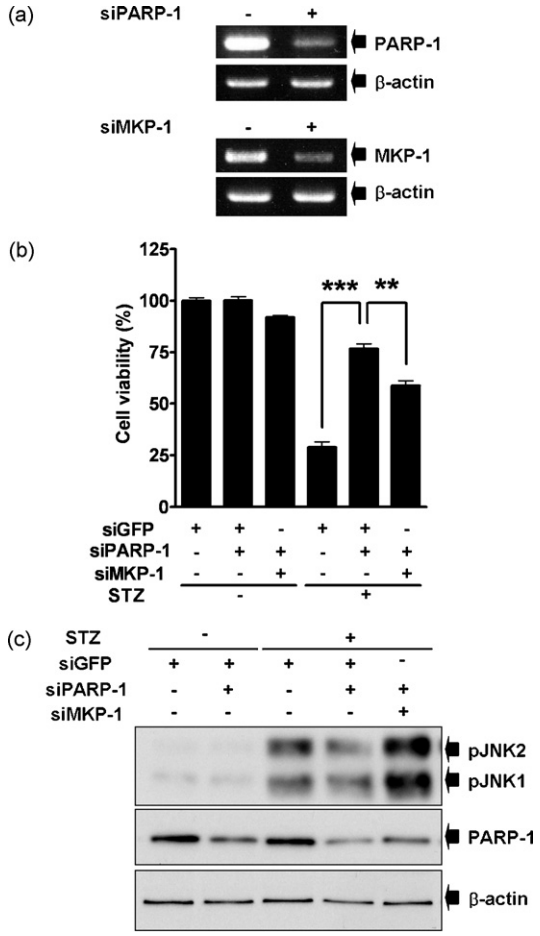

Fig. 5. Effect of PARP-1 or MKP-1 siRNA on STZ-induced JNK activation and cell death. (a) Forty-eight hours after transfection of NIT-1 cells with PARP-1, MKP-1 and GFP siRNA, the expression of PARP-1 and MKP-1 mRNA was determined by RT-PCR. (b) Seventy-two hours after transfection, NIT-1 cells were treated with 10 mmol/l STZ for 24 h. Cell survival/death was evaluated by MTT assay. The data in this figure are presented as the mean ± SEM of three independent experiments. (**$P < 0.01$ and ***$P < 0.001$) (c) Seventy-two hours after transfection, NIT-1 cells were treated with 10 mmol/l STZ for 1 h. Total cell lysates were subjected to immunoblotting using antibodies against PARP-1, phospho-JNK and β-actin.

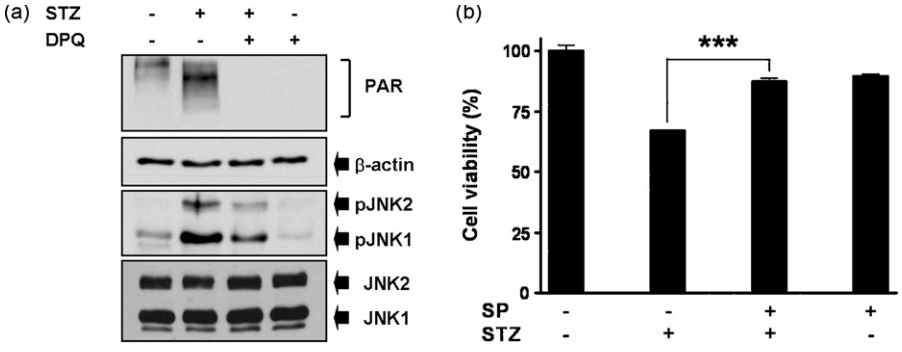

Fig. 6. JNK activation in primary islet cell death by STZ. (a) Pancreatic islet cells isolated from C57BL/6 mice were treated with STZ (10 mmol/l) for 1 h with or without DPQ (50 μmol/l) pretreatment for 1 h. Cell lysates were subjected to immunoblotting using anti-phospho-JNK, -total JNK, -PAR or β-actin antibody. (b) Primary islet cells were pretreated with JNK inhibitor, SP600125 (SP) for 1 h and then exposed to STZ or vehicle. After 24 h, MTT assay was done as in Fig. 1c. (***$P < 0.001$ compared to primary islet cells treated with STZ alone).

