Study of High Glucose-Induced Apoptosis in PC12 Cells: Role of Bax Protein

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Abstract. Hyperglycemia, which occurs under the diabetic condition, induces serious diabetic complications. Diabetic neuropathies, affecting the autonomic, sensory, and motor peripheral nervous system, are among the most frequent complications of diabetes. Little is known about the direct toxic effect of high glucose concentrations on neuronal cells. Therefore in the present study, glucose-induced toxicity was studied in PC12 cells as an in vitro cellular model for diabetic neuropathy using the MTT assay. The possible role of apoptosis was also investigated in this toxicity. The result showed that a 3-fold increase in optimum glucose concentration for PC12 cells (13.5 mg/ml) significantly reduced cell viability after 48 h. In Western blot analysis, the ratio of Bax/Bcl-2 protein expression in cells treated with high glucose was significantly increased compared to controls. Additionally high glucose could induce a DNA ladder pattern in PC12 cells, a hallmark of apoptosis indicating nuclear fragmentation. From our present results, it may be concluded that high glucose can cause PC12 cell death, in which apoptosis plays an important role possibly by the mitochondrial pathway through higher expression of Bax pro-apoptotic protein.

Keywords: PC12, glucose, toxicity, apoptosis

Introduction

Diabetes mellitus is a world wide health problem affecting 1% – 2% of the population causing high morbidity and mortality (1). It is a disorder of glucose metabolism where insulin secretion from β cells and glucose uptake into insulin-consuming cells are deteriorated (2). Hyperglycemia, which occurs under the diabetic condition, induces serious diabetic microvascular complications such as neuropathy, nephropathy, and retinopathy. Diabetic neuropathies, affecting the autonomic, sensory, and motor peripheral nervous system are among the most frequent complications of diabetes. As a result, diabetic neuropathy is the most common cause of non-traumatic amputations and autonomic failure (3, 4). Recent studies suggest that elevated blood glucose (hyperglycemia) is a major cause of nervous system damage (5). Little is known about the direct toxic effect of high glucose concentrations on neuronal cells. Apoptosis could be proposed as a possible mechanism for high glucose-induced neuronal cell death. It has been established that apoptosis contributes to neuronal loss in most neurodegenerative diseases (6). Apoptosis is a gene-regulated phenomenon that is important in both physiological and pathological conditions and characterized by distinct morphological features, including chromatin condensation, cell and nuclear shrinkage, membrane blebbing, and oligonucleosomal DNA fragmentation (7). Two major apoptotic pathways have been identified: 1) the death receptor-mediated pathway and 2) the mitochondrial apoptotic pathway. The mitochondrial pathway involves release of mitochondrial apoptotic proteins such as cytochrome c (8, 9). Although
the mechanism(s) that underlies the release of mitochondrial apoptotic proteins remains uncertain, the Bcl-2 family members play a central role in regulating changes in mitochondrial outer membrane permeability. Studies have shown that the anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL, and Mcl-1 appear to preserve the integrity of the outer mitochondrial membrane by binding to mitochondrial channels. Apoptosis proceeds when the proapoptotic proteins Bax and Bak bind to the mitochondrial outer membrane, where they initiate changes in mitochondrial outer membrane permeability (10, 11).

The pheochromocytoma cells (PC12) derived from a catecholamine-secreting adrenal chromaffin tumor in rats (12) are a suitable model for studying neuronal cell death (13, 14). Therefore, we investigated whether glucose could induce apoptosis in these neuronal cells and studied the role of anti-apoptotic (Bcl-2) and pro-apoptotic protein (Bax) expression in this toxicity.

Material and Methods

Cell culture

PC12 cells were obtained from Pasteur Institute (Tehran, Iran). Cells were maintained at 37°C in a 90% humidified atmosphere containing 5% CO₂. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5% (v/v) fetal bovine serum, 10% (v/v) horse serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The concentration of glucose in DMEM was 4.5 mg/ml. According to the manufacturer’s instructions, PC12 cells were maintained with culture medium containing 4.5 mg/ml glucose. Thus a 3-fold concentration of glucose (13.5 mg/ml) was considered as a hyper-glucose medium.

