Role of Bax Protein and Caspase-3 at High Glucose-Induced Apoptosis in Human Embryonic Kidney (HEK) 293 Cells

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Introduction

Diabetic nephropathy is a major cause of end stage renal disease in many countries and associated with increased morbidity and mortality in diabetic patients [1]. The pathophysiological processes that lead to diabetic nephropathy involve participation of different factors in a complex picture [2]. Current treatments include strict control of glucose concentration and blood pressure. However, these therapeutic strategies still provide imperfect protection against disease progression [3, 4]. Understanding the precise molecular mechanisms of diabetic nephropathy is vital as it may reveal novel drug targets that may be useful in its prevention and in slowing its progression [5, 6].

In recent years, a wide range of studies have been carried out, from various points of view, to understand the progress of renal dysfunction in diabetic nephropathy [7, 8]. However, the etiology of the progressive changes finally resulting in diabetic nephropathy is not fully elucidated yet [9]. It has long been shown that renal cells are uniquely susceptible to a variety of metabolic and hemodynamic factors associated with diabetes, especially hyperglycemia [10].

Hyperglycemia plays a crucial role in the development of serious diabetic microvascular complications such as neuropathy, nephropathy, and retinopathy [11]. Glucose dependent pathways such as oxidative stress and polyol pathway flux are activated within diabetic renal tissues. These pathways result in structural and functional changes of renal cells [12, 13]. With the passage of time, however, diabetic kidneys undergo progressive loss of mass and many studies suggest that high glucose-induced apoptotic cell death may be accelerated in chronic diabetic nephropathy [14-16]. Hyperglycemia has also been shown to induce in vitro apoptosis of several other non-kidney cells [17]. Apoptosis is a genetically controlled type of cell death and is characterized by cell shrinkage, chromatin condensation and fragmentation of nucleosomal DNA [18]. The regulations of apoptosis are complex and two important pathways are involved: 1) the death receptor-mediated pathway and 2) the mitochondrial apoptotic pathway. In the mitochondrial pathway, the loss of mitochondrial transmembrane potential may initiate cytochrome c release, which finally activates caspase cascade [19]. Bcl-2 family members serve as critical regulators of mitochondrial outer membrane permeability. The anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL,
and McI-1 appear to preserve the integrity of the outer mitochondrial membrane by binding to mitochondrial channels [20]. Apoptosis proceeds when the proapoptotic proteins Bax and Bak bind to the mitochondrial outer membrane, where they initiate changes in mitochondrial outer membrane permeability [21].

Caspases, a family of cysteine proteases, are key players in the initiation and execution of apoptosis [22]. Among the family of caspases, caspase-3, in particular, is believed to be one of the most commonly involved mechanisms in the execution of apoptosis in various cell types [23]. Human embryonic kidney 293 cells, also often referred to as HEK 293 cells derived from human embryonic kidney cells grown in tissue culture are a suitable model for studying renal cells [24, 25]. The apoptosis signaling induced by glucose in renal cells remains poorly investigated. Therefore, in this study we examined the effect of high glucose on apoptosis in HEK293 cells and in particular the contributing role of anti-apoptotic (Bcl-2) and proapoptotic (Bax) protein expression, as well as caspase-3, the executioner of apoptosis in this toxicity.

Materials and Methods

HEK 293 cells were obtained from Pasteur Institute (Tehran, Iran). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10% (v/v) horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a 90% humidified atmosphere (5% CO₂). The culture medium was changed every 48 h, and the cells were subcultured every 2-3 days.

MTT assay: After two passages, HEK 293 cells were seeded in 96-well microplates at a density of 5000/well. Control cells were grown in DMEM with 23 mM glucose and the other cells grown in DMEM with 100 mM glucose as high glucose condition. The cell viability was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay [26]. 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 (Dimethyl Sulfoxide), and the absorption was measured at 570 nm by an ELISA reader.

