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Nitric Oxide Prevents Apoptosis of Human Endothelial Cells From High Glucose Exposure During Early Stage

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Abstract Hyperglycemia is a major cause of diabetic vascular disease. High glucose can induce reactive oxygen species (ROS) and nitric oxide (NO) generation, which can subsequently induce endothelial dysfunction. High glucose is also capable of triggering endothelial cell apoptosis. Little is known about the molecular mechanisms and the role of ROS and NO in high glucose-induced endothelial cell apoptosis. This study was designed to determine the involvement of ROS and NO in high glucose-induced endothelial cell apoptosis. Expression of endothelial nitric oxide synthase (eNOS) protein and apoptosis were studied in cultured human umbilical vein endothelial cells (HUVECs) exposed to control-level (5.5 mM) and high-level (33 mM) glucose at various periods (e.g., 2, 12, 24, 48 h). We also examined the effect of high glucose on H_2O_2 production using flow cytometry. The results showed that eNOS protein expression was up-regulated by high glucose exposure for 2–6 h and gradually reduced after longer exposure in HUVECs. H_2O_2 production and apoptosis, which can be reversed by vitamin C and NO donor (sodium nitroprusside), but enhanced by NOS inhibitor (N^G-nitro-L-arginine methyl ether), were collated to a different time course (24–48 h) to HUVECs. These results provide the molecular basis for understanding that NO plays a protective role from apoptosis of HUVECs during the early stage (<24 h) of high glucose exposure, but in the late stage (>24 h), high glucose exposure leads to the imbalance of NO and ROS, resulting to the observed apoptosis. This may explain, at least in part, the impaired endothelial function and vascular complication of diabetic mellitus that would occur at late stages. J. Cell. Biochem. 75:258–263, 1999.

Key words: nitric oxide; high glucose; apoptosis; human endothelial cell

Diabetes mellitus (DM) is the most common serious metabolic disorder. The relationship between DM and premature vascular disease is well established [Kannel and McGee, 1978; Cohen and Tesfamariam, 1992]. Thus, DM is an important risk factor for cardiovascular mortality and mobility [Pyorala et al., 1987]. Early atherosclerotic change and accelerated atherosclerosis are common findings in diabetic patients [Kannel and McGee, 1978; Cohen and Tesfamariam, 1992; Nathan, 1993; Ganda, 1980]. In the early stage of diabetes, decreased peripheral resistance and increased blood flow are common vascular changes [Mogensen, 1971; Kohner et al., 1975; Houben et al., 1992]. Because of the localization and functions in the blood vessel, endothelial cells are considered candidates primarily involved in the pathogenesis of diabetic vascular complications. Acute exposure to high glucose in nondiabetic individuals would induce vasodilation, owing to an increase of endothelium-derived nitric oxide (NO) formation [Tilton et al., 1989, 1992; Sandeman et al., 1996; Houben et al., 1993; Williamson et al., 1993]. However, impairment of endothelium-dependent vascular relaxation in diabetic animals and humans has been shown [Durante et al., 1988; Tesfamariam et al., 1991; Tesfamariam, 1994]. Free radicals were claimed to be involved in inducing diabetic endothelial cell dysfunction [Tesfamariam, 1994]. Under physiologic conditions, glucose is susceptible to oxidation and consequently generates hydrogen peroxide and reactive intermediates, such as hydroxyl-free radicals [Wolff and Dean, 1987]. These findings show that oxidative stress plays an important role in the tissue damage associated with diabetes [Cosentino et al., 1997] and that peroxide formation is increased in an elevated glucose condition [Cosentino et al., 1997; Baynes, 1991; Giugliano et al., 1996; Hunt et

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al., 1990]. However, the mechanisms of the detrimental effect of elevated glucose on endothelium remain to be clarified.

