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αB-crystallin inhibits glucose-induced apoptosis in vascular endothelial cells

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Abstract

Recent studies implicate hyperglycemia as a cause of vascular complications in diabetes. Our study confirmed that high concentration of glucose (30 mM) induces apoptosis in cultures of human umbilical vein endothelial cells. After 5 days of culture TUNEL positive cells in high concentration of glucose were nearly 63% higher when compared to normal concentration of glucose (5 mM). Transfection of pcDNA3-rat αB-crystallin into these cells inhibited high glucose-induced apoptosis by ~36%, such an effect was not observed when cells were transfected with an empty vector. αB-crystallin transfection inhibited by about 35% of high glucose induced activation of caspase-3. High concentration of glucose enhanced formation of reactive oxygen species (ROS) in these cells but this was significantly ($p < 0.001$) curtailed by transfection of αB-crystallin. Results of our study indicate that αB-crystallin effectively inhibits both ROS formation and apoptosis in cultured vascular endothelial cells and provide a basis for future therapeutic interventions in diabetic vascular complications.

Keywords: Endothelial cells; Apoptosis; Diabetes; α-Crystallin; Reactive oxygen species

Vascular complications are a major cause of morbidity and mortality in diabetes. Abnormalities in vascular endothelial cells such as increased permeability, enhanced expression of cell adhesion molecules, and a reduced response to NO are thought to contribute to both micro- and macrovascular complications of diabetes [1,2]. Biochemical mechanisms include formation of advanced glycation end products [3,4], oxidative stress [5], and activation of protein kinase C [6] or the polyol pathway. There is some indication that excessive formation of ROS in mitochondria of vascular cells is the underlying abnormality that activates apoptotic pathways [7], but high concentrations of glucose may be the driving force behind ROS formation. To this point, results from the Diabetes Control and Complications Trial (DCCT) [8] and United Kingdom Prospective Diabetes Study (UKPDS) [9] indicate that it is the persistent hyperglycemia of uncontrolled diabetes that causes vascular complications.

In addition to abnormal vascular function, high concentration of glucose also causes death of vascular cells. Endothelial cells cultured in a high glucose environment die as a result of increased apoptosis [10,11]. Several downstream pathways influenced by hyperglycemia, such as oxidative stress [10–13] and glycation [14,15], are thought to stimulate apoptotic pathways in cultured endothelial cells.

Small heat shock proteins (sHsp) have a high degree of sequence homology, and their expression in cells can be induced by external stimuli, such as hyperthermia and oxidative stress. Two members of this family, Hsp27 and α-crystallin, have been extensively studied. α-Crystallin in the lens of the eye is composed of two

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subunits, αA- and αB-crystallins, in a ratio of 3:1. Whereas αA-crystallin is found primarily in the lens, many other tissues such as kidney, retina, and skeletal muscle contain αB-crystallin [16]. Recent studies show that both Hsp27 and α-crystallin function as chaperones where they inhibit aggregation of denaturing proteins [16,17]. They also limit apoptosis by inhibiting specific steps during the apoptotic process [18]. For example, Hsp27 inhibits apoptosis by binding to cytochrome *c* [19] and preventing activation of caspase-9 and caspase-3 [20].

We wanted to determine if αB-crystallin prevented hyperglycemia-induced apoptosis in glucose-challenged human umbilical vein endothelial cells (HUVEC). We found that transient transfection of rat αB-crystallin into HUVEC inhibited glucose-induced ROS formation and activation of caspase-3, and finally, apoptosis.

Materials and methods

Human umbilical vein endothelial cells (HUVEC) (cc-2517) and EGM-MV medium kits (CC-3125) were obtained from Cambrex (Walkersville, MD). D-(+)-Glucose was from Sigma (St. Louis, MO). In situ Cell Death Detection kit and fluorescein were from Roche (Indianapolis, IN). Caspase-3 substrate (AC-DEVDAFC) was from Calbiochem (La Jolla, CA). Chroromethyl 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was from Molecular Probes (Eugene, OR). Anti-αB-crystallin monoclonal antibody was from Stressgen Biotechnologies (Victoria, BC, Canada). The HUVEC Nucleofector Kit was from Amaxa Biosystems (Koln, Germany). Rat αB-crystallin cDNA was cloned into a pcDNA3.1 mammalian expression vector as described by Liu et al. [21].

