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Regulation of high glucose-induced apoptosis by mitochondrial NADP$^+$-dependent isocitrate dehydrogenase

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Abstract

A high concentration of glucose has been implicated as a causal factor in initiation and progression of diabetic kidney complications, and there is evidence to suggest that hyperglycemia increases the production of free radicals and oxidant stress. Recently, we demonstrated that the control of mitochondrial redox balance and the cellular defense against oxidative damage is one of the primary functions of mitochondrial NADP$^+$-dependent isocitrate dehydrogenase (IDPm) to supply NADPH for antioxidant systems. In this report, we demonstrate that modulation of IDPm activity in HEK293 cells, an embryonic kidney cell line, regulates high glucose-induced apoptosis. When we examined the protective role of IDPm against high glucose-induced apoptosis with HEK293 cells transfected with the cDNA for mouse IDPm in sense and antisense orientations, a clear inverse relationship was observed between the amount of IDPm expressed in target cells and their susceptibility to apoptosis. The results suggest that IDPm plays an important protective role in apoptosis of HEK293 cells induced by a high concentration of glucose and may contribute to various pathologies associated with the long-term complications of diabetes.

Keywords: Diabetes; Isocitrate dehydrogenase; HEK293; Apoptosis; Antioxidant enzyme

Autoxidation of sugars, and of the products of non-enzymatic glycation of proteins is a free radical-mediated reaction that occurs under aerobic conditions. It has been suggested that oxidative stress induced by hyperglycemia is a key factor in the pathogenesis of diabetic complications [1–5]. The glycation proceeds through the formation of a Schiff base between the carbonyl group of sugar and an α- or ε-amino group in a protein together with Amadori rearrangement to yield a relatively stable ketoamine [6,7]. Such a reaction has been expected to occur in various kinds of proteins under physiological conditions. Recently, it has been proposed that generation of reactive oxygen species (ROS) and accumulation of advanced glycation end products (AGE) trigger apoptosis in renal cells in diabetic hyperglycemia [8,9].

Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2 [10], catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides [11]. Glucose 6-phosphate dehydrogenase (G6PD), which is a key enzyme for the generation of NADPH, and glutathione reductase, which is involved in the regeneration of reduced glutathione

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(GSH), are also considered as essential antioxidant enzymes [12]. These enzymes normally act as a team, thus SOD protects catalase and peroxidase against inhibition by O_2^- [13], while catalase and peroxidase provide protection for SOD against inactivation by hydrogen peroxide [14].

The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [15]. NADPH is an essential reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [16,17], both being important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. We recently reported that mitochondrial ICDH (IDPm) is involved in the supply of NADPH needed for GSH production against mitochondrial oxidative damage [18].

In the present report, we demonstrate that modulation of IDPm activity in HEK 293 cells regulated apoptosis induced by a high concentration of glucose. These results suggest that IDPm has an important protective role in hyperglycemia-induced apoptosis, presumably, through acting as an antioxidant enzyme.

Materials and methods

Materials. D-Glucose, β-$NADP^+$, isocitrate, propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI), and xylenol orange were purchased from Sigma Chemical (St. Louis, MO, USA). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) and rhodamine 123 dye were purchased from Molecular Probes (Eugene, OR). Electrophoresis reagents and Bio-Rad protein assay kit were purchased from Bio-Rad (Hercules, CA, USA). Antibodies against Bcl-2, Bax, lamin B, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz (Santa Cruz, CA). A polyclonal antibody against IDPm was prepared from IDPm-immunized rabbit, and the antibody was purified by protein A affinity chromatography.

Cell culture and cytotoxicity. HEK 293 cells, an embryonic kidney cell line, with stable transfections with the cDNA for mouse mitochondrial ICDH in sense and antisense orientations were prepared as described [18]. The HEK293 cell line transfected with LNCX-vector alone was used as a control. HEK293 cells were grown in DMEM containing 10% fetal bovine serum and 50 U/ml penicillin/50 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2.