It has been demonstrated that hyperglycemia is capable of triggering apoptosis in cultured human endothelial cells [Baumgartner-Parzer et al., 1995]. Although some studies have shown that reactive oxygen species (ROS) can trigger apoptosis [Kroemer et al., 1995; Simizu et al., 1996], the relationship between hyperglycemia-induced ROS production and apoptosis in human endothelial cell is unclear. Recently, Cosentino et al. [1997] identified that prolonged exposure to high glucose could increase endothelial nitric oxide synthase (eNOS) expression and superoxide anion generation in human aortic endothelial cells. However, little is known about the role of high glucose-induced eNOS expression on the high glucose-triggered apoptosis in human endothelial cells. In this study, we hypothesized that the increased eNOS expression may play a protective role from oxidative stress-induced apoptosis in early stage of high glucose exposure to human umbilical vein endothelial cells.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured as previously described [Jaffe et al., 1973]. Cells were seeded at a density of 1×10^5 per 75-cm^2 flask in medium 199 (Gibco, Grand Island, NY), supplemented with 20 mM Hepes, 100 μg/ml endothelial cell growth substance (Collaborative Research Inc, Bedford, MA), and 20% fetal calf serum (FCS) (Gibco). The cultures were maintained at 37°C with a mixture of 5% CO_2–95% air . The subcultures were performed using trypsin-EDTA. All media were filtered and supplemented with 5 U/ml heparin, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Medium was changed every 2 days. The endothelial cell monolayers were identified by the presence of factor VIII-related antigen (Histoset Kit, Immunolok, Carpinteria, CA) and the typical "cobblestone" appearance. Endothelial cells of the third to fifth passages in actively growing condition were used in the experiments. HUVECs were treated with media containing 5.5 or 33 mM of glucose for 2–48 h in the presence or absence of ascorbic acid (100 μM), sodium nitroprusside (1 μM), or N^G-nitro-L-arginine methyl ester (1 mM). Furthermore, in order to validate the effects of high glucose, rather than hyperosmolarity, we used a concentration of mannitol in culture medium similar to that described by Cosentino et al. [1997].

Detection of Apoptosis

The apoptosis was determined by using the cell death detection enzyme-linked immunosorbent assay (ELISA) method (Boehringer Mannheim, Indianapolis, IN) [Wang and Phang, 1995]. This method is based on a quantitative sandwich enzyme immunoassay principle. It can detect histone-associated DNA fragments in one immunoassay, demonstrating the internucleosomal degradation of genomic DNA occurring during apoptosis. For fluorescence microscopy, cells were collected and fixed in methanol/acetone (1:3, v/v) solution for 5 min and washed with phosphate-buffered saline (PBS). Fixed cells were then stained with 0.1 ng/ml Hoechst 33258 for 10 min under dark conditions to counterstain nuclei. Cells were observed and photographed with a Nikon fluorescence microscope.

Western Blot Analysis

Treated HUVECs were lysed in a lysis buffer (50 mM Tri-HCl, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40 [NP-40], 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 mg/ml aprotinin, and leupeptin, at pH 7.4) for 20 min on ice. The lysates were centrifuged at 14,500 rpm for 20 min at 4°C; the concentration of protein in each cell lysate was measured with a commercial BCA kit (Pierce Co.). A 30-μg protein of cellular lysates was subjected to electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) to detect eNOS. The samples were then electroblotted onto nitrocellulose paper. After blocking, blots were incubated with anti-eNOS antibody (Transduction Laboratories, USA) in PBS/Tween 20 and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, USA) for 30 min. The level of eNOS protein expression was determined using the enhanced chemiluminescence (ECL) kit (Amersham), and chemiluminescence was exposured by the filters of Kodac X-Omat films.

Fluorescence-Activated Cell Sort (FACS) Analysis

Intracellular hydrogen peroxide (H_2O_2) production was monitored by flow cytometry using DCFH-DA [Hunt et al., 1990]. Briefly, cells ($2 \times$

10^5) were co-incubated with 50 mM DCFH-DA in the absence or presence of glucose at 37°C for various time intervals. After incubation cells were resuspended in ice-cold PBS and placed on ice at dark for flow cytometry analysis.

