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# Up-Regulation of the Association Between Heat Shock Protein 90 and Endothelial Nitric Oxide Synthase Prevents High Glucose-Induced Apoptosis in Human Endothelial Cells

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**Abstract** Hyperglycemia is the hallmark of diabetes mellitus. Poor glycemic control is correlated with increased cardiovascular morbidity and mortality. High glucose can trigger endothelial cell apoptosis by de-activation of endothelial nitric oxide synthase (eNOS). eNOS was recently demonstrated to be extensively regulated by Akt and heat shock protein 90 (HSP90). Yet, little is known about the molecular mechanisms that regulate eNOS activity during high glucose exposure. The present study was designed to determine the involvement of protein interactions between eNOS and HSP90 in high glucose-induced endothelial cell apoptosis. The protein interaction of eNOS/HSP90 and eNOS/Akt were studied in cultured human umbilical vein endothelial cells (HUVECs) exposed to either control-level (5.5 mM) or high-level (33 mM) glucose for different durations (2, 4, 6, and 24 h). The results showed that the protein interactions between eNOS and HSP90 and between eNOS and Akt and the phosphorylation of eNOS were up-regulated by high glucose exposure for 2–4 h. With longer exposures, these effects decreased gradually. During early hours of exposure, the protein interactions of eNOS/HSP90 and eNOS/Akt and the phosphorylation of eNOS were all inhibited by geldanamycin, an HSP90 inhibitor. High glucose-induced endothelial cell apoptosis was also enhanced by geldanamycin and was reversed by NO donors. LY294002, a phosphatidylinositol 3 ($PI_3$) kinase inhibitor, inhibited the association of eNOS/Akt and the phosphorylation of eNOS but had no effect on the interaction between eNOS and HSP90 during early hours of exposure. From our results we propose that, in HUVECs, during early phase of high glucose exposure, apoptosis can be prevented by enhancement of eNOS activity through augmentation of the protein interaction between eNOS and HSP90 and recruitment of the activated Akt. With longer exposure, dysregulation of eNOS activity would result in apoptosis. The present study provides a molecular basis for the effects of eNOS in the prevention of endothelial cells apoptosis during early phase of high glucose exposure. These observations may contribute to the understanding of the pathogenesis of vascular complications in diabetes mellitus. J. Cell. Biochem. 94: 194–201, 2005. 

**Key words:** heat shock protein 90; endothelium; apoptosis; hyperglycemia

Atherosclerosis is one of the major complications of diabetes mellitus. Recent large-scale clinical trials have correlated poor glycemic control with an increased incidence of both microvascular and macrovascular diseases in diabetic patients [The Diabetes Control, and Complications Trial Research Group, 1993; Kuusisto et al., 1994; Beks et al., 1995].

Impairment of endothelium-dependent vasorelaxation is an early marker for the development of atherosclerosis which can be produced

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by elevation in blood glucose levels, as demonstrated in both animals [Oyama et al., 1986; Tesfamariam et al., 1989; Bucala et al., 1991] and humans [Ting et al., 1996; Williams et al., 1996]. In cultured human endothelial cells, hyperglycemia also has been found to be capable of triggering apoptosis [Baumgartner-Parzer et al., 1995; Ho et al., 2000]. Endothelial nitric oxide synthase (eNOS) dysregulation [Liang et al., 2001; Tomasz et al., 2002] and free radical generation [Tesfamariam, 1994; Wolff and Dean, 1998] were claimed to be involved in the induction of diabetic endothelial cell dysfunction. However, it has been well known that in the early stage of diabetes, the peripheral resistance is decreased and the blood flow increased. [Mogensen, 1971; Houben et al., 1992]. In nondiabetic individuals, acute exposure to high glucose also induces vasodilatation through increased secretion of endothelium-derived nitric oxide (NO) [Tilton et al., 1989; Williamson et al., 1993; Sandeman et al., 1996]. In our previous study, in which cultured human endothelial cells were exposed to high glucose, we demonstrated a biphasic response of an early up-regulation and a later down-regulation of eNOS expression, resulting in an imbalance between NO and free radical generation [Ho et al., 1999]. We proposed that this process might contribute to the high glucose-induced apoptosis in human endothelial cells.

