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17β-Estradiol Antagonizes the Down-Regulation of Endothelial Nitric-Oxide Synthase and GTP Cyclohydrolase I by High Glucose: Relevance to Postmenopausal Diabetic Cardiovascular Disease

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ABBREVIATIONS: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; BH4, tetrahydrobiopterin; GTPCH-I, GTP cyclohydrolase I; 17β-E2, 17β-estradiol; BAEC, bovine aortic endothelial cell; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; ER, estrogen receptor; ROS, reactive oxygen species; PKC, protein kinase C; HAEC, human aortic endothelial cells; HRT, hormone replacement therapy

ABSTRACT

In postmenopausal women, the risk of diabetic cardiovascular disease drastically increases compared to that of men or premenopausal women. However, the mechanism of this phenomenon has not yet been clarified. We hypothesized that the beneficial effects of estrogen on endothelial function may be relevant to protection against hyperglycemia-induced vascular derangement. Bovine aortic endothelial cells were incubated for 72 h in the presence and absence of the physiological concentration of 17β-estradiol (17β-E2) under normal and high-glucose conditions. The presence of 17β-E2 significantly counteracted the reduction in basal NO production under high glucose conditions. This finding was associated with the recovery of eNOS protein expression, BH4 levels, and the activity and gene expression of GTP cyclohydrolase I (GTPCH-I), a rate-limiting enzyme for BH4 synthesis. Both the gene transfer of estrogen receptor α (ERα) using adenovirus and treatment with the protein kinase C inhibitor bisindolylmaleimide I significantly enhanced the effects of 17β-E2 treatment under high glucose conditions, while these effects were completely abolished by the estrogen receptor antagonist ICI 182,780. Transfection of small interfering RNA targeting eNOS resulted in a marked reduction in GTPCH-I mRNA under both normal and high glucose conditions, but this reduction was strongly reversed by 17β-E2. These results suggest that the activation of ERα with 17β-E2 can counteract high glucose-induced down-regulation of eNOS and GTPCH-I in endothelial cells. Therefore, estrogen deficiency may result in an exaggeration of hyperglycemia-induced endothelial dysfunction, leading to the development of cardiovascular disease in postmenopausal diabetic women.

Introduction

Impaired NO bioavailability results in endothelial dysfunction, which is believed to be a characteristic feature of vascular diseases such as diabetic macroangiopathy (Cai and Harrison, 2000; Calles-Escandon and Cipolla, 2001; Creager et al., 2003). NO plays a central role in maintaining vascular homeostasis through its effects on endothelial cells, smooth muscle cells, leukocytes, and platelets (Albrecht et al., 2003). eNOS is only fully functional in a dimeric form, and the functional activity of the eNOS dimer is dependent on the number of bound BH4 molecules. BH4 levels are principally regulated by *de novo* synthesis, in which GTPCH-I is a rate-limiting enzyme (Gesierich et al., 2003).

Several lines of evidence have suggested the link of excess vascular oxidative stress with impaired NO activity in patients with diabetes (Tesfamariam and Cohen, 1992; Hayashi et al., 2005). It has been reported that supplementation with BH4 improves endothelial-dependent vasodilation by increasing NO activity in patients with type II diabetes mellitus (Heitzer et al., 2000). Thus, hyperglycemia and insulin resistance may be pathogenic factors that lead to decreased vascular relaxation via an imbalance of NO/O_2^- due to a relative deficiency of BH4 in endothelial cells (Shinozaki et al., 1999). Previously we reported that a high glucose concentration enhanced oxidative stress by eNOS dysfunction and activation of NADPH oxidase in BAECs (Ding et al., 2004).

Diabetes and abnormal glucose tolerance are associated with increased risk of cardiovascular disease (Kannel and McGee, 1979). The effect of diabetes on cardiovascular disease appears to be worse in women than in men (Barret-Connor et al., 1991). Since the risk of cardiovascular disease dramatically increases with age, especially after menopause (Gordon et al., 1978), the influence of postmenopausal hormone use on cardiovascular disease in postmenopausal women has been a subject of interest. Recent reports from HERS (Kanaya et al., 2003) and WHI (Margolis et al., 2004) randomized trials indicate that postmenopausal hormone replacement therapy (HRT) reduces the risk of

diabetes. Therefore, it remains important to gain a better understanding of the molecular mechanisms that confer cardiovascular-protective effects of hormone replacement therapy in postmenopausal women with diabetes.

Laboratory evidence suggests that the vascular endothelium is an important target of estrogen. It has been shown that estrogen activates eNOS via genomic and non-genomic mechanisms, leading to an increase in NO (Hayashi et al., 1995; Simoncini et al., 2000). We thus hypothesized that the profound effect of estrogen on eNOS-associated endothelial function may be relevant for obtaining protection against hyperglycemia-induced vascular impairment. In the present study, we demonstrate that treatment with 17β-E2 at the physiological concentration can significantly counteract the down-regulation of eNOS and GTPCH-I in BAECs under high glucose conditions. We found that 17β-E2 increased GTPCH-I expression independently of eNOS expression, despite the finding that GTPCH-I expression was strongly regulated by the presence of eNOS.

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Materials and Methods

Materials. We used 17β-estradiol (Sigma, St. Louis, MO), D-glucose, D-mannitol (Wako, Osaka, Japan), bisindolylmaleimide I (Calbiochem, Darmstadt, Germany), Takara One Step RNA PCR Kit (Takara, Kyoto, Japan), eNOS monoclonal antibody (BD Biosciences, San Jose, CA), estrogen receptor α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-actin antibody (Abcam, Cambridge, UK), siGENOME set of four duplexs (siRNAs targeting eNOS) (GE Healthcare Bio-Science Corp., Piscataway, USA), and control (non-sil) siRNA fluorescein (Qiagen, Miami, FL). Lipofectamine2000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA). ICI 182,780 was kindly provided by Astra-Zeneka Pharmaceuticals (Macclesfield, UK). GTPCH-I antibody was kindly provided from Prof. H. Ichinose (Tokyo Institute of Technology, Yokohama, Japan) who made it using recombinant full length human GTPCH-I.