Statistical Analysis

Results were expressed as mean±SEM. n indicates the number of experiments. Statistical evaluation of the data was performed by the use of the unpaired Student's *t*-test and analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of High Glucose on eNOS Expression

The eNOS protein was measured after the exposure of HUVECs to a high level of glucose (33 mM) for various time intervals (2, 6, 24, and 48 h). The expression of eNOS after high glucose treatment was progressively increased to reach a maximum at 6 h and then gradually decreased after 24 h and 48 h compared with control (5.5 mM) (Fig. 1).

Effect of High Glucose on Hydrogen Peroxide Production

In treating HUVECs with high glucose, the production of H_2O_2 was not significantly affected after 24-h exposure but was markedly increased at 48 h (Fig. 2). The increase of H_2O_2 production by high glucose was inhibited by ascorbic acid (100 μM) and sodium nitroprusside (SNP; 1 μM; a NO donor) and enhanced by N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM; a NOS inhibitor) (Fig. 2). Ascorbic acid, SNP, and L-NAME by themselves did not affect the H_2O_2 production. Moreover, in mannitol-treated cells, it did not affect the H_2O_2 production (99.5 ± 1.7% of control, n = 3).

Induction of HUVECs Apoptosis by High Glucose

As shown in Figure 3, treatment with high glucose for 48 h, but not at 24 h, markedly induced apoptosis in HUVECs. High glucose-induced apoptosis was enhanced by L-NAME (1 mM) but was inhibited by ascorbic acid (100 μM) and SNP (1 μM). Moreover, the exposure to osmotic control mannitol for 48 h did not induce the apoptosis (135.2 ± 10.3% of control, n = 3).

DISCUSSION

Hyperglycemia is generally believed to be the major cause of vascular complications in diabetic patients [Kannel and McGee, 1978; Cohen and Tesfamariam, 1992; Nathan, 1993; Ganda, 1980; Diabetes Control and Complications Trial Research Group, 1993]. High glucose-induced functional disorder in vascular endothelial cells can be a key event in the development of dia-

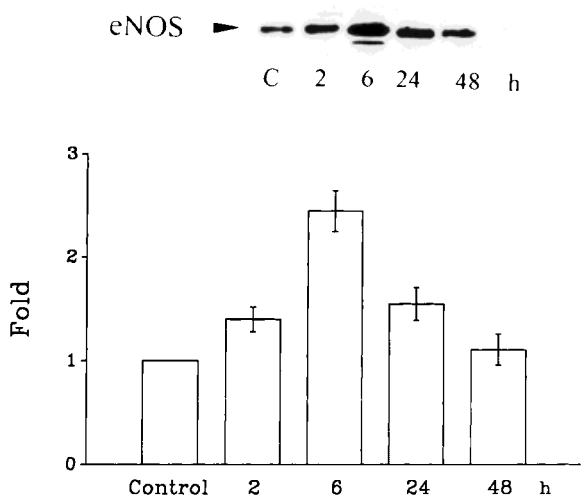

Fig. 1. Effect of glucose on eNOS protein expression in human umbilical vein endothelial cells (HUVECs) exposed to control level (5.5 mM) and high levels of glucose (33 mM) at different time periods (2, 6, 24, and 48 h). The eNOS proteins were determined by Western blot and densitometric quantification as described in Methods. Data are presented as mean ±SEM (n = 4).