Recently, eNOS was reported to be highly regulated by post-translational modification and protein interaction [Fulton et al., 1999]. Studies demonstrated that eNOS activity can be regulated by the post-translational modifications such as phosphorylation by Akt/protein kinase B [Fulton et al., 1999] and by the interactions with several regulatory proteins, such as heat shock protein 90 (HSP90) [Garcia-Cardena et al., 1998; Brouet et al., 2001]. HSP90 also plays important roles in the balance between NO and superoxide. Geldanamycin, an HSP90 inhibitor, can uncouple eNOS and increase eNOS-dependent superoxide anion production [Pritchard et al., 2001; Dikalov et al., 2002]. These findings suggest that the association of HSP90 with eNOS is critical in the process of eNOS-associated NO production. However, little is known about the effect of elevated glucose on the regulation of eNOS function.

In the present study, we tested the hypothesis that, in human umbilical vein endothelial cells (HUVECs), the protective effects of eNOS in the early stage of high glucose exposure is mediated through eNOS\HSP90\Akt protein interaction.

## MATERIALS AND METHODS

### Cell Culture

HUVECs were cultured as previously described [Jaffe et al., 1973; Maciag et al., 1979]. Cells were seeded at a density of $1 \times 105$ per 75 $cm^2$ flask in medium 199 (Gibco, Grand Island, NY), supplemented with 20 mmol/L HEPES, 100 μg/ml endothelial cell growth supplement (Collaborative Research, Inc., Bedford, MA), and 20% fetal calf serum (Gibco). The cultures were maintained at 37°C with a gas mixture of 5% CO2–95% air. Subcultures were performed with trypsin-EDTA. All media were supplemented with 5 U/ml heparin, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Medium was refreshed every third day. Endothelial cells of the third to fifth passages in actively growing condition were used for experiments. HUVECs were treated with media containing either 5.5 or 33 mM of glucose for 2–24 h in the presence or absence of sodium nitroprusside (1 μM), *S*-nitroso-*N*-acetylpenicillamine (1 μM), geldanamycin (1 μg/ml) or LY294002 (10 μM), a phosphatidylinositol 3 ($PI_3$) kinase inhibitor.

### Western Blot

Cells were lysed in the lysis buffer (50 mM Tris-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, pH 7.4) on ice for 20 min. The protein concentrations of the cell lysates were measured with a commercial BCA kit (Pierce Co., Rockford, IL). Protein (30 μg) of cellular lysates were subjected to electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) for the detection of eNOS, phospho-eNOS, HSP90, and caspase-3. The samples were then electrotransferred and immunoblotted with anti-eNOS, anti-phospho-eNOS, anti-HSP90, or anti-caspase-3 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After blocking, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Durham, NC). The protein expression was determined with the enhanced chemiluminescence (ECL) kit (Amersham, Bucks, UK), and exposed by the filters of Kodak X-Omat films. Beta-actin was

incorporated in the immunoblot as a control for the loading protein amount. Protein gel was scanned with a densitometer and was analyzed by a commercialized software (Molecular Dynamic Densitometer and ImageQuant version 3.3 software, ITC-Academic Computing Health Sciences, University of Virginia). The data was reported as the ratio of the band of interest to actin.

### Immunoprecipitation

Cell lysates were incubated with anti-eNOS or anti-Akt (StressGen) polyclonal antibody and protein A/G (Santa Cruz Biotechnology) to immunoprecipitate eNOS or Akt, and then immunoblotted with anti-HSP90 (StressGen Biotechnologies, Victoria, Canada) or anti-eNOS antibody to determine the amount of HSP90/eNOS in association with eNOS/Akt. Immunoglobulin G heavy chain was also immnoblotted as a control for the loading protein amount.