Cell culture. BAECs were obtained from a fetal calf as described previously (Hayashi et al., 1995), and cultured in DMEM (Invitrogen) with 10% (v/v) of Calf Serum (CS), 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine. Cells were allowed to reach 80% confluence, and were then stimulated with different concentrations of D-glucose (5.5, 10.5, 30.5 mM) as well as other reagents in phenol-red free DMEM with 2% (v/v) of CS. We defined the control as the status under 5.5mM glucose concentration, which is the same concentration as normal human plasma. Mannitol was used as a control to rule out the effect of osmotic pressure. Subconfluent cell monolayers were studied at six to eight passages.

Determination of intracellular BH4 levels and GTPCH-I activity. BAECs were harvested with trypsin, pelleted by centrifugation and frozen at -80°C. BH4

measurements were performed following the high performance liquid chromatography procedure described by Fukushima and Nixon (1980). GTPCH-I activity was assayed as described by Sawada et al. (1986), based on the quantification of D-erythro-neopterin by high performance liquid chromatography after the conversion of enzymaticaly formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythro-neopterin by sequential iodine oxidation and dephosphorylation.

RT-PCR analysis of GTPCH-I mRNA and eNOS mRNA. Total RNA was isolated from BAECs with TRIZOL reagent (Invitrogen) following the manufacturer's protocol. One μg of total RNA was reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (200 U/μl), and the cDNA samples were analyzed for GTPCH-I and eNOS by PCR. PCR was performed in 25 μl of reaction volume containing 80.2 mM each of Ex-Taq polymerase buffer and deoxynucleotide phosphate, as well as mixture and 81.5 mM of $MgCl_2$, 2.5 U Ex-Taq DNA polymerase, oligonucleotide primers (0.5 μM of each), and the cDNA. The programmed cycles were as follows: 1 cycle of 50°C x 30 min and 94°C x 2 min followed by 40 cycles of 94°C x 30 sec, 60°C x 30 sec, and 72°C x 1 min. The PCR amplified product was analyzed by 1% agarose gel electrophoresis. The bovine GTPCH-I primer sequences were 5' CCGCCTACTCGTCCATCCTGA 3' (sense) and 3' ACCTCGCATTACCATACACAT 5' (antisense). The bovine eNOS primer sequences were 5' CCGTGTCCAACATGCTGCT 3' (sense) and 3' ACCTCGCATTACCATACACAT 5' (antisense). The bovine β-actin primer sequence were 5' CGAGCATTCCCAAAGTTCTACAGTG 3' (sense) and 3' CTACATACTTCCGAAAACCAGGGG 5' (antisense).

Western blot analysis of eNOS and ERα protein. Total protein was extracted by

adding lysis buffer (10 mM Tris (pH 7.4), 1% SDS, and 1 mM sodium carbonate). Protein was quantified and 10 μg of protein was loaded into each lane of 7.5% (12.5% for β-actin) polyacrylamide gel. The protein was electrophoresed and transferred to polyvinylidene difluoride membrane, blocked with 2% skimmed milk powder, and then incubated with primary antibody overnight at 4 °C and horseradish peroxidase-conjugated anti–mouse IgG antibody for 1 h. The bands were developed in the dark on the film (Fuji Medical X-Ray Film, Tokyo, Japan), and measured densitometrically by an NIH Image analyzer. Equal protein loading was confirmed by Coomassie Brilliant Blue and Amido Black staining of protein in each lane of the same blot.

Measurement of nitrite. As described in our previous report (Ding et al., 2004), the concentration of nitrite:metabolites of NO in 10 μl of culture medium was determined with an automated NO detector HPLC system (ENO10; EICOM, Kyoto, Japan), where nitrite was detected based on Griess reaction to form a purple azo dye, then absorbance at 540 nm was detected. Values for nitrite level were normalized for cell protein. The incubated medium was not completely free from nitrite; therefore, an aliquot of medium underwent the same process as the medium obtained from the cultured cells. We usually used the nitrite value obtained with the medium alone as a blank, and it was subtracted from all the samples. Our preliminary study confirmed that co-incubation with the NOS inhibitor N^G-nitro-L-arginine methyl ester (1 mM) decreased a nitrite level after subtraction to less than 5 % of the previous subtracted value, indicating that nitrites in the medium where BAECs are present are mostly from NOS-mediated NO production.

Construction of an adenovirus vector carrying ERα and transfer into cultured ECs. Human ERα cDNA cloned into pBR322 was inserted into pAxCAwt.

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Recombinant adenoviruses were constructed by Takara. Adenoviruses carrying an *Escherichia coli* lacZ gene encoding a nucleus-localized variant of β-galactosidase were also used. We grew $5x10^5$ BAECs in a 6-well plate for 24 h, then incubated cells with adenoviruses at a multiplicity of infection of 20 for 24 h.

Preparation of siRNAs and transfection of siRNAs into EC. Four siRNAs targeting human eNOS with the following sense and antisense sequences were used: Nos.1, (antisense), Nos.2, CGGAACAGCACAAGAGUUAUU (sense), 5'-PUAACUCUUGUGCUGUUCCGUU (antisense), Nos.3, CGAGGAGACUUCCGAAUCUUU (sense), 5'-PAGAUUCGGAAGUCUCCUCGUU (antisense), Nos.4, AGGAGAUGGUCAACUAUUUUU (sense), 5'-PAAAUAGUUGACCAUCUCCUUU (antisense). Control(non-sil.) siRNA, Fluorescein (20 μM) was used for negative control. The siRNAs were disolved in siRNA buffer (20 mM KCl, 6.0 mM HEPES (pH 7.5) and 0.2 mM $MgCl_2$) to prepare a 20 μM stock solution. We grew $3x10^5$ BAECs in a 6-well plate for 24 h, then transfected with siRNAs in 1.5 ml of serum and antibiotics-free medium using Lipofectamine2000. 5 μl of Lipofectamine2000 in 245 μl of Opti-MEM was incubated at room temperature for 5 min, then added in 5μl of siRNA stock solution in 245 μl of Opti-MEM. After 20 min incubation at room temperature, siRNAs-lipid complex solution was added to the cells in serum. After incubation for 6 h at 37 °C, the medium was replaced with 2 % CS and 1 % PS contained medium and cultured for 48 h.