Enzyme assay. Cells were collected at 1000*g* for 10 min at 4 °C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris–Cl, pH 7.4). Cell homogenate was centrifuged at 1000*g* for 5 min and the supernatants were further centrifuged at 15,000*g* for 30 min. The supernatants were added by 1/10 volume of 10× PBS containing 1% Triton-X 100, which finally made the solution 1× PBS containing 0.1% Triton-X 100. The supernatants were used to measure the activities of several cytosolic enzymes. The precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1× PBS containing 0.1% Triton-X 100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000*g* for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad. Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the decrease in absorbance at 240 nm. SOD activity in cell extracts was assayed spectrophotometrically using a pyrogallol assay, where 1 U of activity is defined as the quantity of enzyme which reduces the superoxide-dependent color change by 50%. Glutathione reductase activity was quantified by the GSSG-dependent loss of NADPH as measured at 340 nm ($\varepsilon = 6.67\ mM^{-1}\ cm^{-1}$). Reaction mixture contained 0.1 mM NADPH, cell-free extract, 1 mM GSSG, 1 mM EDTA, and 0.1 M potassium phosphate, pH 7.4, in a final volume of 1.5 ml. G6PD activity was measured by following the rate of $NADP^+$ reduction at 340 nm using the procedure described [18]. For the measurement of IDPm activity, mitochondrial extract was added to 1 ml Tris buffer, pH 7.4, containing 2 mM $NADP^+$, 2 mM $MgCl_2$, and 5 mM isocitrate. Activity of IDPm was measured by the production of NADPH at 340 nm at 25 °C [18]. One unit of IDPm activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min.

Immunoblot analysis. Proteins were separated on 10% SDS–polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham–Pharmacia Biotech).

DAPI staining. DAPI staining was used for apoptotic nuclei determination. HEK 293 cells were collected at 2000*g* for 5 min, washed once with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, and stained with 0.8 μg/ml DAPI in the dark state [19]. The morphological changes of apoptotic cells were analyzed by the Zeiss Axiovert 200 microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).

FACS. To determine the portion of apoptotic cells, cells were analyzed with PI staining [20]. Untreated or glucose-treated HEK293 cells were collected at 2000*g* for 5 min and washed once with cold PBS, fixed in 70% ethanol, decant ethanol by centrifuge, and stained with 1 ml solution containing 50 mg/ml PI, 1 mg/ml RNase A, and 1.5% Triton X-100 for at least 1 h in the dark at 4 °C. Labeled nuclei were subjected to flow cytometric analysis and then gated on light scatter to remove debris, and the percentage of nuclei with a sub-G_1 content was considered apoptotic cells.

Cellular redox status. NADPH was measured using the enzymatic cycling method as described by Zerez et al. [21]. Briefly, the reaction mixture, which combined 100 mM Tris (pH 8.0), 5 mM EDTA, 2 mM phenazine ethosulfate, 0.5 mM MTT, 1.3 U glucose 6-phosphate dehydrogenase, and appropriate amounts of the cell extracts was preincubated for 5 min at 37 °C. The reaction was started by the addition of 1 mM glucose 6-phosphate. The absorbance at 570 nm was measured for 3 min. The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$) as the method described by Akerboom and Sies [22], and oxidized glutathione (GSSG) was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine [23]. Hydrogen peroxide oxidizes ferrous (Fe^{2+}) to ferric ion (Fe^{3+}) selectively in dilute acid and the resulting ferric ions can be determined using a ferric sensitive dye, xylenol orange, as an indirect measure of hydrogen peroxide concentration. Mitochondrial fractions were added to FOX solution (0.1 mM xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H_2SO_4) and incubated at room temperature for 30 min, and absorbance was measured at 560 nm. Hydrogen peroxide was used to draw standard curve as described [24]. Intracellular peroxide production was measured using DCFH-DA and spectrofluorimetry as described [25].

Membrane potential transition. Mitochondrial membrane potential transition (MPT) was measured by the incorporation of rhodamine 123 dye into the mitochondria, as previously described [26]. Cells

(1×10^6) grown on poly-L-lysine coated slide glasses were exposed to ionizing radiation. Cells were then treated with 5 μM rhodamine 123 for 15 min and excited at 488 nm with an argon laser. Cells were double-stained with 100 nM MitoTracker Red, which is a morphological marker of mitochondria. The fluorescence images at 520 nm were simultaneously obtained with a laser confocal scanning microscope.

Replicates. Unless otherwise indicated, each result described in the paper is representative of at least three separate experiments.