Cell culture. HUVEC were cultured in an EGM-MV medium kit (containing 5% fetal calf serum). The medium was changed every other day. All experiments used nearly confluent cells between passages 4 and 6.

DNA fragmentation detected by TdT-mediated dUTP nick end labeling. Cultures of 80% confluent HUVEC (5×10^4 cells) were subcultured on coverslips in 24-well plates in EGM-MV medium containing 1% fetal calf serum for 5 days in the presence or absence of 30 mM glucose described above. After incubation we detected DNA fragmentation with an in situ cell death detection kit. Briefly, the cells on coverslips were washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. Then all samples were rinsed with PBS and permeabilized on ice in a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 5 min. After washing, 50 μl of TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture was added to each sample, and incubation was continued in the dark in a humidified atmosphere for 90 min at 37 °C. The samples were analyzed using a fluorescence microscope.

Western blotting for αB-crystallin. HUVEC were treated with high glucose for 1 week in EGM-MV medium containing 1% FCS to measure α-crystallin. Adherent and detached cells were collected and washed twice with ice-cold PBS and lysed on ice in 20 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. The cell lysate was dispersed by sonication for 20 s in a Branson Sonifier (Danbury, CT) set at 20% amplitude. The suspension was centrifuged at 7500*g* for 10 min at 4 °C. Samples from the supernatant fraction (2.5 μg protein) were applied to 15% mini gels and separated by SDS–PAGE. The proteins were transferred electrophoretically to nitrocellulose membranes and treated with a monoclonal antibody to αB-crystallin (diluted 1:1000). The samples were incubated for 16 h at 4 °C and then reacted with HRP-conjugated secondary antibody (rabbit antimouse, 1:2000 dilution) for 1 h at room temperature. The membrane was washed with buffer A and developed with the Pierce Enhanced Chemiluminescence (ECL) kit (Pierce, Rockford, IL).

Transient transfection of αB-crystallin into HUVEC. We first transfected HUVEC (5×10^5) using pCMS-EGFP using a Nucleofector kit. The cells were cultured for 24 h on coverslips placed in 6-well plates. After washing with PBS, we fixed the attached cells in 4% paraformaldehyde for 30 min at room temperature. We confirmed transfection by fluorescence microscopy and estimated transfection efficacy to be 50–60%. We then transiently transfected HUVEC with α-crystallin using an Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany). Cells were harvested, washed once in PBS, and suspended in 100 μl of the electroporation buffer, then 5 μg of plasmid DNA (pcDNA3-rat αB-crystallin or pcDNA3 vector alone) was mixed, transferred to a cuvette, and nucleofected. We confirmed the transfection by Western blotting with a monoclonal antibody to αB-crystallin as described above. Transfected and non-transfected HUVEC were grown on coverslips placed in 24-well culture plates with media containing 5 or 30 mM glucose at 37 °C for 5 days. The medium (from the EGM-MV medium kit) contained 1% fetal calf serum and was changed every other day. Apoptosis was measured by the TUNEL assay as described above.

Caspase-3 assay. HUVEC with pcDNA3.1(−) alone and pcDNA3.1(−) containing rat αB-crystallin cDNA were cultured in 6-well plates for 3 days at 37 °C with 5 or 30 mM glucose in the EGM-MV medium (1% fetal calf serum), and the medium was changed every day. The treated cells were lysed on ice in 200 μl lysis buffer containing 0.71% NP-40, 71 mM Tris (pH 7.5), 0.71 mM EDTA, and 212 mM NaCl. Samples corresponding to 40 μg protein were applied to a black plastic 96-well microplate and incubated for 1 h at 37 °C in 21 mM Hepes buffer (pH 7.4) that contained 105 mM NaCl, 5.25 mM DTT, and 50 μM Ac-DEVD-AFC (total volume of 200 μl). A microplate fluorescence reader (Gemini XS, Molecular Devices) measured the fluorescent product at 505 nm (excitation—400 nm).