Statistical analysis. Data were obtained from three or four different experiments. Results are expressed as the mean of independent experiments (mean ± S.D.). Overall differences between groups were analyzed using a two-way ANOVA followed by the Fisher's post hoc test for determining differences between means when more than two

groups were compared. An independent *t*-test was used when only two groups were compared. In all tests, a probability level of $P<0.05$ was used as the decision rule for significance testing.

Results

Effects of a high glucose levels and 17β-E2 on BH4 levels and GTPCH-I activity and expression

Abnormally low levels of cofactor BH4 may be a factor involved in eNOS dysfunction; therefore, we measured BH4 levels in BAECs cultured for 72 h under high glucose conditions. Under the two high-glucose conditions (10.5 and 30.5 mM), BH4 levels were significantly decreased by 20% compared with those under normal glucose conditions (5.5 mM) (Fig. 1A). On the other hand, no change was observed in the number of cells with exposure to high glucose (95.3±4.8% and 92.2±6.1% of the control [5.5 mM glucose] with 10.5 and 30.5 mM glucose, respectively). To evaluate whether 17β-E2 is effective at ameliorating eNOS dysfunction, BAECs were treated with 17β-E2. The addition of 17β-E2 (1 nM) significantly increased BH4 levels, not only under normal, but also under high glucose conditions (Fig. 1A). Even under hyperglycemic conditions, almost the same amount of BH4 was obtained as under normal glucose conditions. To rule out an osmotic effect, we added 25 mM mannitol to 5.5 mM glucose and 20 mM mannitol to 10.5 mM glucose. It was confirmed that mannitol did not affect BH4 levels (102.3±2.4% and 107.5±6.2% of control [5.5 mM glucose] with and without 17β-E2, respectively) or GTPCH-I activity (98.3±2.9% and 103.5±6.1% of the control 5.5 mM glucose with and without 17β-E2, respectively).

Furthermore, we measured the activity and mRNA expression of GTPCH-I, a rate-limiting enzyme required for BH4 synthesis. GTPCH-I activity also decreased under high glucose conditions and was increased by 17β-E2 (Fig. 1B). The basal expression level of GTPCH-I mRNA under high glucose conditions did not significantly differ from that under normal glucose conditions. Treatment with 17β-E2 resulted in a significant increase in GTPCH-I mRNA under both normal and high glucose conditions (Fig. 1C).

Interestingly, a more marked effect of 17β-E2 treatment on GTPCH-I mRNA was observed under high glucose conditions.

Effects of high glucose and 17β-E2 on eNOS protein expression

To determine whether a longer period of exposure to high glucose can affect the levels of expression of eNOS, we analyzed the expression levels of eNOS protein in BAECs cultured for 72 h under high glucose conditions. In this study, a high glucose concentration was found to reduce the level of expression of eNOS protein, with a decrease of 19% at 10.5 mM and of 36% at 30.5 mM (Fig. 2A) after 72 h. We previously reported that 17β-E2 (10^{-10}~10^{-8} M) increases eNOS expression in BAECs (Hayashi et al., 1995). In this study, 17β-E2 (1 nM) caused 1.4- and 2.1-fold increases in eNOS protein expression under normal and high glucose conditions, respectively (Fig. 2B). Interestingly, an even more marked effect of 17β-E2 treatment on eNOS protein expression was observed under high glucose conditions; this effect was similar to that of 17β-E2 treatment on the expression of GTPCH-I, which is involved in the synthesis of cofactor BH4.

Effects of high glucose levels and 17β-E2 on the production of nitrite

Given the finding that the expression levels of BH4 and eNOS protein were low in BAECs cultured for 72 h under high glucose conditions and that normal levels were restored by treatment with 17β-E2, we measured the levels of stable NO metabolites, nitrite and nitrate, in the culture medium. Under high glucose conditions, nitrite production was decreased by 15% compared to that under normal glucose conditions, whereas the addition of 17β-E2 (1 nM) restored nitrite production to normal glucose levels (Fig. 3). The decrease in nitrite levels in a high glucose environment may be associated with an increase in the production of ROS.

Roles of ERα and PKC in the effects of 17β-E2 on eNOS under high glucose conditions

To determine the mechanism underlying the effects of 17β-E2 under high glucose conditions, we investigated the role of estrogen receptors and PKC. Two types of ER have been identified to date (ERα and ERβ) (Walter et al., 1985; Kuiper et al., 1996). ERα is known to have a protective effect on vascular tissues, and a deficiency in the expression of ERα in endothelial cells is known to accelerate atherosclerosis (Sudhir et al., 1997; Lubanyi et al., 1997). In contrast, ERβ is more widely present in tissues, and in females it is abundantly expressed in smooth muscle cells (Mendelsohn, 2002). In this study, we examined the role of ERα in eNOS expression in BAECs under high glucose conditions. Although ERα protein levels tended to become lower under high glucose conditions, as compared with those under normal glucose conditions, gene transfer of ERα resulted in a successful increase in ERα under both conditions (Fig. 4A). The reduction in eNOS protein expression by exposure to high levels of glucose was counteracted by gene transfer of ERα using adenovirus, as well as by the administration of 17β-E2 (Fig. 4A and B). The upregulating effect of 17β-E2 was abolished by incubation of cells with ICI 182,780, a specific antagonist of ER, for 72 h (Fig. 4C). Furthermore, ICI 182,780 significantly decreased eNOS expression in the presence of 17β-E2 under normal glucose conditions. It is believed that PKC might be activated by hyperglycemia and thereby reduce eNOS expression (Srinivasan et al., 2004). The PKC inhibitor bisindolylmaleimide I significantly enhanced the protein expression of eNOS in the presence of 17β-E2 under high glucose conditions without affecting that under normal glucose conditions (Fig. 4C).