### Detection of Apoptosis

Apoptosis of the treated HUVECs was detected by the ELISA method of cell death detection (Roche Applied Science, Indianapolis, IN). This assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. It allows specific determination of mono- and oligonucleosomes in the cell lysates.

### Analysis of DNA Fragmentation

Oligonucleosomal banding was demonstrated by harvesting total cellular DNA. After the indicated treatment, an amount of $1 \times 106$ cells, both adherent and detached, were harvested and washed with phosphate-buffered saline (PBS), and then lysed in 50 mM Tris, pH 7.5, 10 mM EDTA, 0.5% Triton X-100, and 0.5 mg/ml proteinase K for 16 h at 50°C. DNA was then extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. The pellet was resuspended in Tris/EDTA and 10 μg/ml RNase A and the DNA was separated by electrophoresis on a 1.8% agarose gel stained with ethidium bromide. The gel was photographed with UV transillumination.

### Statistical Analysis

Results were expressed as mean ± SEM. n indicates the number of experiments. Data with various periods of exposure were compared by unpaired Student's $t$-test only if the analysis of variance (ANOVA) showed significant different. A $P$ value $< 0.05$ was considered statistically significant.

## RESULTS

### Effect of High Glucose on eNOS Expression and eNOS/HSP90 Interaction

After exposing the HUVECs to either 5.5 or 33 mM glucose for various time intervals (2, 4, 6, 24 h), the protein levels of eNOS and HSP90 in the cell lysates were measured. Proteins from the same cell lysates were subjected to Western blot and immunoprecipitation for eNOS and HSP90. The results showed that, after high glucose treatment, the e-NOS-associated HSP90 protein progressively increased to reach a peak level at 4 h, and then gradually decreased up to 24 h (Fig. 1A), while the expression of eNOS was increased with the highest level at 6 h and then gradually decreased up to 24 h (Fig. 1B). The expression of total HSP90 in cell lysates remained unchanged (Fig. 1C).

### Effect of High Glucose on eNOS/Akt Interaction and eNOS Phosphorylation

Lysates of HUVECs were immunoprecipitated with anti-Akt antibody. Immunoprecipitates were then Western blotted for eNOS and Akt. As shown in Figure 2A, the association between eNOS and Akt was up-regulated to reach a maximum at 4 h and then decreased up to 24 h after high glucose exposure (Fig. 2A). The phosphorylation of eNOS was also increased, with the maximum level at 4 h and gradually decreased up to 24 h (Fig. 2B).

### Effects of Geldanamycin and LY294002 on the High Glucose-Induced eNOS/HSP90 and eNOS/Akt Interactions

After pretreatment with geldanamycin or LY294002, HUVECs were subjected to high glucose exposure for 4 h and then the cell were harvested. The lysates were immunoprecipitated with anti-eNOS or anti-Akt antibody and the immunoprecipitates were immunoblotted for HSP90 and eNOS. The lysates were also immunoblotted with anti-phospho-eNOS antibody. After high glucose treatment for 4 h, the protein interaction between