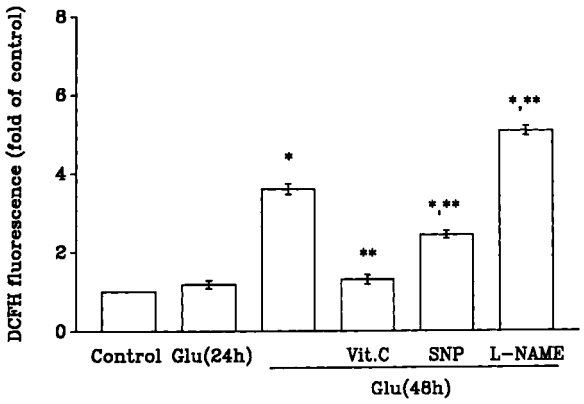

Fig. 2. High glucose-induced intracellular hydrogen peroxide (H_2O_2) in human umbilical vein endothelial cells (HUVECs). The HUVECs were exposed to high levels of glucose (33 mM) for 24 and 48 h in the presence or absence of ascorbic acid (vitamin C; 100 μM), sodium nitroprusside (SNP; 1 μM) or N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM). Control level of glucose is 5.5 mM. The intracellular H_2O_2 production was determined by the fluorescence of DCFH-DA as described in Methods. Data are presented as mean ±SEM (n = 6). *$P < 0.05$, as compared with control group. **$P < 0.05$, as compared with the group of high glucose treatment for 48 h.

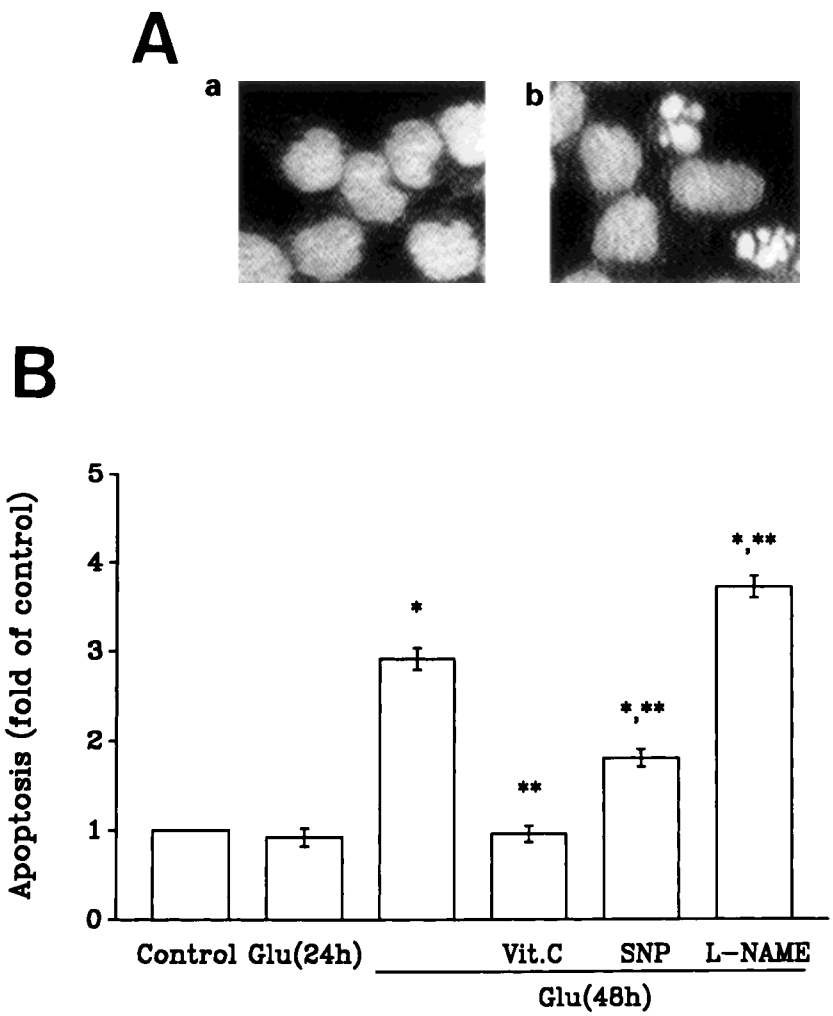

Fig. 3. Apoptosis induced by high glucose in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with high level of glucose (33 mM) for 24 h and 48 h, respectively, in the presence or absence of ascorbic acid (vitamin C; 100 μM), sodium nitroprusside (SNP; 1 μM) or N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM). Control level of glucose is 5.5 mM. The apoptosis was determined by using fluorescent dye Hoechst 33258 method (**A-a**, control; **A-b**, high glucose exposure for 48 h) and cell death detection enzyme-linked immunosorbent assay (ELISA) method **(B)** as described in Materials and Methods. Data are presented as mean±SEM (n = 4). $*P < 0.05$, as compared with control. $**P < 0.05$, as compared with the group of high glucose treatment for 48 h.