Effect of siRNAs targeting eNOS on GTPCH-I mRNA levels under normal and high glucose conditions

To determine whether or not there is a correlation between the expression levels of eNOS and GTPCH-I, we used siRNA to specifically ablate eNOS mRNA in BAECs. Two of four siRNAs targeting eNOS (Nos. 2 and 3) successfully silenced the expression of eNOS mRNA (Fig. 5A) and protein (Fig. 5C), as compared with that of the negative control (non-sil) siRNA 48 h after transfection. The expression levels of GTPCH-I mRNA were significantly down-regulated by the elimination of eNOS mRNA (Fig. 5A). This finding suggests that the expression of GTPCH-I is greatly affected by the presence of eNOS. Under normal and high glucose conditions, the transfection of eNOS siRNA (No. 2) decreased GTPCH-I mRNA by 34% and 54%, respectively. The addition of 17β-E2 slightly but significantly increased GTPCH-I mRNA expression under normal and high glucose conditions. Although the ablation of eNOS by siRNA markedly depressed GTPCH-I expression under both normal and high glucose conditions, the presence of 17β-E2 strongly reversed this depression (Fig. 5B).

Discussion

In the present study, the impairment of endothelial function under hyperglycemic conditions was detected as a decrease in NO production resulting from the down-regulation of eNOS expression, BH4 levels, and the GTPCH-I expression and activity in BAEC. This decrease in NO production could lead to increased oxidative stress and impaired vascular dilation. It is thus most likely that an enhancement of BH4 synthesis and NO bioavailability may be important in preventing diabetic cardiovascular complications. Previously we reported that under high glucose conditions, the activation of NADPH oxidase and the uncoupling of eNOS due to a BH4 deficiency increased ROS (Ding et al., 2004).

We inferred that 17β-E2 may reverse hyperglycemia-induced endothelial dysfunction, because premenopausal women have a lower incidence of cardiovascular disease than do postmenopausal women and men of a similar age (Fujishima et al., 1996; Gordon et al., 1978). Our present results showed that treatment with the physiological concentration of 17β-E2 (1 nM) in endothelial cells simultaneously exposed to high levels of glucose increased, not only the eNOS expression level but also intracellular BH4, as well as GTPCH-I activity and gene expression levels. Interestingly, 17β-E2 was found to be more effective under high glucose conditions. The characteristic effects of 17β-E2 on hyperglycemia have been noted in a previous clinical study (Colacurci et al., 1998), although the underlying mechanisms have not yet been investigated. It has been reported that 17β-E2 elevates mRNA levels of GTPCH-I and intracellular BH4 levels in microvascular endothelial cells in the brain (Serova et al., 2004). However, the overall pattern of effects of 17β-E2 on BH4 and GTPCH-I observed in that study appears to differ somewhat from the pattern observed in BAECs in the present study, as based on differences between microvessels and muscular and elastic arteries. Statins

(3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) and insulin have also been shown to increase both BH4 levels and GTPCH-I activity in endothelial cells (Ishii et al., 2001; Hattori et al., 2003). However, to the best of our knowledge, the present study is the first to indicate that the beneficial effect of 17β-E2 on eNOS under high glucose conditions may be associated with a recovery of GTPCH-I in endothelial cells.

There are a number of reports showing the profound effect of high levels of glucose on eNOS expression. Shrinivasan *et al.* (2004) have documented that in HAECs, acute elevated glucose (4 h) led to the up-regulation of eNOS expression; however, chronic elevated glucose (1~7 days) was associated with decreases in eNOS expression and nitrite production, as well as to increases in the mitochondrial production of ROS. Our previous study demonstrated that high glucose exposure for 24 h significantly increased eNOS mRNA expression (Ding et al., 2004). Noyman et al. (2002) have found reduced levels of eNOS expression and activity with a decrease in insulin sensitivity in BAECs cultured under high glucose conditions for 2 weeks. In contrast, one previous study revealed that eNOS expression and NO production are increased in HAECs cultured under high glucose conditions for 2 weeks. Possible reasons for this apparent discrepancy may include the use of different types of endothelial cells and different culture conditions. In agreement with recent results (Noyman et al., 2002; Srinivasan et al., 2004), the present results revealed decreases in eNOS protein expression and nitrite production in BAECs cultured under high glucose conditions for 1 ~3 days.

This study supports our previous reports showing a gender difference in NO production in the aortas of animals and humans (Hayashi et al., 1992, 1995, 2002). We have reported that atherosclerosis developed more slowly in female rabbits than in male rabbits fed a high-cholesterol diet, which may have been caused by a difference in the basal release, as well as the stimulated release of NO (Hayashi et al. 1995, 2000, 2002). The

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results of the present study also suggest that estrogen contributes to the improvement of endothelial function and provides protection against the progression of atherosclerosis. We have also shown that basal eNOS can be activated by 17β-E2 at physiological concentrations (10^{-12}~10^{-8} M) in young cells (passaged fewer than 6 times), whereas ERα is decreased in old cells (passaged more than 12 times) (Hayashi et al., 1995). We preliminarily observed that the expression of ERα at passages 6 to 8 tended to be lower under high glucose than under normal glucose conditions (Fig. 4A). Accordingly, the gene transfer of ERα may be important for allowing estrogen to act effectively against the type of endothelial dysfunction caused by aging and hyperglycemia. Treatment with 17β-E2 following the gene transfer of ERα using adenovirus resulted in a large increase in eNOS expression in BAECs. This effect was abolished by the addition of ICI 182,780, an ER antagonist, which confirmed the involvement of genomic activity.