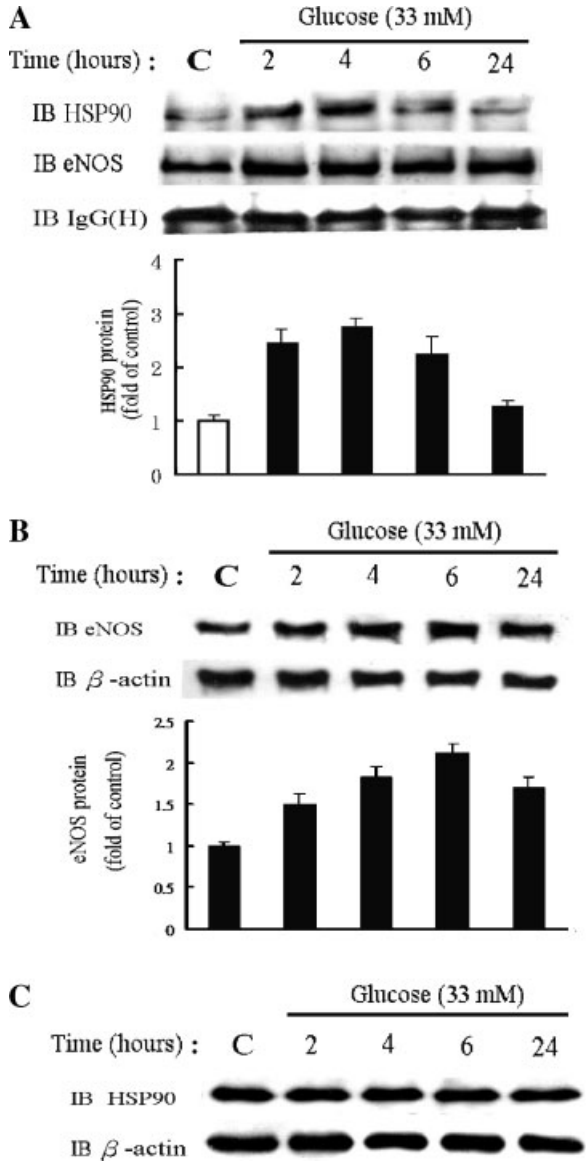


**Fig. 1.** Association of eNOS with HSP90 and eNOS protein expression in response to high glucose exposure. Human umbilical vein endothelial cells (HUVECs) were exposed to control (5.5 mM) or high glucose (33 mM) for different periods (2, 4, 6, 24 h). **A**: Lysates from treated HUVECs were immunoprecipitated with polyclonal antibody against eNOS. Immunoprecipitates were then Western blotted for eNOS and HSP90. **B**: The lysates were Western blotted with antibodies against eNOS. **C**: The lysates were Western blotted with antibodies against HSP90. Data are presented as mean ± SEM (n = 3).

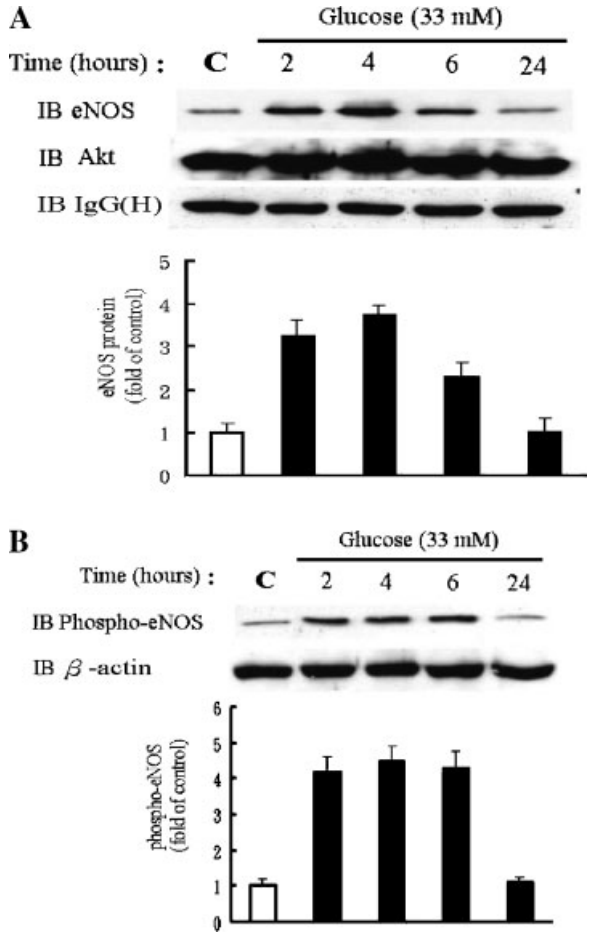


**Fig. 2.** Association of Akt with eNOS and phospho-eNOS protein expression during high glucose exposure. **A**: Lysates from HUVECs were immunoprecipitated with polyclonal antibody against Akt. Immunoprecipirates were Western blotted for eNOS. **B**: Lysates from HUVECs were Western blotted with antibodies against phospho-eNOS. Data are presented as mean ± SEM (n = 3).