Intracellular reactive oxygen species. Transfected cells were treated with normal and high glucose as described above. Samples (1×10^5 cells) were loaded with 10 μM CM-H_2DCFDA in 1.0 ml HBSS by shaking for 45 min at 37 °C. The samples were centrifuged at 200*g* for 5 min at room temperature, the supernatant was discarded, and 800 μl of HBSS was added to the cell pellet. Fluorescence at 530 nm (excitation—480 nm) was measured using a microplate spectrofluorometer.

Protein measurement. We measured proteins with a Bio-Rad protein assay kit using bovine serum albumin (BSA) as the standard.

Statistical analyses. We used ANOVA (Statview 5.0, SAS Institute, Cary, NC) to evaluate differences among treatment groups with $p < 0.01$ considered to be significant.

Results

We measured high glucose-induced apoptosis by TUNEL assay. Cells cultured in the high glucose environment exhibited nearly 2.5-fold increase in TUNEL positive cells over normal glucose controls (Fig. 1). These results are in agreement with the previous observation of Ido et al. [11]. Because small heat shock proteins have anti-apoptotic properties, we wanted to determine how a high glucose environment affects the

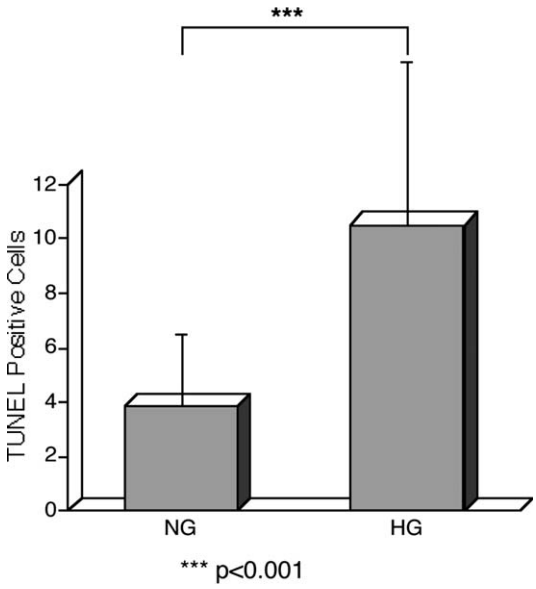

Fig. 1. Hyperglycemia-induced apoptosis in endothelial cells. Endothelial cells (HUVEC) were grown for 5 days in EGM-MV (1% FCS) 5 mM (NG) or 30 mM D-glucose (HG). Apoptosis was assessed by TUNEL staining.

levels of αB-crystallin in HUVEC. As can be seen in Fig. 2, we detected only traces of αB-crystallin in HUVEC cultured in either normal or high glucose medium.

We wanted to establish whether over-expression of αB-crystallin in HUVEC could protect cells from apoptosis caused by a high glucose environment. We established the transfection efficiency of our procedure by using a vector that contained enhanced green fluorescent protein (EGFP) as a marker. As can be seen in Fig. 3, EGFP was transfected efficiently and transfection was estimated as 50–60%. We then transfected HUVEC with rat αB-crystallin, and Western blotting established an over-expression of αB-crystallin in the transfected cells (Fig. 3).

The TUNEL assay showed the effect of high glucose on apoptosis in HUVEC and confirmed the anti-apoptotic effect of αB-crystallin. Transfection of αB-crystallin significantly reduced apoptosis in HUVEC that were incubated for 5 days with 30 mM glucose ($p < 0.05$) (Fig. 4). We observed a nearly twofold reduction in apoptosis in cells transfected with αB-crystallin. This phenomenon was limited to the αB-crystallin-transfected cells, since

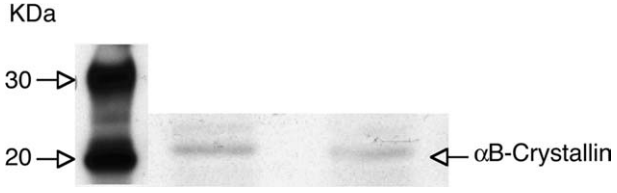

Fig. 2. Western blotting for small heat shock proteins. HUVEC were grown in 5 or 30 mM D-glucose for one week, lysed, and the cytosolic proteins were Western blotted for α-crystallin as described in Materials and methods.