PKC may be yet another important factor in eNOS regulation in hyperglycemia. Ohara et al. (1995) have shown that PKC inhibits eNOS expression in BAECs. It has been suggested that the decrease in eNOS expression observed in hyperglycemia is mediated by PKC and mitochondrial ROS via the activation of oxidative stress transcription factor AP-1 (Srinivasan et al., 2004). eNOS activated by the phosphorylation of Ser 1177 through the phosphatidylinositol 3-kinase/Akt pathway is inactivated by the phosphorylation of Thr 495 and dephosphorylation of Ser 1177 through PKC (Matsubara et al., 2003). Hyperglycemia appears to alter the signaling pathway activating eNOS via modification at the Akt site (Du et al., 2001) and the activation of PKC (Ishii et al., 2001; Naruse et al., 2006). In the present study, the PKC inhibitor bisindolylmaleimide I enhanced the effects of 17β-E2 on eNOS expression under high glucose conditions, suggesting that the recovery effect of 17β-E2 on high glucose-induced eNOS down-regulation is regulated by PKC.

Furthermore, this study is the first to demonstrate that eNOS expression levels

affect GTPCH-I expression levels in endothelial cells. We thus found that transfection with siRNA-targeting eNOS reduced the expression of GTPCH-I mRNA. This suggests that the decrease in eNOS observed under pathological conditions such as inflammation, atherosclerosis, or diabetes accelerates endothelial dysfunction by decreasing levels of the cofactor BH4. However, even when eNOS siRNA was transfected, 17β-E2 was capable of increasing GTPCH-I expression levels. Thus, this effect of 17β-E2 was more clearly demonstrated in an experiment using eNOS siRNA, in which 17β-E2 enhanced the level of GTPCH-I mRNA expression, regardless of whether or not GTPCH-I was down-regulated by high-glucose conditions.

In diabetes, elevated blood glucose, altered insulin signaling, increased ROS, enhanced PKC, and inflammation can together or separately, lead to a decrease in NO bioavailability (Endemann et al., 2004). High glucose levels reduced eNOS expression, GTPCH-I expression, and BH4 levels in BAECs. Finally, accelerated NO degradation by ROS might result in a decline in NO bioavailability, although we failed to confirm the possible involvement of oxidative stress in the high glucose-induced decrease in eNOS expression in BAECs. Importantly, 17β-E2 treatment ameliorated these endothelial cell abnormalities under high glucose conditions, which appeared to be related to the activation of ERα and masking by PKC.

The results of the present study may contribute to an understanding of the anti-atherogenic effects of estrogen in preventing endothelial dysfunction in diabetes mellitus (Fig. 6). If estrogen also prevents endothelial NO dysfunction under high glucose conditions in humans, premenopausal diabetic women may be protected against the progression of atherosclerosis. In fact, the prevalence of coronary heart disease in premenopausal women is much lower than that in the same generation of males or that of postmenopausal women. HRT in postmenopausal diabetic women may provide the benefit of reduced risk of

developing cardiovascular disease. However, it should be noted that WHI (Pradhan et al., 2002) and WHIMS (Brinton et al., 2005) indicated the risk associated with HRT (0.625 mg of conjugated equine estrogen and 2.5 mg of medroxyprogesterone acetate per day) for stroke, coronary heart disease, and Alzheimer's Disease, even in diabetic patients. However, most of the patients in the study had another coronary risk factor such as obesity (66%), smoking habit (50%), and hypertension (35%). In fact, the data from the WHI from patients without a uterus who had been prescribed only conjugated equine estrogen showed no increase in CHD. It is generally thought that estrogen may be atheroprotective (Hayashi et al. 2002); however, in most clinical cases, concomitant administration of progesterone, which masks the beneficial effects of estrogen, is necessary to prevent uterine cancer. Therefore, we cannot conclude that estrogen has a negative effect, simply based on the results of the WHI study. The present study conclusively demonstrates that the activation of ERα with 17β-E2 can counteract the high glucose-induced down-regulation of eNOS and GTPCH-I in endothelial cells. Therefore, estrogen may be of potential use in the reversal of hyperglycemia-induced endothelial dysfunction, in turn retarding the development of cardiovascular disease in postmenopausal diabetic women. The clinical implications of the present findings will need to be addressed in future studies.

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Footnotes

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Legends for Figures

Fig. 1. Effect of 17β-E2 on BH4 levels (A), GTPCH-I activity (B), and GTPCH-I mRNA expression (C) in BAECs under different glucose-level conditions. Cells were incubated for 72 h with different concentrations of D-glucose (5.5, 10.5 or 30.5 mM) in the absence or presence of 1 nM 17β-E2. Mannitol was added to rule out an osmotic pressure effect. It was confirmed that mannitol exerted no effect on either BH4 levels or GTPCH-I activity. In panel C, representative RT-PCR data are shown in the upper image. Note that there is no apparent difference in β-actin mRNA, which was used as an internal control. Each result is expressed as the percentage of the value compared to that of the band obtained under normal (5.5 mM) glucose conditions without 17β-E2. *P<0.05 compared with normal (5.5 mM) glucose in the absence of 17β-E2. $^{\dagger}P$<0.05 compared to samples treated with 10.5 mM glucose in the absence of 17β-E2. $^{\#}P$<0.05 compared to samples treated with 30.5 mM glucose in the absence of 17β-E2 (A, B) or to those treated at a normal glucose concentration in the presence of 17β-E2 (C), as determined by ANOVA followed by Fisher's post hoc test. NG = normal glucose. HG = high (30.5 mM) glucose. Man = mannitol (25 mM).

Fig. 2. (A) Changes in eNOS protein expression in BAECs under different glucose-level conditions. (B) Effects of 17β-E2 on the high glucose-induced decrease in eNOS protein expression in BAECs. Cells were incubated for 72 h with different concentrations of D-glucose (5.5, 10.5, or 30.5 mM) in the absence or presence of 1 nM 17β-E2. Mannitol was added to the samples in order to rule out an osmotic pressure effect. Each densitometric result is expressed as the relative percentage of the band obtained under abnormal glucose conditions, as compared to the band obtained under normal (5.5 mM) glucose conditions in the absence of 17β-E2 taken as 100%. Representative immunoblots are shown in the upper trace of each panel. *P<0.05 compared with the sample exposed to a normal glucose concentration in the absence of 17β-E2. $^{\#}P$<0.05 compared with the sample exposed to a high (30.5 mM) glucose concentration and in the absence of 17β-E2, as

determined by ANOVA, followed by Fisher's post hoc test. NG = normal glucose. HG = high glucose. Man = mannitol (25 mM).