HSP90 and eNOS was inhibited by geldanamycin but not by LY294002 (Fig. 3A), while the protein interaction between Akt and eNOS was inhibited by both geldanamycin and LY294002 (Fig. 3B). The eNOS phosphorylation elicited by high glucose exposure was also inhibited by both geldanamycin and LY294002 (Fig. 3C).

### Enhancement of High Glucose-Induced Apoptosis at Early Stage by Geldanamycin

As shown in Figure 4A, in the presence of geldanamycin (1 μg/ml), treatment of HUVECs with high glucose for 4 h markedly enhanced apoptosis. The apoptosis was partially inhibited by NO donors (sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine). The caspase-3 cleavage product was present in the combined glucose and geldanamycin-treated HUVECs (Fig. 4B). Apoptosis of the treated HUVECs was also confirmed by DNA fragmentation analysis (Fig. 4C).

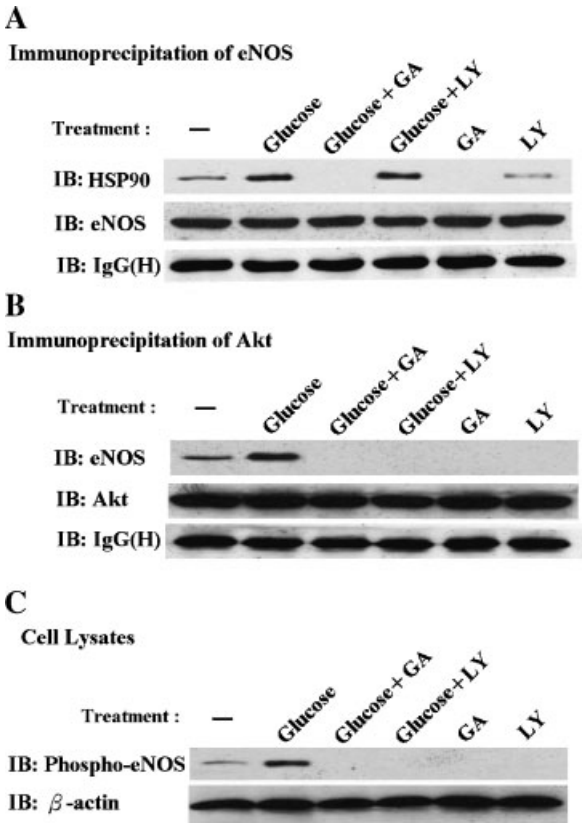


**Fig. 3.** Effects of geldanamycin and LY294002 on hyperglycemia-induced protein interactions of eNOS. Cells were treated with high glucose for 4 h. **A**: Geldanamycin inhibited the protein interaction between eNOS and HSP90 in high glucose exposure. **B**: Hyperglycemia-induced protein interaction between eNOS and Akt was inhibited by both geldanamycin and LY294002. **C**: Hyperglycemia-induced phosphorylation of eNOS was inhibited by both geldanamycin and LY294002.

## DISCUSSION

Vascular endothelium plays important roles in maintaining vascular tone and function, in part by the synthesis and release of NO [Vallance et al., 1989]. High glucose-induced endothelial dysfunction is generally believed to contribute to the development of diabetic complications [Lorenzi and Cagliero, 1991]. Reactive oxygen species (ROS)-induced de-activation of eNOS was found to produce endothelial cell dysfunction in diabetes [Tesfamariam,