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3.6. Suppression of islet cell death by JNK inhibitors

We finally tested whether JNK activation by STZ plays a role in the death of primary islet cells as well as that of insulinoma cells. STZ treatment of primary murine islet cells from C57BL/6 mice for 1 h induced a strong activation of JNK, which was inhibited by pretreatment with DPQ in doses that inhibited PARP-1 activation (Fig. 6a), which is similar to the treatment of insulinoma cells with STZ and supports that JNK activation is downstream of PARP-1 activation. Similar to the case of NIT-1 insulinoma cells, STZ treatment for 24 h exerted cytotoxicity on islet cells which were significantly inhibited by SP600125 (Fig. 6b). These results indicated that JNK activation plays an important role in STZ-induced death not only of insulinoma cells but also of primary islet cells.

4. Discussion

In this investigation to study the role of JNK activation in STZ-mediated β-cell injury, we observed that JNK activation occurs downstream of PARP-1 and contributes to the β-cell death by STZ. Detail mechanism of JNK activation downstream of PARP-1 activation after STZ treatment of β-cells is not clearly elucidated. ROS production by PARP-1 activation appears to contribute to the JNK activation after STZ treatment of β-cells because ROS were produced in response to STZ and ROS are able to activate JNK by inhibiting phosphatases. MKPs that directly dephosphorylate JNK or other phosphatases that target the upstream of JNK may be involved in STZ-induced JNK activation (Kamata et al., 2005; Sakon et al., 2003). These results implicating the role for ROS in STZ-induced JNK activation probably through inhibition of phosphatases are consistent with previous papers showing the role for PARP-1 as an amplifier of ROS production in the course of cell death (Virag et al., 1998) and our data showing that phosphatase inhibitors not only induced JNK activation themselves but also abrogated the suppression of STZ-induced JNK activation by PARP inhibitors. Our data using chemical inhibitors of PARP or phosphatases do not appear to be due to their nonspecific effects since PARP-1 or MKP-1 siRNA has similar effects on JNK activation and insulinoma cell death. In contrast, a recent paper reported a role for MEKK-1, a MAPK3 kinase upstream of JNK rather than MKP in β-cell death by ROS or STZ (Mokhtari et al., 2008). While these reports implicated a role for ROS in β-cell injury by STZ, ROS may not be the only mediators inducing JNK activation in STZ-mediated β-cell injury because several ROS scavengers did not inhibit JNK activation after treatment of insulinoma cells with STZ (data not shown). For instance, PAR that is produced abundantly after STZ treatment has been reported to contribute to cell death (Yu et al., 2006).

While the main function of PARP-1 activation would be to maintain genomic DNA stability (de Murcia et al., 1997; Wang et al., 1997), new functions of PARP-1 are being recognized. PARP-1 appears to be critically involved in the regulation of transcriptional factors such as NF-κB, p53, AP-1 (Chiarugi, 2002) and HIF-1α (Martin-Oliva et al., 2006) since activation of such transcriptional factors is impaired in *PARP-1*-null cells or by PARP inhibitors. In glial cells, activation of transcriptional factors and production of inflammatory mediators were abrogated by targeted disruption of *PARP-1*, while such findings appear to be tissue-specific (Ha, 2004). Abrogation of JNK activation in *PARP-1*-null mouse embryonic fibroblasts (Xu et al., 2006) or by PARP inhibitors in our study may also be related to the role of PARP-1 in the activation of transcriptional factors as JNK is critically involved in the activation of c-Jun, a subunit of AP-1.

Our results demonstrated a role of JNK activation in STZ-induced β-cell death and diabetes. JNK plays an important role in the pathogenesis of diabetes. In type 2 diabetes, JNK activation is critically involved in insulin resistance (Hirosumi et al., 2002; Solinas et al., 2006). In β-cells of type 2 diabetes models, JNK activation decreases insulin production from them (Kaneto et al., 2002) and induces lipotoxicity (Martinez et al., 2008). While mechanistically different, JNK is also involved in type 1 diabetes. JNK has been reported to play a role in the differentiation of Th1/Th2 cells and cytokine-mediated β-cell injury (Bonny et al., 2001; Jaeschke et al., 2005). A recent paper reported that the incidence of diabetes after multiple low-dose STZ treatment, an artificial model of type 1 diabetes, was lower in *JNK1*-null mice, which was attributed to decreased cytokine production in those mice (Fukuda et al., 2008).

In conclusion, these results suggest that PARP-1 plays important roles in the activation of various transcriptional factors or MAP kinases such as JNK and production of inflammatory mediators or ROS in addition to its classical role in the protection of genomic DNA. In the course of such intracellular processes, JNK appears to be activated downstream of PARP-1 activation which contributes to the execution of cell death by STZ.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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