Fig. 3. Effect of 17β-E2 on the nitrite concentrations in the cultured medium of BAECs under normal and high glucose conditions. Cells were incubated for 72 h with 5.5 or 30.5 mM glucose in the absence (open bar) or presence (solid bar) of 1 nM 17β-E2. The nitrite concentrations are expressed as percentage of the value obtained under normal glucose conditions in the absence of 17β-E2. *$P<0.05$ compared with normal glucose in the absence of 17β-E2. $^{\#}P<0.05$ vs. high glucose in the absence of 17β-E2, by ANOVA followed by the Fisher's post hoc test. Each result is expressed as percentage of the value under normal (5.5 mM) glucose conditions. NG = normal glucose. HG = high glucose. Man = mannitol (25 mM).

Fig. 4. (A) Effects of ERα overexpression by gene transfer of adenovirus vector (Ad ERα) on ERα protein expression in BAECs under normal and high glucose conditions. Where indicated, cells were treated with 1 nM 17β-E2. (B) Effects of ERα overexpression by gene transfer of adenovirus vector (Ad ERα) on eNOS protein expression in BAECs under normal and high glucose conditions. The cells transfected with adenovirus carrying an *Escherichia coli* lacZ gene (Ad LacZ) were served as control. Where indicated, cells were treated with 1 nM 17β-E2. * $P<0.05$ compared with normal glucose, Ad LacZ transfection. $^{\#}P<0.05$ compared with normal glucose, Ad LacZ transfection in the presence of 17β-E2. † $P<0.05$ compared with high glucose, Ad LacZ transfection, by ANOVA followed by the Fisher's post hoc test. (C) Effects of ICI 182,780 and bisindolylmaleimide I on eNOS protein expression in BAECs treated with 17β-E2 under normal and high glucose conditions. Cells were incubated for 72 h with 1 μM ICI 182,780 and 3 μM bisindolylmaleimide I. ***$P<0.001$ compared with normal glucose in the presence of 17β-E2. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with high glucose in the presence of 17β-E2. $^{\ddagger}P<0.05$ compared with normal

glucose in the presence of 17β-E2 and bisindolylmaleimide I, by ANOVA followed by the Fisher's post hoc test. In both A and B, each result is expressed as percentage of the control value under normal glucose conditions. Representative immunoblots are shown in the upper trace of each panel. Note that ERα is usually detected as a very faint band. NG = normal (5.5 mM) glucose. HG = high (30.5 mM) glucose.

Fig. 5. Effect of siRNAs targeting eNOS on GTPCH-I mRNA expression in BAECs. (A) Representative RT-PCR data showing that two of four eNOS siRNAs (Nos. 2 and 3) successfully eliminated eNOS mRNA 48 h after transfection and reduced GTPCH-I mRNA. Neg = Negative control. (B) Representative immunoblot data showing no detection of eNOS protein 48 h after transfection of successful eNOS siRNA(Nos. 2 and 3). (C) Representative immunoblot data showing no detection of eNOS protein and GTPCH-1 protein 48 h after transfection of successful eNOS siRNA. (D) Modulation by 1 nM 17β-E2 of the effect of successful eNOS siRNA (No. 2) on the mRNA expression level of GTPCH-I in BAECs under normal (5.5 mM) and high (30.5 mM) glucose conditions. **P*<0.05 compared with control. $^{\#}$*P*<0.05 compared with transfection of eNOS siRNA in the absence of 17β-E2, by ANOVA followed by the Fisher's post hoc test.

Fig. 6. Diagram of the possible mechanism underlying the effect of 17β-E2 on hyperglycemia-induced endothelial dysfunction. As depicted in the left panel, hyperglycemia would cause endothelial dysfunction by decreasing eNOS expression and BH4 synthesis and by activating PKC. The decrease in eNOS expression could lead to a decrease in GTPCH-I expression. PKC may inactivate eNOS possibly due to phosphorylation of Thr 495 and dephosphorylation of Ser 1177. As shown in the right panel, 17β-E2 via activation of ERα could increase eNOS expression and BH4 synthesis even in hyperglycemia, and activate eNOS by phosphorylating Ser 1177 through the

phoaphatidylinositol (PI) 3-kinase/Akt pathway. The increase in eNOS expression by 17β-E2 may be masked by PKC

