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Glycation-induced Inactivation of Antioxidant Enzymes and Modulation of Cellular Redox Status in Lens Cells

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Oxidative mechanisms are thought to have a major role in cataract formation and diabetic complications. Antioxidant enzymes play an essential role in the antioxidant system of the cells that work to maintain low steady-state concentrations of the reactive oxygen species. When HLE-B3 cells, a human lens cell line were exposed to 50-100 mM glucose for 3 days, decrease of viability, inactivation of antioxidant enzymes, and modulation of cellular redox status were observed. Significant increase of cellular oxidative damage reflected by lipid peroxidation and DNA damage were also found. The glycation-mediated inactivation of antioxidant enzymes may result in the perturbation of cellular antioxidant defense mechanisms and subsequently lead to a pro-oxidant condition and may contribute to various pathologies associated with the long term complications of diabetes.

Key words: Glycation, Diabetes, Antioxidant enzymes, Redox status, Lens cells

INTRODUCTION

Autoxidation of sugars, and of the products of nonenzymatic 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 (Brownlee, 2000; Nishikawa *et al.*, 2001; Vincent *et al.*, 2002). There have been many reports that glycated proteins, such as hemoglobin (Brownlee *et al.*, 1984), albumin (Biemel *et al.*, 2002) and lens crystallines (Duhaiman *et al.*, 1990), undergo changes in their structures or functions. It has been proposed that complications in diabetes and aging, such as cataracts, atherosclerosis and microvascular diseases, could be partly due to glycation process (Myint *et al.*, 1995).

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 (McCord and Fridovich, 1969), catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides (Chance *et al.*, 1979). These enzymes normally act as a team, thus superoxide dismutase protects catalase and peroxidase against inhibition by O_2^-, while catalase and peroxidase provide protection for superoxide dismutase against inactivation by hydrogen peroxide (Tabatabaie and Floyd, 1994). Glucose 6-phosphate dehydrogenase (G6PD) and $NADP^+$-dependent isocitrate dehydrogenase (ICDH), key enzymes for the generation of NADPH, and glutathione reductase, which is involved in the regeneration of reduced glutathione, are also considered as essential antioxidant enzymes (Kil *et al.*, 2004). The damage brought about by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are damaged and inactivated by glycation-induced oxidative stress.

In this study, we evaluated the susceptibility of antioxidant enzymes to glycation in HLE-B3 cells, a human lens epithelial cell line. The data demonstrate that antioxidant enzymes are susceptible to inactivation by glycation-induced oxidative stress and inactivation of antioxidant enzymes resulted in the perturbation of cellular redox status.

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MATERIALS AND METHODS

Materials

D-Glucose, β-$NADP^+$, isocitrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), xylenol orange, and avidin-conjugated tetramethylrhodamine isothiocyanate (TRITC) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 2',7'-Dichlorofluorescin diacetate (DCFHDA), *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloro-methylcoumarin (CMAC), and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Molecular Probes (Eugene, OR, U.S.A.).

Cell culture and cytotoxicity

HLE-B3 cells, human lens epithelial cells, were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 20% fetal calf serum at 37°C in an atmosphere of 5% CO_2. The viability of B3 cells was determined by MTT assay. Cell viability is expressed as a percentage of the absorbance seen in the untreated control cells.

Enzyme assay

Cells were collected at 1,000 × 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 1,000 × g for 5 min and the supernatants further centrifuged at 15,000 × g for 30 min. The supernatants were added by 1/10 volume of 10X PBS containing 1% Triton-X100, which finally made the solution 1X PBS containing 0.1% Triton-X100. The supernatants were used to measure the activities of several cytosolic enzymes. 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 one unit 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. The activity of ICDH was measured by the production of NADPH at 340 nm. The reaction mixture for ICDH activity contained 50 mM MOPS, pH 7.2, 5 mM threo-DS-isocitrate, 35.5 mM triethanolamine, 2 mM NAD^+, 1 mM ADP, 2 mM $MgCl_2$, and 1 μg/mL rotenone. One unit of ICDH activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min.