Results

To study the relationship between IDPm activity and high glucose-induced apoptotic cell death, the three kinds of HEK293 transfectant cells were constructed. The HEK293 cells were transfected with the LNCX containing either an IDPm gene as a sense orientation, IDPm(+), as an antisense orientation, IDPm(−), or the LNCX alone, control. Chromosomal integration of the transfected IDPm constructs was confirmed by polymerase chain reaction (PCR) (data not shown). The IDPm activity of IDPm(+) cells was increased sixfold compared with that of the control cells. In contrast, IDPm(−) cells exhibited 30% less IDPm activity when compared with that of the control (Fig. 1A). Immunoblot analysis using anti-IDPm antibody further confirmed the correlation between the amount of IDPm enzyme measured in cell extracts by immunoreaction and the corresponding levels of enzyme activity (Fig. 1B). Increased expression of IDPm(+) or reduced expression of IDPm(−) did not significantly alter the

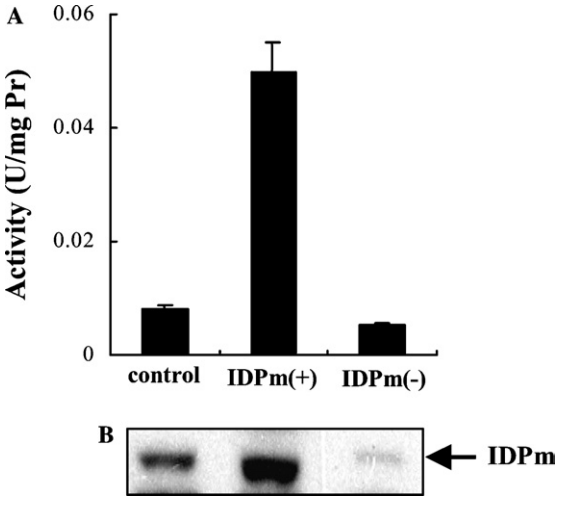

Fig. 1. (A) Activity of IDPm in HEK293 transfectant cells. Control, IDPm(+), and IDPm(−) denote the cells expressing LNCX-vector alone, LNCX-sense IDPm, and LNCX-antisense IDPm, respectively. The results shown are means ± SD of three separate experiments. (B) Immunoblot analysis of IDPm protein expressed in HEK293 transfectant cells. The mitochondrial fractions (20 μg protein) from cultured cells were separated on 10% SDS–polyacrylamide gel, transferred to nitrocellulose membrane, and then subjected to immunoblot analysis using anti-IDPm IgG.

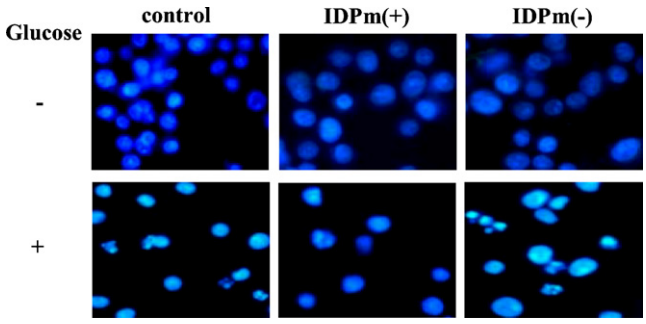

Fig. 2. High glucose-induced nuclear condensation and fragmentation in HEK293 transfectant cells. IDPm transfectant HEK293 cells were exposed to 50 mM glucose for 3 days, and then harvested, fixed, permeabilized, and loaded with 0.8 μg/ml DAPI for 5 min. The morphological changes of cells were analyzed by fluorescence microscopy (excitation, 351 nm; emission, 380 nm).

activities of other antioxidant enzymes such as SOD, catalase, G6PD, and glutathione reductase (data not shown), suggesting that the transfection of IDPm cDNA did not affect the activities of other enzymes involved in antioxidation.

Exposure of HEK293 cells to 50 mM glucose for 3 days caused shrinkage of the cell and plasma membrane blebbing that was apparent by light microscopy (data not shown). To assess whether these changes were attributable to apoptotic changes, nuclear morphology was assessed by fluorescence microscopy using DAPI and flow cytometry using PI. As shown in Fig. 2, nuclear condensation and fragmentation were apparent in IDPm(−) cells treated with glucose when compared to control and IDPm(+) cells. Fig. 3 shows a typical cell cycle plot of HEK293 transfectant cells that were untreated or treated with 50 mM glucose for 3 days. Apoptotic cells were estimated by calculating the number of subdiploid cells in the cell cycle histogram. When cells were exposed to glucose, apoptotic cells were increased markedly in IDPm(−) cells as compared to IDPm(+) cells.