After two passages, PC12 cells were plated at the density of 5000 per well in a 96 micro plate well for the MTT assay. Control cells were grown in DMEM with 4.5 mg/ml glucose and the other cells grown in DHEM with 13.5 mg/ml glucose. For protein and DNA extraction, cells were seeded in a 75-cm² plastic cell culture flask and allowed to attach and grow for 24 h. Then the cells were incubated with 13.5 mg/ml of glucose for 24 h. Cells were detached using cold 0.25% trypsin in phosphate-buffered saline (PBS).

MTT assay

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay (15). In brief, MTT solution (5 mg/ml in DMEM) was added to the 96-well plates and the cells were allowed to incubate for 1 h at 37°C. After removing of medium, the cells and dye crystals were solubilized by adding 100 µl of DMSO, and the absorption was measured at 570 nm by an ELISA reader.

Western blotting

Western blot analysis was performed as described previously (16). Briefly, proteins were measured with the Bio-Rad protein assay method. Equal amounts of protein (15 µg/well) were loaded and separated by SDS-polyacrylamide gel (15%) electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was incubated with the first antibodies (Bax and Bcl-2) and second antibody (anti-rabbit IgG conjugated with alkaline phosphatase). Bands were visualized using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro blue tetrazolium.

DNA laddering analysis

DNA extraction was performed as described by Gurr (17). Briefly, the cells (5 × 10⁶) were fixed with 70% ethanol and then stored at −20°C for 24 h. Then they were incubated with 40 µl of phosphate-citric acid buffer at room temperature for 30 min, centrifuged (1,500 × g, 5 min), and the supernatant was dried in a concentrator. The powder was incubated with 7 µl of 0.25% Nonidet P-40 and 7 µl of 1 mg/ml RNase, at 37°C for 30 min. A 7-µl aliquot of 1 mg/ml proteinase K was added to the solution and incubated at 37°C for another 30 min. The mixture was loaded on a 2% agarose gel and electrophoresed at 85 V.

Statistical analyses

All results evaluated using the mean ± S.E.M. The results were analyzed by ANOVA and Bonferroni’s test. A probability level of P<0.05 was considered significant.

Results

Effect of glucose on cell viability

The effects of high concentration of glucose on PC12 cells viability were examined using the MTT assay. After the initial 24-h attachment period, the cells were exposed to glucose at the concentration of 13.5 mg/ml for 24, 48, 72, and 96 h. As shown in Fig. 1, glucose (13.5 mg/ml) could decrease the viability of PC12 cells after 48 h. This toxicity was time-dependently increased, so increasing the incubation time caused higher toxicity (Fig. 1).

Expression of Bax and Bcl-2 proteins

A 72-h incubation with glucose at 13.5 mg/ml enhanced expression of pro-apoptotic Bax protein
compared to the control (Fig. 2). While the expression of Bcl-2 protein did not change significantly (Fig. 2), the Bax/Bcl-2 ratio increased in glucose treated cells after 72 h (Fig. 3). There were no significant changes in expression of Bcl-2 protein compared to the control (Fig. 2), but the Bax/Bcl-2 ratio increased in the high glucose-treated samples (Fig. 3).

**DNA fragmentation**

A 72-h incubation with high glucose (13.5 mg/ml) induced nucleus fragmentation in the cells. As shown in Fig. 4, while glucose has produced a DNA ladder pattern that is characteristic of the apoptotic process in the treated cells, there was no fragmentation in the control cells.

**Discussion**

Despite considerable investigations, the precise mechanism(s) of diabetic neuropathy is not clearly known (18). The present study demonstrated that high concentration of glucose could induce neuronal cell death. Based on the previous study, the optimal glucose concentration for PC12 cell-culture (in vitro) was 4.5 mg/ml (19), which was higher than the human serum glucose concentrations. Therefore, to prepare the condition of high glucose concentration for PC12 cells, 13.5 mg/ml glucose was used as previously being shown by Koshimura et al. (19).