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Figure 1

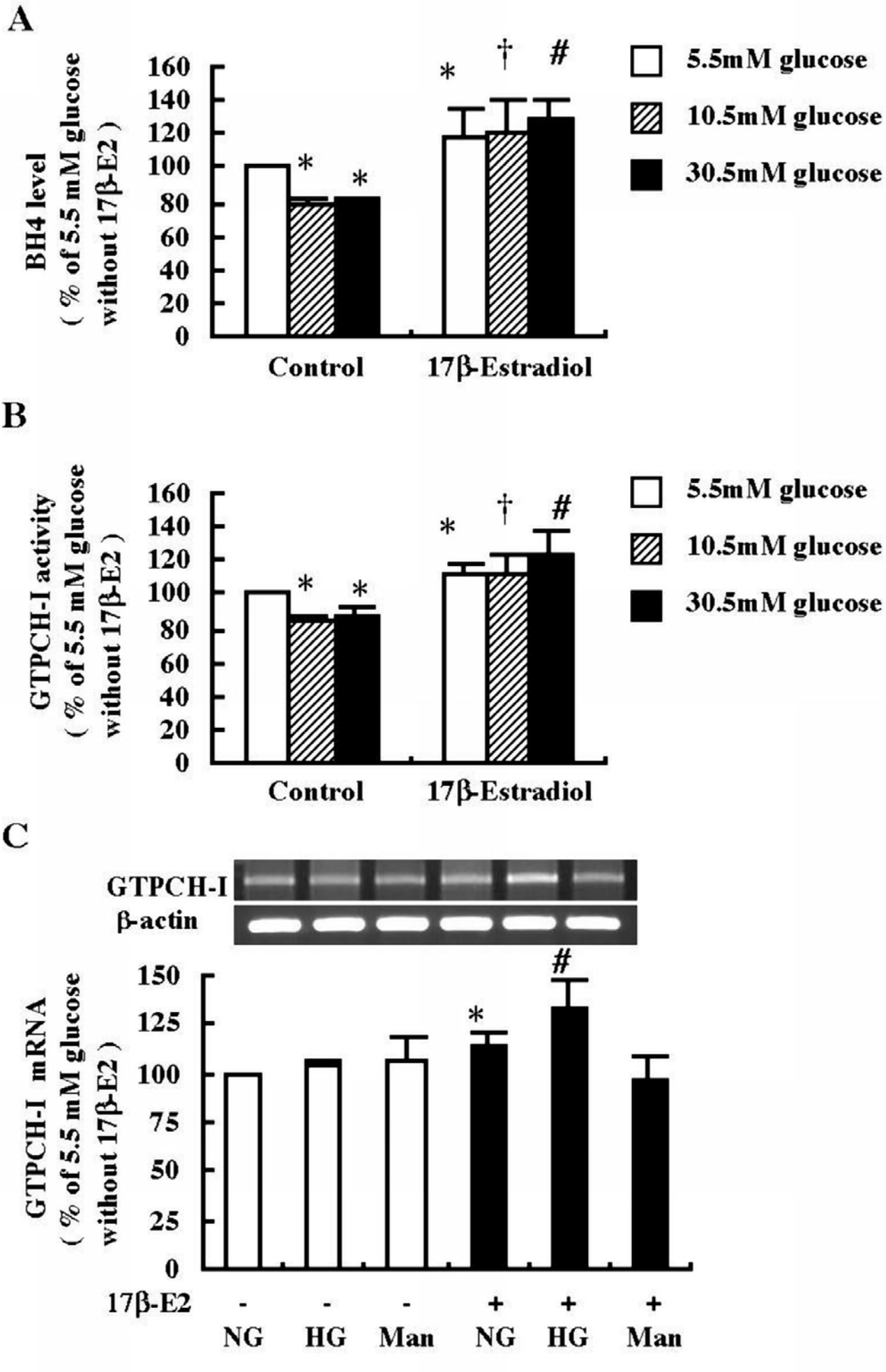

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Figure 2

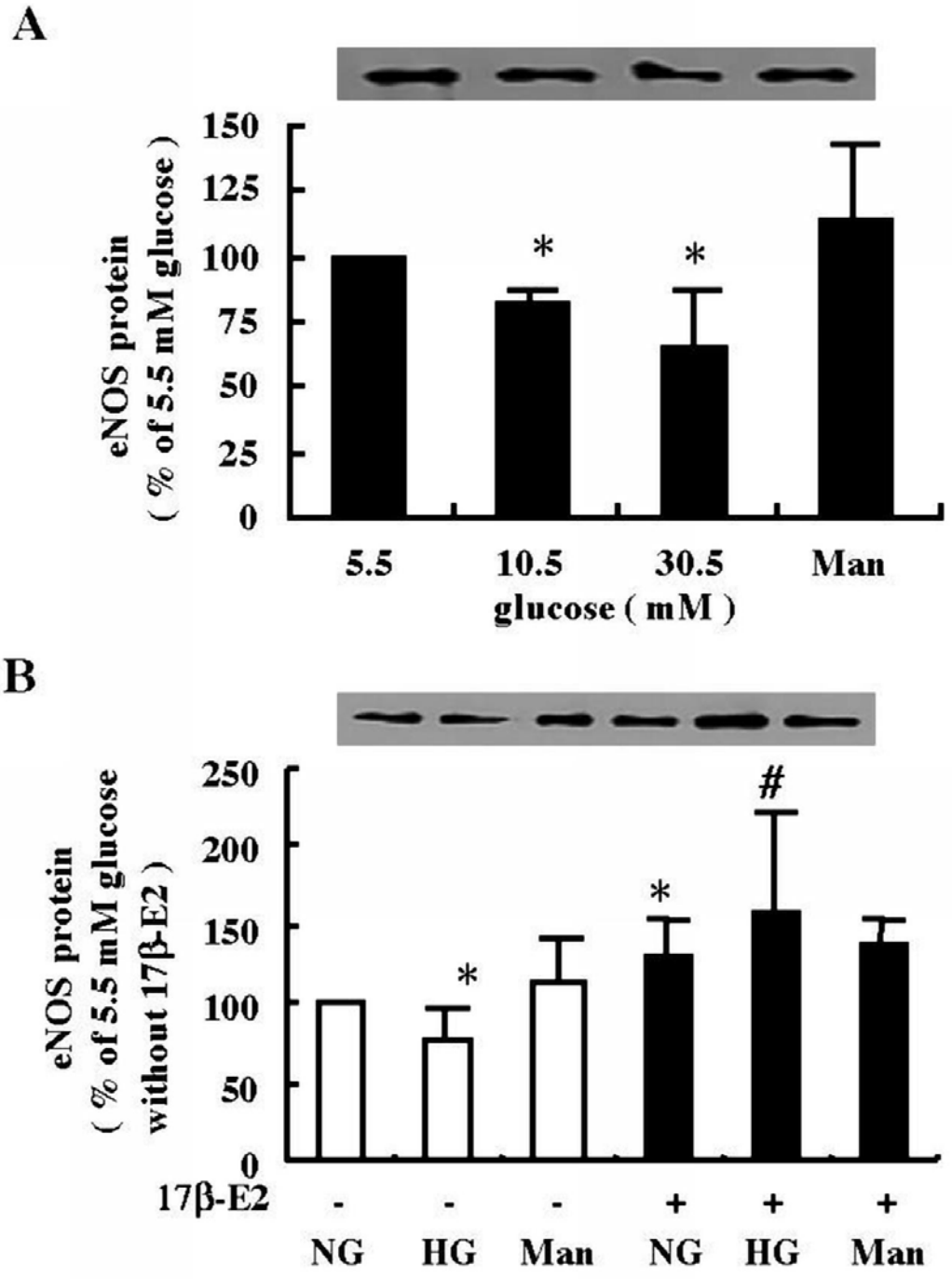