To investigate the role of IDPm in cellular defense against high glucose-induced oxidative stress, we determined the cellular redox status in HEK293 transfectants unexposed or exposed to high glucose. As shown in Fig. 4A, the treatment of 50 mM glucose for 3 days resulted in a significantly higher intracellular level of hydrogen peroxide in IDPm(−) cells when compared with those of control and IDPm(+) cells. The levels of intracellular peroxides in HEK293 cells were evaluated with an oxidant-sensitive probe DCFH-DA. As shown in Fig. 4B, an increase in DCF fluorescence was observed in IDPm(−) cells when they were exposed to 50 mM glucose for 3 days. The increase in fluorescence was significantly reduced in IDPm(+) cells. NADPH, required for GSH generation by glutathione reductase, is an essential factor for the cellular defense against

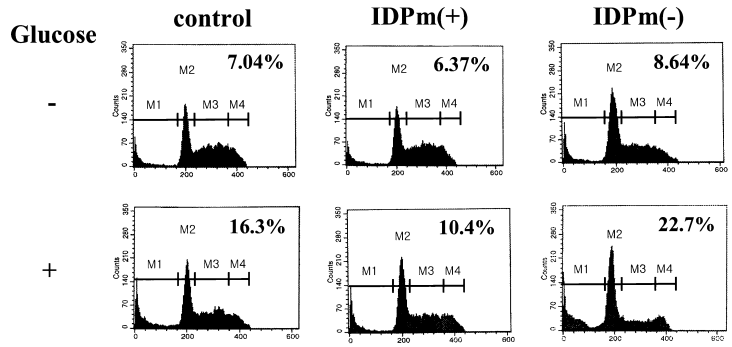

Fig. 3. High glucose-induced apoptosis in HEK293 transfectant cells. Cell cycle analysis with cellular DNA content was examined by flow cytometry. The sub-G_1 region (presented as 'M1') includes cells undergoing apoptosis. The number of each panel refers to the percentage of apoptotic cells.

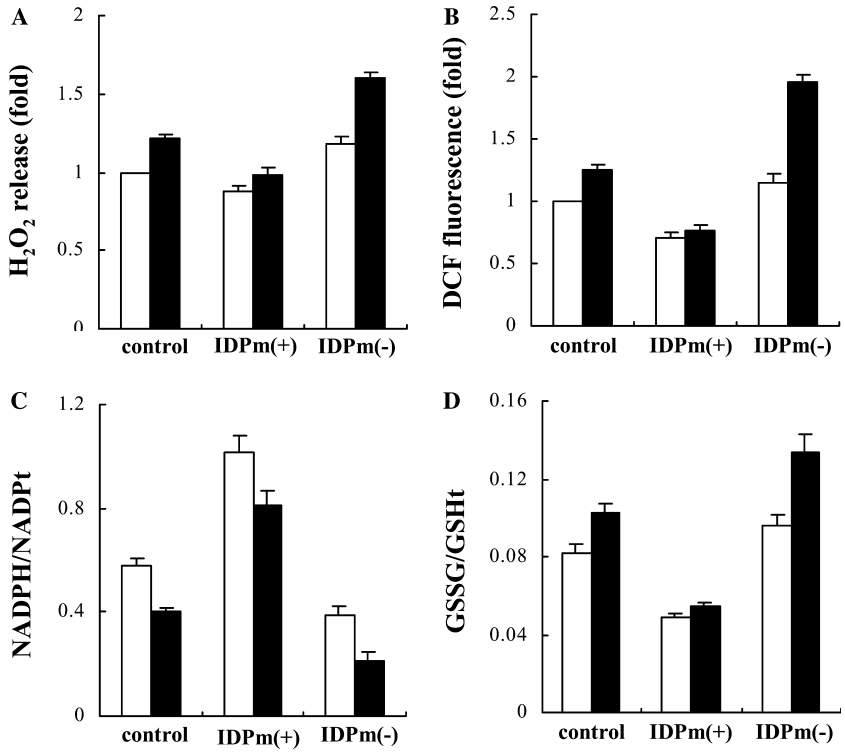

Fig. 4. (A) Production of hydrogen peroxide in HEK293 transfectant cells exposed to high glucose was determined by the method described under Materials and methods. The results shown are means ± SD of five separate experiments. (B) Measurement of in vivo molecular oxidation. DCF fluorescence was measured in HEK293 transfectant cells exposed to high glucose. Fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. The results shown are means ± SD of five separate experiments. Ratios of NADPH versus total NADP pool (C) and GSSG versus total GSH pool (D) in HEK293 transfectant cells. The results shown are means ± SD of five separate experiments. Open and shaded bars represent the cells unexposed and exposed to high glucose, respectively.