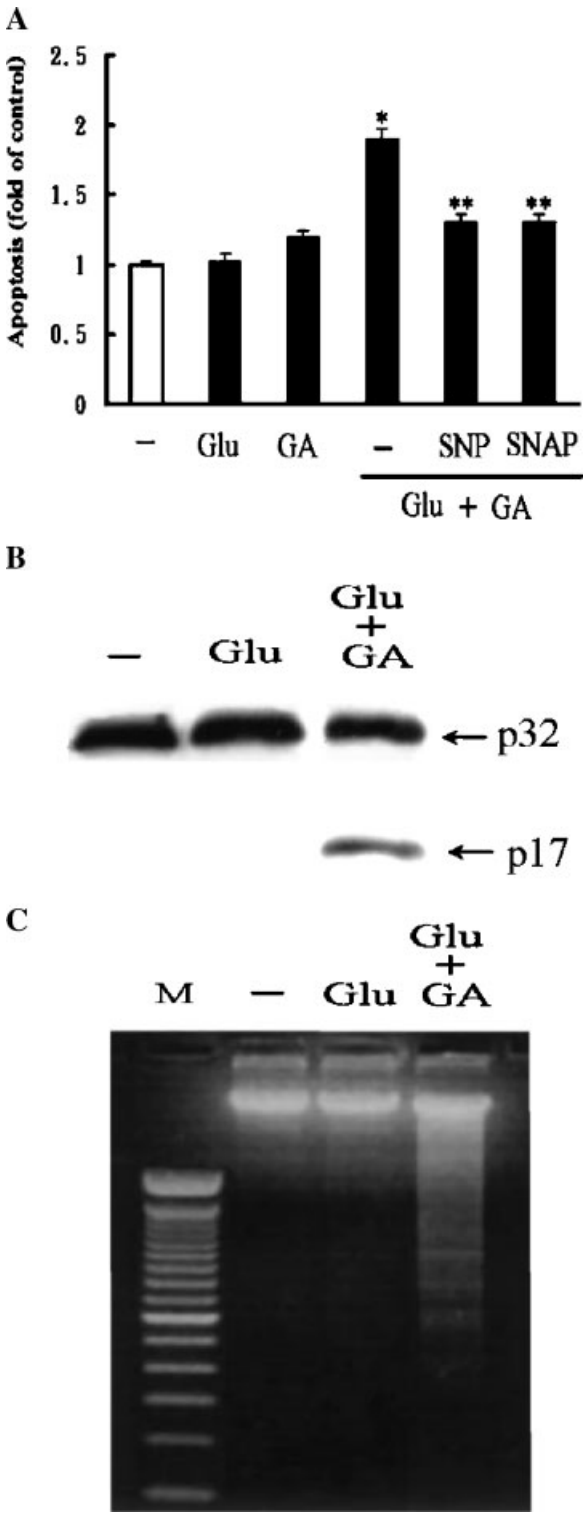


**Fig. 4.** Effects of geldanamycin and NO donors on high glucose-induced apoptosis in HUVECs. **A**: HUVECs were treated with 33 mM glucose for 4 h, in the presence or absence of sodium nitroprusside (1 μM, SNP), *S*-nitroso-*N*-acetylpenicillamine (1 μM, SNAP), or geldanamycin (1 μg/ml). Data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$, as comparing with control. $^{**}P < 0.05$, as comparing with the treatment of high glucose plus geldanamycin without addition of either SNP or SNAP. **B**: Lysates from treated HUVECs were immunoblotted with polyclonal antibody against caspase-3. **C**: HUVECs were treated with 33 mM glucose for 4 h, in the presence or absence of geldanamycin (1 μg/ml). Apoptosis of the treated HUVECs was detected by DNA fragmentation analysis. **Lane M**: DNA size marker was multiples of 100 bp.

1994; Wolff and Dean, 1998; Liang et al., 2001; Tomasz et al., 2002]. In cultured endothelial cells, hyperglycemia was shown to be able to induce apoptosis [Baumgartner-Parzer et al., 1995; Ho et al., 2000] and this process could be inhibited by NO [Ho et al., 1999]. Our previous study showed that, in cultured HUVECs, high glucose exposure could trigger a response to enhance eNOS expression to prevent cells from apoptosis in early phase (<24 h). But, with longer exposure (>24 h), the eNOS was gradually down-regulated and cell apoptosis ensued [Ho et al., 1999]. All these observations pointed to a pivotal role of the dysregulation of eNOS in the pathogenesis of diabetic endothelial dysfunction.