The present result showed that the viability of PC12 cells was significantly decreased starting after 48 h of incubation with 13.5 mg/ml of glucose in the culture medium and continued to decrease in a time dependent manner. To exclude the role of osmolarity on cell toxicity, we referred to Koshimura et al. who showed
and suggested that the neurotoxicity of glucose is not related to osmolarity; instead, it is a direct effect of glucose on the cell, so that L-glucose, a non-utilizable form of glucose, does not have a toxic effect, while D-glucose, the natural form of glucose, could cause cell toxicity (19). In contrast to the current results, Koshimura et al. (19) reported that the viability of differentiated PC12 cells cultured in 13.5 mg/ml of glucose decreased after 7 days of culture, but not at 3 days of culture. Therefore, compared to the previous report, the present study has indicated that undifferentiated PC12 cells were possibly more sensitive to glucose toxicity than differentiated cells.

In present study, the role of apoptosis in glucose-induced toxicity was studied by two different and distinct methods: DNA fragmentation, a hallmark of apoptosis and expression levels of pro- and anti-apoptotic proteins involved in apoptosis (Bax and Bcl-2). The morphological characteristics of apoptosis are frequently accompanied by cleavage of DNA into 180 – 200 base pair multimers. The oligonucleosomal-sized fragments can be visualized as a characteristic DNA ladder using agarose gel electrophoresis (20).

The current result for the first time to our knowledge showed that Bax expression was significantly increased by a glucose concentration of 13.5 mg/ml after 72 h, whereas the expression of Bcl-2 was not significantly altered. The Bax/Bcl-2 ratio, an important index of apoptotic cell death was significantly increased, indicating glucose-induced apoptosis in PC12 could possibly be mediated by the mitochondrial pathway (21, 22).

To elucidate the possible mechanism of high glucose-induced neurotoxicity, it has previously been shown that high glucose could elevate ROS in PC12 cells (21). It has also been indicated that high glucose could elevate nitric oxide synthase and in turn nitric oxide production due to elevation of intracellular calcium in PC12 cells (19, 22). Indeed, the concomitant generation of sueroxide and nitric oxide favors the production of peroxynitrite (ONOO⁻).

There is compelling evidence that three major signals cause the release of apoptogenic mitochondrial mediators, including a proapoptotic member of the BCL2 family, elevated level of intracellular calcium, and reactive oxygen species or free radicals (23). It has previously been shown that both ROS and NO can change mitochondrial membrane potential by opening the mitochondrial permeability transition pores (mPTP), releasing cytochrome C, and subsequent caspase 9 and 3 activation and eventually causing cell death. Members of the BCL2 family are known to be pro and anti-apoptotic. The balance between pro and anti-apoptotic signals from this family has a crucial role in the release of cytochrome C and its subsequent consequences.

Based on the results obtained in present study and referring to the previous reports, it could be speculated that high glucose induced by elevation of oxidative and nitrosative stresses, including ROS, NO, and also intracellular calcium, causes opening of mPTP. Therefore apoptosis may be induced by mPTP opening caused by the direct effect of ROS, NO, and intracellular calcium and/or by higher expression of Bax protein, an apoptotic facilitating protein, leading to release of mitochondrial mediators such as cytochrome C, which is a caspase-dependent pathway. On the other hand, apoptosis may also be induced by endonuclease G causing DNA fragmentation, which is a non-caspase-dependent pathway (23 – 26).

It may finally be concluded that the high glucose can cause PC12 cell death, in which apoptosis plays an important role by over-expression of Bax protein possibly through opening of mPTP and mitochondrial mediator release, a caspase-dependent pathway, and also fragmentation of DNA possibly by activation of endonuclease G, a non-caspase-dependent pathway.

References