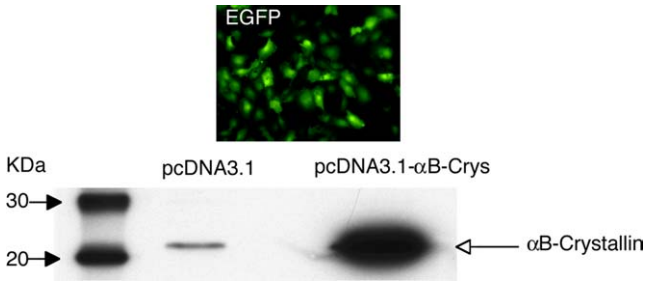

Fig. 3. Transient transfection of rat αB-crystallin. HUVEC were transiently transfected with either empty vector or vector containing cDNA for rat αB-crystallin. Western blotting detected αB-crystallin.

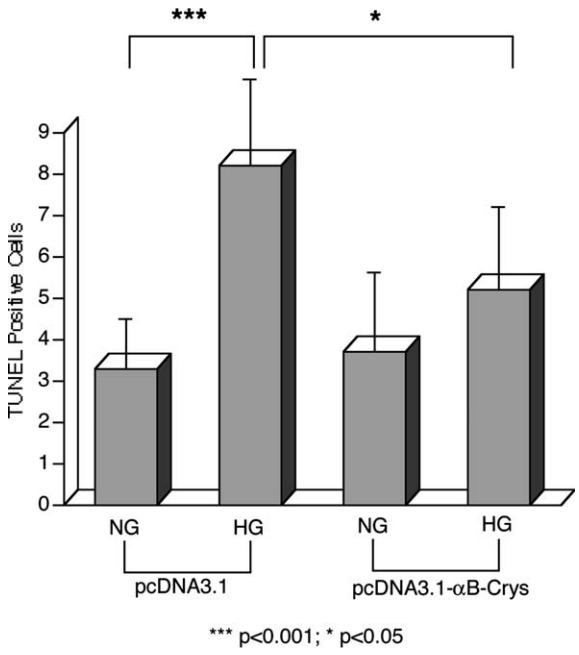

Fig. 4. Hyperglycemia-induced apoptosis in αB-crystallin-transfected cells. HUVEC were transiently transfected with either empty vector or vector containing cDNA for rat αB-crystallin and grown in EGM-MV medium supplemented with either 5 or 30 mM glucose for 5 days. Apoptosis was assessed by TUNEL staining.

apoptosis was similar in empty vector-transfected and non-transfected cells cultured with high glucose. We also assessed caspase-3 activity in all of the cell treatment groups. Caspase-3 activity increased significantly in cells cultured in a high glucose environment, but it was significantly reduced in αB-crystallin-transfected cells (Fig. 5).

High glucose increases oxidative stress in endothelial cells, and increased oxidative stress has been linked to apoptosis. In order to determine if αB-crystallin inhibits apoptosis by reducing intracellular ROS, we measured ROS content by loading the cells with 10 μM CM-H_2DCFDA. As we found for caspase-3 activity, a high glucose environment enhanced ROS production in empty vector-transfected cells, but this response was significantly curtailed in αB-crystallin-transfected cells (Fig. 6).