For protein 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 exposed to normal (23 mM) or high glucose (100 mM) glucose for 72 h. Adherent and floating cells were collected and homogenized in ice-cold lysis buffer (1×PBS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.1 mg/ml phenylmethylsulphonyl fluoride (PMSF), 30 µl/ml aprotinin). Total cell lysates were incubated on ice for 1 h after a further addition of PMSF (10 µl of 10 mg/ml solution), and centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was collected and stored at -80°C until analysis. The protein concentration was determined using the Bradford method [27].

Western blot analysis was performed as described previously [28]. Briefly, equal amounts of proteins, 60 µg from each sample, were loaded and separated by SDS-PAGE (PolyAcrylamide Gel Electrophoresis) and transferred onto a PVDF (Polyvinylidene difluoride) membrane which was incubated with primary (Bax, Bcl-2 and procaspase-3) and secondary antibodies (anti-mouse IgG conjugated with horse-radish peroxidase). To control the protein loading, the membranes were probed with a rabbit anti- β-Actin antibody. Bands were visualized using ECL (Elec tro Chemiluminescence) detection reagents. The bands were quantified by densitometric analysis.

Caspase-3 activity was measured by colorimetric assay based on the recognition of specific amino acid sequences by this caspase. In the assay system, the tetrapeptide substrate is labeled with the chromophore p-nitroaniline (pNA). pNA is released from the substrate upon cleavage by the caspase. Free pNA produces a yellow color that is monitored by an ELISA reader at 405 nm. The amount of yellow color produced upon cleavage is proportional to the amount of caspase activity present in the sample [29]. Briefly, total cell lysates were incubated in caspase assay buffer (100 mM HEPES (Hydroxyethyl pipерazine ethanesulfonic acid), pH 7.4, 0.5 Mm EDTA (Ethylene diamine tetraacetic acid), 20% glycerol, 5 mM dithiothreitol) containing 100 µM of the substrate (Ac-DEVD-pNA: Acetyl-Asp-Glu-Val-Asp-pNA) for 4 h at 37°C. Thereafter, the absorbance was measured at 405 nm. Caspase activity was defined as nmol pNA released per hour per mg of protein (nmol/h/mg protein), using a pNa calibration curve.

Data values in this report are expressed as mean±SD. Statistical differences between control and treated groups were determined by non-paired Student’s t-test. Differences with a value of p<0.05 were considered to be significant.

Results

The effects of high concentration of glucose on HEK 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 100 mM for 24, 48 and 72 h. High glucose (100 mM) could significantly decrease the HEK cells viability in a time dependent manner, starting from 48 h (Fig. 1). A 72-h incubation with glucose at 100 mM 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 (p<0.001) (Fig. 3).

The caspase activity was measured using specific chromogenic substrates. The activity of caspase-3 was increased in HEK cells exposed to glucose (100 mM) for 72 h, suggesting that caspase-3 was involved in high glucose-induced apoptosis (Fig. 4).
Caspases are synthesized as pro-enzymes that are converted to active form following proteolytic cleavage [22]. Therefore, to correlate the enzymatic activities of the caspase-3 with respect to changes in protein expression, its precursor protein (procaspase-3) was evaluated by Western blotting analysis. A 72-h incubation with high glucose (100 mM) led to a significant decrease in procaspase-3 level \((p<0.01)\) (Fig. 5) which, corresponds to data obtained from the activity assay, confirming the activation of this caspase (Fig. 6).

**Figure 1.** Effect of glucose (100 mM) (HG) on HEK cell viability after 24, 48 and 72-h incubation. For each time course, there were control samples (C) that remained untreated. Results are reported as the Mean ± SD. (N=8). *\(p<0.05\), **\(p<0.01\), compared to the controls.

**Figure 2.** Western blot analysis to determine the expression of Bax and Bcl-2 in extracts from control cells (C) and cells treated with high glucose (100 mM) for 72 h (HG).

**Figure 3.** Bax/Bcl-2 ratio of control cells and cells treated with high glucose (100 mM) for 72 h. Bar graph indicates the Mean±SD. ***\(p<0.001\), compared to control group.

**Figure 4.** Effect of glucose (100 mM) on enzyme activity of caspase-3 in HEK293 cells, after 72-h incubation. Bar graph indicates the Mean±SD. **\(p<0.01\), compared to control group.