The interaction between eNOS and HSP90 has recently been shown to be critical in the regulation of eNOS function [Garcia-Cardena et al., 1998; Fulton et al., 1999; Brouet et al., 2001; Pritchard et al., 2001; Dikalov et al., 2002]. If not stimulated, eNOS is associated with caveolin and remains in a membrane-bound, inactive state [Michel et al., 1997; Prabhakar et al., 1998]. When stimulated with vascular endothelial growth factor (VEGF), the eNOS-caveolin complex is disrupted by $Ca^{2+}$/calmodulin and the association between eNOS and HSP90 is promoted [Brouet et al., 2001]. The eNOS-bound HSP90 can then recruit VEGF-activated Akt to the complex to induce phosphorylation of eNOS [Garcia-Cardena et al., 1998; Fulton et al., 1999; Brouet et al., 2001]. The binding of HSP90 to eNOS ensures the transition from the early $Ca^{2+}$-dependent to the late phosphorylation-dependent activation of the eNOS [Brouet et al., 2001]. Failure of this binding can cause eNOS uncoupling and increase eNOS-dependent superoxide anion production [Pritchard et al., 2001; Dikalov et al., 2002].

In the present study, we demonstrated that the association between eNOS and HPS90 was up-regulated during early phase of high glucose exposure. We found that, with high glucose treatment, eNOS expression reached a maximum level at 6 h while HSP90 protein level in immunoprecipitates of eNOS from the same cell lysates started to decrease at the same time point (Fig. 1). These observations exclude the possibility that the increase in association between HSP90 and eNOS is merely due to increased eNOS protein amount. Also, we demonstrated that the expression of HSP90 remained unchanged during early phase of high glucose exposure, which excludes the change of half life or increased expression of HSP90 as an etiology of this association.

In this study, we showed that the protein interaction between eNOS and HSP90 during early high glucose exposure was inhibited by geldanamycin (Fig. 3A). Failure of the association between HSP90 and eNOS leaded to the inhibition of Akt recruitment (Fig. 3B) and eNOS phosphorylation (Fig. 3C), which in turn resulted in enhancement of apoptosis (Fig. 4). We also showed that the increased apoptosis, which was mediated through caspase-3 activation, could be partially reversed by NO donors (Fig. 4A). These observations indicate that, without protein interaction with HSP90, eNOS alone would not be able to exert the protecting effects for HUVECs from apoptosis in response to high glucose exposure.

Our results demonstrated that, with high glucose exposure, the eNOS-Akt interaction and the eNOS phosphorylation were inhibited by LY294002, a PI3 kinase inhibitor (Fig. 3B,C). These findings suggest that high glucose can trigger a PI3 kinase-dependent activation (phosphorylation) of Akt, which is necessary for the recruitment of Akt to eNOS-HSP90 complex and the subsequent activation (phosphorylation) of eNOS.

Finally, we demonstrated that in the later phase of high glucose exposure, the protein interaction between eNOS and HSP90 and the recruitment of activated Akt were down-regulated, leading to uncoupling and de-activation of eNOS, NO/ROS imbalance and cell apoptosis [Ho et al., 1999]. One recent report showed that statin can promote eNOS/HSP90/Akt interaction and eNOS phosphorylation in endothelial cells [Agnés et al., 2001]. This endothelial cell protection mechanism may partially explain the effects of statins in the prevention of late vascular complications in diabetic patients [Taku et al., 2001; Heart Protection Study Collaborative Group, 2003].

In conclusion, the present study provides a molecular basis for eNOS in preventing endothelial cells from apoptosis during early phase of high glucose exposure. In the later stage, prolonged high glucose exposure leads to dysregulation of eNOS and enhanced apoptosis. This observation may explain, at least in part, the impaired endothelial dysfunction and vascular complications in diabetic mellitus.

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