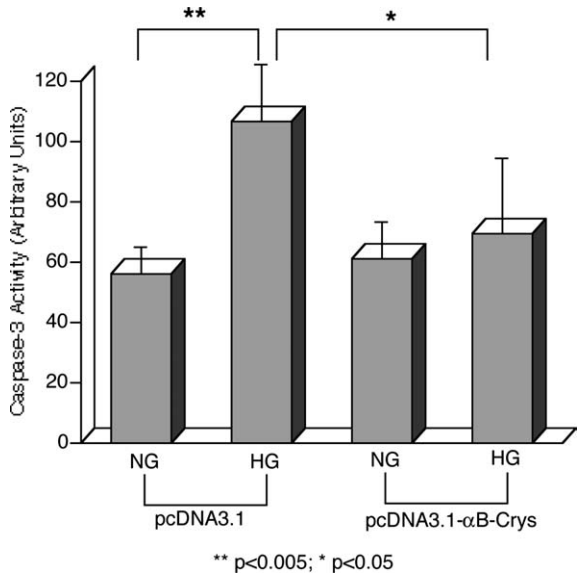

Fig. 5. Caspase-3 activity in αB-crystallin-transfected cells. Caspase-3 activity in the cytosolic was assessed using a fluorogenic substrate DEVD-AFC, as described in Materials and methods. Other details are same as for Fig. 4.

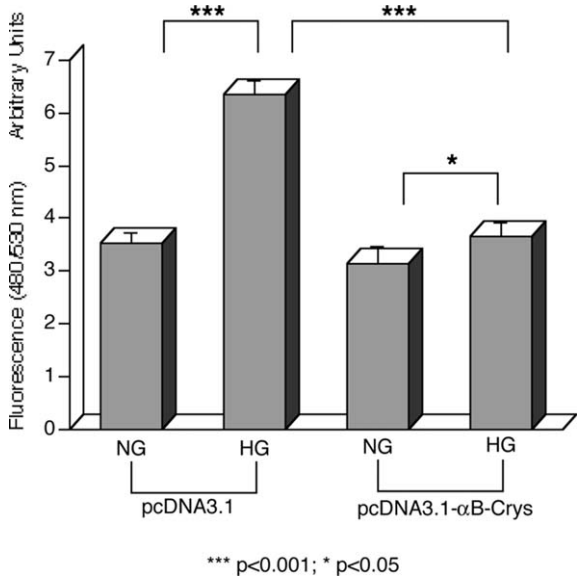

Fig. 6. Reactive oxygen species (ROS) in αB-crystallin-transfected cells. ROS was measured by loading the cells with 10 μM CM-H_2DCFDA and measuring the resulting fluorescence in a spectrofluorometer.

Discussion

Recent studies indicate that small heat shock proteins (sHsp) function as anti-apoptotic proteins. In fact, overexpression of either α-crystallin or Hsp27 inhibits apoptosis in a variety of different cells [20,22,23]. We initiated the present study to determine if αB-crystallin could prevent apoptosis in vascular endothelial cells caused by exposure to high levels of glucose. Both αA- and αB-crystallins inhibit apoptosis in cultured lens epithelial cells, although αA-crystallin appears to be the more effective of the two [23,24].

We detected only trace amounts of αB-crystallin in normally cultured HUVEC, and the level of this protein did not rise when the cells were grown in medium supplemented with 30 mM glucose. However, when we transfected HUVEC with αB-crystallin and challenged the cells with staurosporine, we found that transfection rendered HUVEC resistant to apoptosis. Although we do not know exactly how αB-crystallin inhibits apoptosis in HUVEC, we can suggest several possibilities based on the findings by others. αB-crystallin can inhibit activation of caspase-3 [25] and inhibit the translocation of two pro-apoptotic proteins, Bcl-XS and Bax, during the process of apoptosis [26]. These properties could contribute to its anti-apoptotic function.

Hsp27 was shown to reduce ROS formation and resist glutathione loss in cells stressed by apoptotic agents [27,28]. We found that αB-crystallin shares this property; it inhibited ROS formation in HUVEC cultured under high glucose conditions. Whether the inhibition results from prevention of glutathione loss during hyperglycemia remains to be established, although α-crystallin expression in lens epithelial cells correlates positively with the glutathione content of the cells [29].

In summary, we showed that an elevation of αB-crystallin in endothelial cells through transfection inhibits glucose-induced apoptosis. This finding has important implications for diabetic individuals with uncontrolled hyperglycemia, since their entire vascular endothelium is exposed to a high glucose environment. Our model of αB-crystallin-transfected HUVEC invites further studies of how hyperglycemia injures the vasculature, and it could help design therapeutic strategies for early intervention in diabetic complications.

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