Cellular redox status

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. Cell homogenates 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 in a room temperature for 30 min, and absorbance was measured at 560 nm. Hydrogen peroxide was used to draw standard curve as described (Jiang *et al.*, 1992). Intracellular reactive oxygen species (ROS) production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy (Lee *et al.*, 2002). The intracelluar GSH level was also determined by using a GSH-sensitive fluorescence dye CMAC. B3 cells (1×10^6 cells/mL) were incubated with 5 μM CMAC cell tracker for 30 min. The images of CMAC cell tracker fluorescence by GSH was analyzed by the Zeiss Axiovert 200 inverted microscope at fluorescence 4, 6-diamidino-2-phenylindole (DAPI) region (excitation, 351 nm; emission, 380 nm) (Tauskela *et al.*, 2001).

Cellular oxidative damage

Lipid peroxidation was estimated by using a fluorescent probe DPPP as described by Okimoto *et al.* (2000). B3 cells were incubated with 5 μM DPPP for 15 min in the dark and the images of DPPP fluorescence by reactive species were analyzed by the Zeiss Axiovert 200 inverted microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). 8-OH-dG levels of B3 cells were estimated by using a fluorescent binding assay as described by Struthers *et al.* (1998). After B3 cells were exposed to glucose, cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 in PBS for 1 h) for fluorescent microscope with 488 nm excitation and 580 nm emission

Quantitation of relative fluorescence

The average of fluorescence intensity from fluorescence images were calculated as described (Sundaresan *et al.*, 1995).

Replicates

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

RESULTS AND DISCUSSION

Oxidative stress involved in the pathogenesis of diabetic complications and prevention of synthesis and tissue accumulation of oxidative-derived end products could constitute a major advance in the treatment of diabetic complications (Baynes, 1991). The lens is a tissue that does not turn over, and studies on the senile cataractous lenses isolated from human diabetes have shown a significant elevation in the levels of sugars (Varma and Kinoshita, 1974). As shown in Fig. 1, when HLE-B3 lens cells were exposed to glucose a dose-dependent decrease in cell viability was observed.

Despite their role in the cellular defense mechanism, the antioxidant enzymes are susceptible to inactivation by ROS. Previous study demonstrated that oxidative processes result in the loss of key antioxidant enzymes, which may exacerbate oxidative stress-induced cytotoxicity. Antioxidant enzymes provide a substantial defense network against the accumulation of ROS. Because formation of ROS is probably inevitable under diabetic conditions, the glycation-mediated reaction with proteins, including antioxidant enzymes, could also be unavoidable. Therefore, it is implied that the inactivation of antioxidant enzymes by glycation may lead to the perturbation of the antioxidant defense system, which may be responsible for diabetic complications. As shown in Fig. 2, when cells were exposed to 50-100 mM glucose for 3 days the activity of antioxidant enzymes was significantly decreased.

To investigate whether or not the difference in viability of B3 cells upon exposure to glucose is associated with ROS formation, the intracellular peroxides in B3 cell line were measured by confocal microscope with the oxidant-sensitive probe DCFH-DA. As shown in Fig. 3A and Fig. 3B, a 4- to 5- fold increase in fluorescence was observed in the B3 cells when they were exposed to 100 mM glucose. We also demonstrated the level of intracellular H_2O_2 in cells exposed to glucose. The treatment of glucose

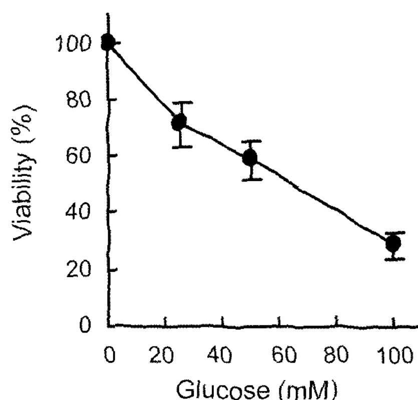

Fig. 1. Viability of B3 cells upon exposure to glucose. B3 cells were exposed to various concentrations of glucose for 3 days and viability of cells was determined by using an MTT assay. Survival of untreated cells was expressed as 100%. Results shown are means ± S.D. of five independent experiments.