oxidative damage. The ratio for [NADPH]/[$NADP^+$ + NADPH] was significantly decreased in IDPm(−) cells treated with high glucose, however, the decrease in this ratio was much less pronounced in IDPm(+) cells (Fig. 4C). One important parameter of GSH metabolism is the ratio of GSSG/total GSH (GSH_t) which may reflect the efficiency of GSH turnover. When the cells were exposed to 50 mM glucose for 3 days, the ratio for cellular [GSSG]/[GSH_t] was significantly higher in IDPm(−) cells compared to those of control and IDPm(+) cells (Fig. 4D). These data indicate that GSSG in IDPm(−) cells was not reduced as efficiently as in IDPm(+) cells. Taken together, these results strongly suggest that the decrease in the efficiency of GSH recycling may be responsible for the higher concentration of intracellular peroxides in IDPm(−) cells treated with high glucose.

Mitochondrial changes occur in cells undergoing apoptosis, and some of the alterations may be causally involved in the cell death process. MPT associated with the opening of large pores in the mitochondrial

membranes [27]. To answer whether IDPm modulates the MPT upon exposure to high glucose, we determined the change in MPT by intensity of fluorescence emitting from a lipophilic cation dye, rhodamine 123. Significantly less rhodamine 123 dye was taken up by the mitochondria of IDPm(−) cells, compared with IDPm(+) cells (Fig. 5).

Caspase-3 activation in HEK293 cells was assessed by immunoblot analysis of lysates from cells that had been exposed to high glucose. As shown in Fig. 6, the appearance of the apoptotic-cleaved product of caspase-3 was identified in HEK293 cells treated with high glucose, and the cleavage was more pronounced in IDPm(−) cells when compared to that in IDPm(+) cells. High glucose also induced the formation of fragments which represents proteolytic fragments of PARP and lamin B, indicating an oncoming apoptotic process. The cleaved products of PARP (a 89-kDa band) and lamin B (96- and 64-kDa bands) increased markedly in IDPm(−) cells compared to IDPm(+) cells upon exposure to high glucose. Taken together, glucose-induced cleavage of procaspase-3 into the active form of caspase-3 and caspase-3 induces degradation of PARP or lamin B. The results also indicate that IDPm exhibits a protective effect on the high glucose-induced apoptosis. The role of mitochondrial pathway of apoptosis in the high glucose-induced death of HEK293 cells was examined by immunoblot analysis of the abundance of Bcl-2, an antiapoptotic protein, and of Bax, an proapoptotic protein. As shown in Fig. 6, the abundance of Bcl-2 in HEK 293 cells was significantly decreased in IDPm(−) cells as compared to that of IDPm(+) cells when exposed to high glucose. The amount of Bax was increased after treatment with high glucose, and it was significantly increased in IDPm(−) cells as compared to that of IDPm(+) cells.

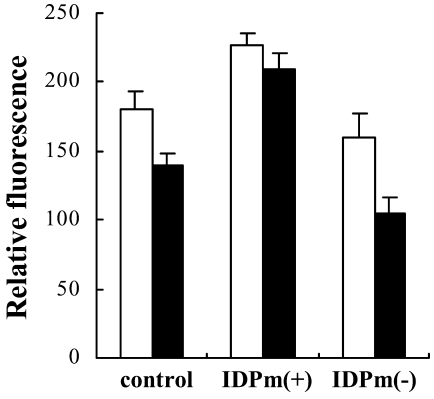

Fig. 5. MPT of HEK293 transfectant cells was measured by the incorporation of rhodamine 123 dye into the mitochondria. The average of fluorescence intensity was calculated as described [37]. Open and shaded bars represent the cells unexposed and exposed to high glucose, respectively. The results shown are means ± SD of three separate experiments.