**Figure 5.** Western blotting analysis of procaspase-3 protein expression levels in normal glucose control and high-glucose-treated HEK293 cells for 72 h.

**Figure 6.** Procaspase-3/β-Actin ratio of control cells and cells treated with high glucose (100mM) for 72 h. Bar graph indicates the Mean±SD. **\(p<0.01\), compared to control group.

**Discussion**

In fact, apoptosis of kidney mesangial cells, epithelial cells, and tubular cells has been demonstrated in cell cultures under the expression of glucose-induced apoptotic signals [16, 30]. In the present study, the role of Bax and caspase-3 proteins in glucose-induced cytotoxicity [28] was studied in HEK cells, as an in vitro model for diabetic nephropathy. Results showed that the viability of HEK cells was significantly decreased starting after 48 h of incubation with 100 mM of glucose in the
culture medium and continued to decrease in a time dependent manner. Bax expression was significantly increased by a glucose concentration of 100 mM after 72 h in HEK cells which is similar to studies in mesangial cells and epithelial cells [9, 10]. The expression of Bcl-2 was not significantly altered in contrast to results of previous study in epithelial cells [10]. This may be due to the different extent of protein expression in HEK293 cells (which are in embryonic stage) and other fully developed cells. However, similar to other studies, the Bax/Bcl-2 ratio, an important index of apoptotic cell death was significantly increased indicating glucose-induced apoptosis in HEK cells could possibly be mediated by the mitochondrial pathway.

Overexpression of Bax from one side may increase mitochondrial membrane permeability, and release of cytochrome-c leading to activation of caspase-9 and, subsequently, caspase-3 [21]. Alternatively, it may initiate caspase-independent apoptosis. In this case, it has been proposed that AIF (Apoptosis-Inducing Factor) can function independent of caspases [31, 32]. Thus, in order to unravel the possible downstream event of Bax, we examined activation of caspase-3.

Caspases are a class of cysteine proteases that play a fundamental role in the execution of apoptosis [22]. They are synthesized as inactive pro-enzymes and converted to the active form as a consequence of cleavage after receiving death signal. Proteolytic processing of procaspase-3 (32 kDa) produces its active form [23]. Therefore, the reduction of procaspase expression levels would be an indicator of the corresponding caspase activation. Treatment of HEK cells with high glucose led to increased enzyme activity of caspase-3, which was paralleled by a decrease in expression of procaspase-3 at 72 h post-treatment. Our present results are consistent with previous reports that high glucose results in apoptosis of different kidney cells in vivo and in vitro via activation of caspase-3 and Bax protein.

Regarding the possible upstream event responsible for hyperglycemia-induced nephrotoxicity, it was reported that, the initial cellular response to high glucose challenge is the generation of reactive oxygen species (ROS) in renal cells [33, 34]. This early and critical event in high glucose-induced cell death has several key stages beginning with the activation of key enzymes in the polyol pathway that may be linked to glucose transporters at the cell membrane. The glucose induced polyol and hexosamine pathways may or may not be directly linked to the generation of ROS, but they certainly contribute to the overall oxidative burden [35].

It is well documented 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 apoptotic cell death [34]. 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 [21]. Based on the results obtained in present study and referring to the previous reports, it could be speculated that hyperglycemia promotes opening of mPTP through elevation of oxidative and nitrosative stresses and also intracellular calcium. Therefore, under hyperglycemic conditions, apoptosis may be elicited, in part, by mPTP opening caused by the direct effect of oxidative stress, and intracellular calcium and/or by higher expression of Bax protein, leading to release of mitochondrial apoptotic mediators, which is a caspase dependent pathway [34].

It may finally be concluded that the high glucose can cause HEK cells death, in which apoptosis plays an important role by over-expression of Bax protein possibly through opening of mitochondrial permeability transition pores, mitochondrial mediators release and a caspase-3 dependent pathway.

**Authors’ Contributions**
All authors had equal role in design, work, statistical analysis and manuscript writing.

**Conflict of Interest**
The authors declare no conflict of interest.

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**References**