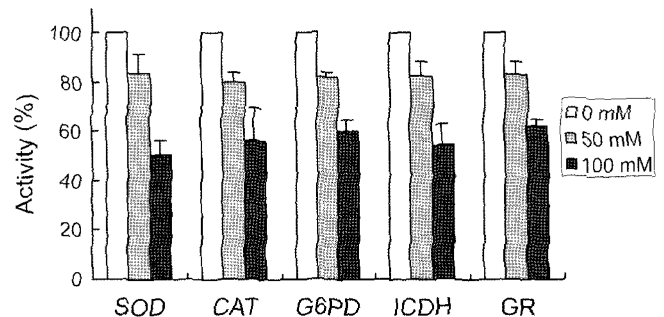

Fig. 2. Activity of antioxidant enzymes in B3 cells exposed to glucose for 3 days. Activity of untreated cells is expressed as 100%. Results shown are means ± S.D. of five independent experiments. SOD, superoxide dismutase; CAT, catalase; G6PD, glucose 6-phosphate dehydroegnase; ICDH, NADP$^+$-dependent isocitrate dehydrogenase; GR, glutathione reductase.

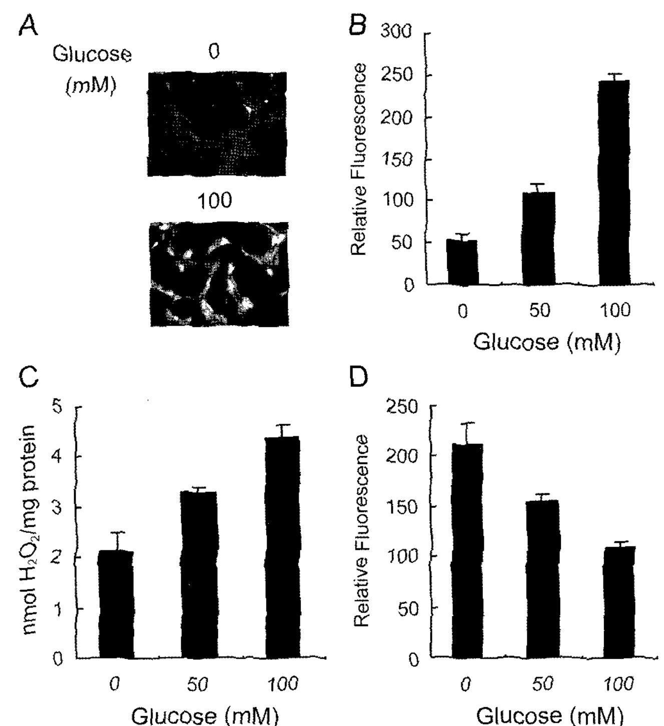

Fig. 3. Glycation-induced modulation of cellular redox status in B3 cells. (A) Relative intensity of DCF fluorescence in B3 cells exposed to glucose for 3 days. Fluorescence images were obtained under laser confocal microscopy. (B) The averages of fluorescent intensity were calculated from each image. (C) Intracellular hydrogen peroxide generation in B3 cells exposed to glucose for 3 days. (D) Effect of glycation on GSH levels in B3 cells exposed to glucose for 3 days. Fluorescence images of CMAC-loaded cells were obtained under microscopy. The averages of fluorescence intensity were calculated as described (Sundaresan *et al.*, 1995). Results shown are means ± S.D. of three independent experiments.