betic complications [Lorenzi and Cagliero, 1991]. Baumgartner-Parzer et al. [1995] have reported that high ambient glucose (30 mM) induced apoptosis in cultured HUVECs; the increase in apoptosis was approximately 20% after 48 h and 46% after long-term (13 ± 1 days) high glucose exposure. ROS has been shown to play a role in the induction of apoptosis in many cell lines [Kroemer et al., 1995; Simizu et al., 1996]. The involvement of superoxide anions in diabetes-induced changes in blood vessel response has been suggested in many studies [Tesfamariam, 1994; Cosentino et al., 1997; Graier et al., 1997]. Moreover, a recent study conducted by Cosentino et al. [1997] showed that prolonged exposure (5 days) to high glucose increased eNOS expression, nitrite release, and production of $O_2^{\cdot -}$ in human aortic endothelial cells. However, the role of nitric oxide (NO) in ROS-induced apoptosis during high glucose exposure in human endothelial cells remains unclear.

Our present studies demonstrated that the marked increase of eNOS expression in HUVECs was obtained at short-term (2–24 h) of high glucose (33-mM) exposure and gradually down-regulated after 24 h exposure. However, the increased intracellular H_2O_2 generation and

apoptosis were significantly appeared only after 24- to 48-h exposure of high glucose in HUVECs. Induction of apoptosis is associated with a marked concomitant increase of H_2O_2 generation. In this condition, the H_2O_2 generation may be converted from $O_2^{\cdot -}$ by superoxide dismutase (SOD). Indeed, SOD, a scavenger of $O_2^{\cdot -}$, prevents the high glucose-induced apoptosis (data not shown). Experiments with mannitol also certainly rule out an effect of osmolarity. Moreover, the increased intracellular H_2O_2 generation and apoptosis by high glucose exposure for 48 h, could be inhibited by antioxidant (ascorbic acid) and NO donor (SNP), but enhanced by NOS inhibitor (L-NAME). These results suggest that the early expression of eNOS during high glucose exposure (< 24 h) may play a protective role in high glucose-induced endothelial cells damage.

On the basis of these findings, we hypothesize that the potential mechanism may be explained as follows. During high glucose treatment, the increased production of $O_2^{\cdot -}$, via autoxidation of glucose and nonenzymatic protein glycosylation, may disrupt cellular functions and cause oxidative damage [Wolff and Dean, 1987; Hunt et al., 1990]. NO may rapidly react with $O_2^{\cdot -}$ to form inactive nitrite and can be considered as an O_2^{-} scavenger. In this way, NO may provide a chemical barrier to the cytotoxic effects of $O_2^{\cdot -}$ [Tesfamariam, 1994]. Although the NO–$O_2^{\cdot -}$ interaction may produce "peroxynitrite," which is considered a highly toxic radical, the HUVECs have not yet shown apparent damage within 24-h exposure of high glucose in our experimental condition. The NO, therefore, may play an important role for protecting endothelial cell injury in early stage of high glucose exposure (< 24 h). However, in the late stage (> 24 h), the amplification of oxidative stress by a continuing cycle of metabolic stress leads to increased ROS production and compromises free radical inhibitory and scavenge system. The oxidative stress is exacerbated, leading to cell death and tissue damage [Baynes, 1991].

In conclusion, the results of the present work provide the molecular basis for understanding that NO may play a protective role from apoptosis during thr early stage of high glucose exposure. In the late stage, high glucose exposure leads to an imbalance of NO and ROS, inducing apoptosis. This phenomenon may explain, at least in part, the impaired endothelial function and vascular complications of diabetic mellitus that occur at later stages.

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