Discussion

Both AGE accumulation and oxidative stress are involved in the pathogenesis of diabetic complications such as nephropathy, retinopathy, and neuropathy

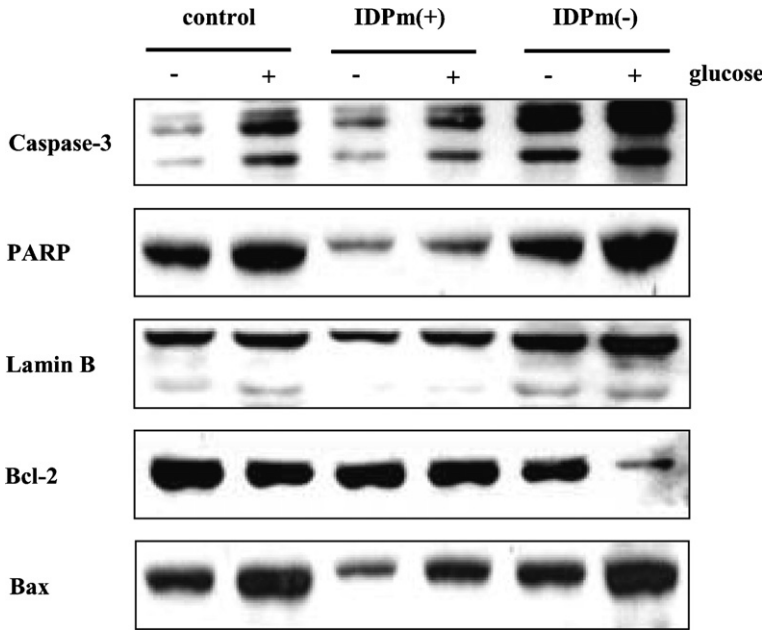

Fig. 6. Immunoblot analysis of various apoptosis-related proteins in HEK293 transfectant cells untreated or treated with 50 mM glucose for 3 days. Cell extracts were subjected to 10–12.5% SDS–PAGE and immunoblotted with antibodies against cleaved caspase-3, cleaved PARP, lamin B, Bcl-2, and Bax.

[1,28]. Prevention of synthesis and tissue accumulation of AGE- or oxidative-derived end products could constitute a major advance in the treatment of diabetic complications. Our observations are consistent with the hypothesis that ROS play a pivotal role in apoptosis and that reductants can block or delay this process [29]. In the present study, we examined the apoptotic pathway initiated by high glucose in HEK293 cells. A temporal pattern of events was observed, starting from perturbation of redox status reflected by the modulation of intracellular ROS generation, GSH recycling, and NADPH pool, followed by MPT alteration, caspase-3 activation, cleavage of caspase target proteins, and finally DNA fragmentation. The IDPm expression significantly improved redox status and inhibited the whole apoptotic pathway.

IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria. NADPH is an essential cofactor for the regeneration of GSH, the most abundant low-molecular-mass thiol in most organisms, by glutathione reductase in addition to its critical role for the activity of NADPH-dependent thioredoxin system [16,17]. The oxidized form of thioredoxin, with a disulfide bridge between the half-cystines, can be reduced by NADPH in the presence of a flavoprotein, thioredoxin reductase [30]. Reduced thioredoxin may provide reducing equivalents to at least two enzymes, thioredoxin peroxidases, which remove hydrogen peroxide using hydrogen provided by the NADPH-dependent thioredoxin system [16,17], and methionine sulfoxide reductase, which can reactivate damaged proteins at their methionine residues [30], presumably, involved in the defense against oxidative stress. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppressed the oxidative stress and concomitant ROS-mediated damage. It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states caused by genetic mutations as well as exogenous compounds or agents [31]. Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase [32], which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and cofactor for mitochondrial thioredoxin peroxidase family/peroxiredoxin family including peroxiredoxin III/protein SP-22 [33–35] and peroxiredoxin V/AOEB166 [36]. Therefore, any mitochondrial NADPH producer, if present, becomes critically important for cellular defense against ROS-mediated damage.

In conclusion, IDPm plays an important protective role by suppressing mitochondrial dysfunctions in high glucose-induced apoptosis of HEK293 cells and may contribute to various pathologies associated with the long-term complications of diabetes.

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