resulted in a significantly higher intracellular level of H_2O_2 as compared to that of the untreated control with the exposure to glucose (Fig. 3C). GSH is a well-known antioxidant, which is usually present as the most abundant low-molecular-mass thiol in most organisms. It has various functions in the defense against oxidative stress and xenobiotic toxicity (Meister and Anderson, 1983). It can act as the electron donor for glutathione peroxidase in animal cells and also directly react with ROS. GSH is readily oxidized to glutathione disulfide (GSSG) by the glutathione peroxidase reaction, as well as the reaction with ROS, which may subsequently cause the reduction of GSH level. Determination of changes in GSH concentration provides an alternative method of monitoring oxidative stress within cells. It has been shown that GSH-sensitive fluorescent dye CMAC can be employed as a useful probe to evaluate the level of intracellular GSH (Tauskela *et al.*, 2001). Cellular GSH levels in B3 cells treated with 50-100 mM glucose for 3 days was significantly decreased (Fig. 3D). In our study, glycation affected the glutathione redox status and the intracellular ROS was increased in the same condition. These results indicate that glycation is able to modulate the cellular redox balance presumably by depleting GSH. Consequently, the perturbation of the balance between oxidants and antioxidants leads to a pro-oxidant condition.

We evaluated the induction of lipid peroxidation in membrane upon exposure to glucose. Recently, it has been shown that DPPP is a suitable fluorescent probe to monitor lipid peroxidation specifically within the cell membrane. DPPP reacts with lipid hydroperoxides stoichiometrically to yield highly fluorescent product DPPP oxide (Okimoto *et al.*, 2000). As shown in Fig. 4A, DPPP fluorescent intensity had increased markedly in B3 cells treated with glucose as compared to the untreated control. The reaction of intracellular ROS with DNA resulted in numerous forms of base damage, and 8-OH-dG is one of the most abundant and most studied lesions generated. Because 8-OH-dG causes misreplication of DNA (Shibutani *et al.*, 1991), it has been implicated as a possible cause of mutation and cancer. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* (Park and Floyd, 1992). Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC (Struthers *et al.*, 1998). The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased when B3 cells were exposed to either 100 mM glucose for 3 days (Fig. 4B).

In summary, these results suggest that high concentrations of glucose resulted in a pro-oxidant condition of B3 lens cells by depletion of GSH and inactivation of antioxidant enzymes which consequently leads decrease in survival and oxidative damage to lipid and DNA. The results indicate that the inactivation of antioxidant enzymes is probably one of the important intermediary events in glycation-induced cellular damage.

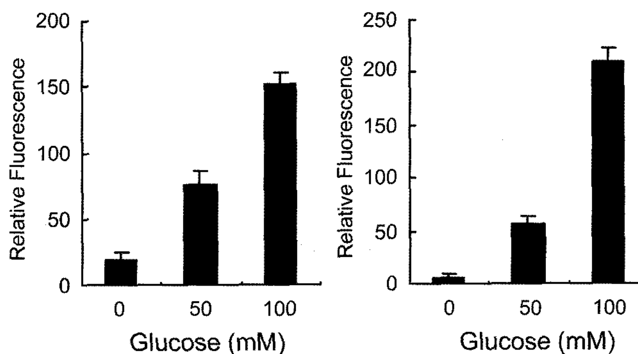

Fig. 4. Glycation-induced oxidative damage of B3 cells. (A) Visualization of lipid peroxidation in B3 cells exposed to glucose for 3 days. Cells (1×10^6 cells/mL) were stained with 5 μM DPPP for 15 min. Fluorescence images were obtained under microscopy. The relative intensity of DPPP fluorescence was calculated as described (Sundaresan *et al.*, 1995). (B) 8-OH-dG levels in B3 cells exposed to glucose for 3 days. Cells were fixed and permeabilized immediately after exposure to glucose. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope with 488 nm excitation and 580 nm emission. The averages of fluorescence intensity were calculated as described (Sundaresan *et al.*, 1995). Results shown are means ± S.D. of three independent experiments.

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