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Figure 3

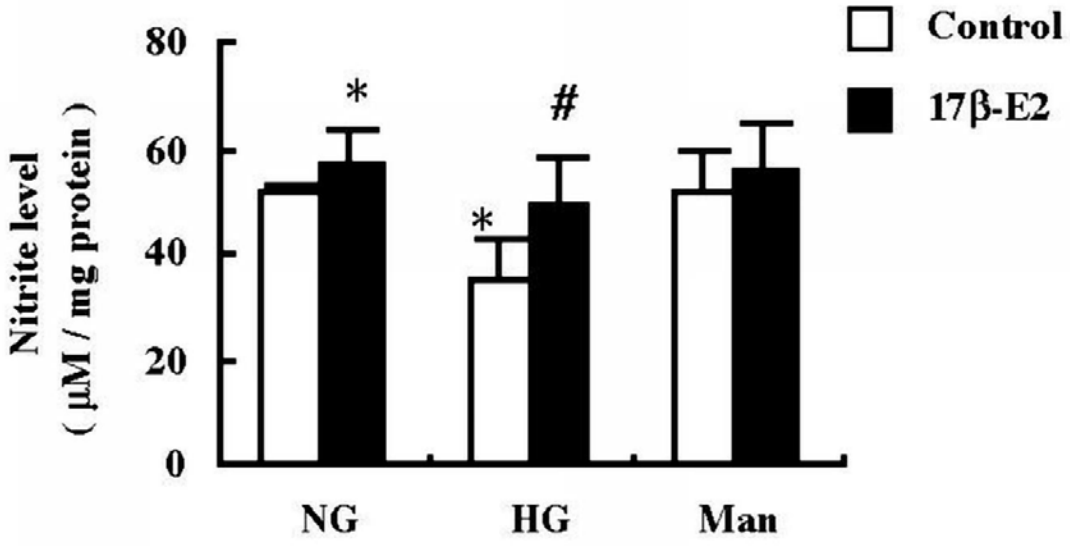

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Figure 4

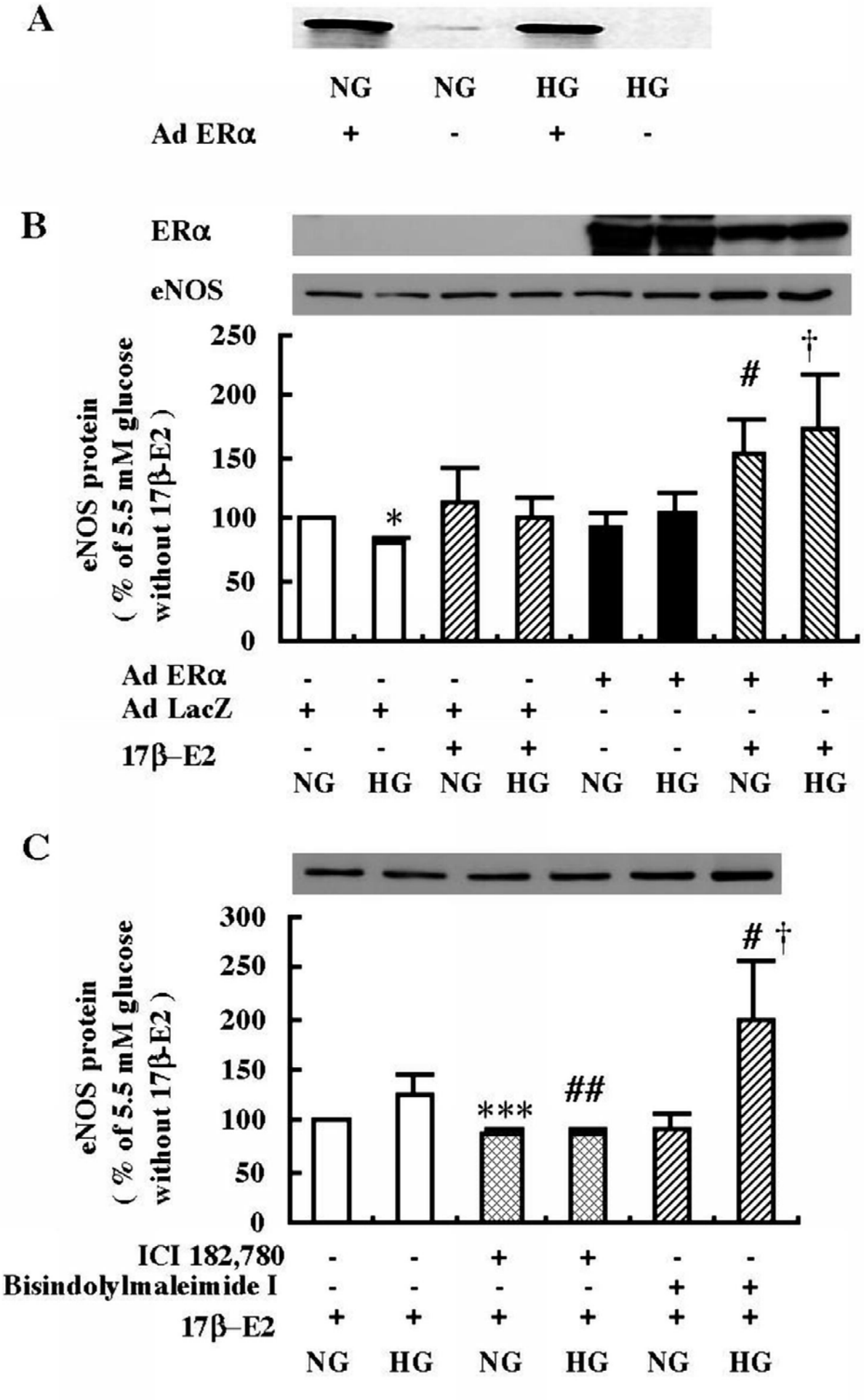

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Figure 5

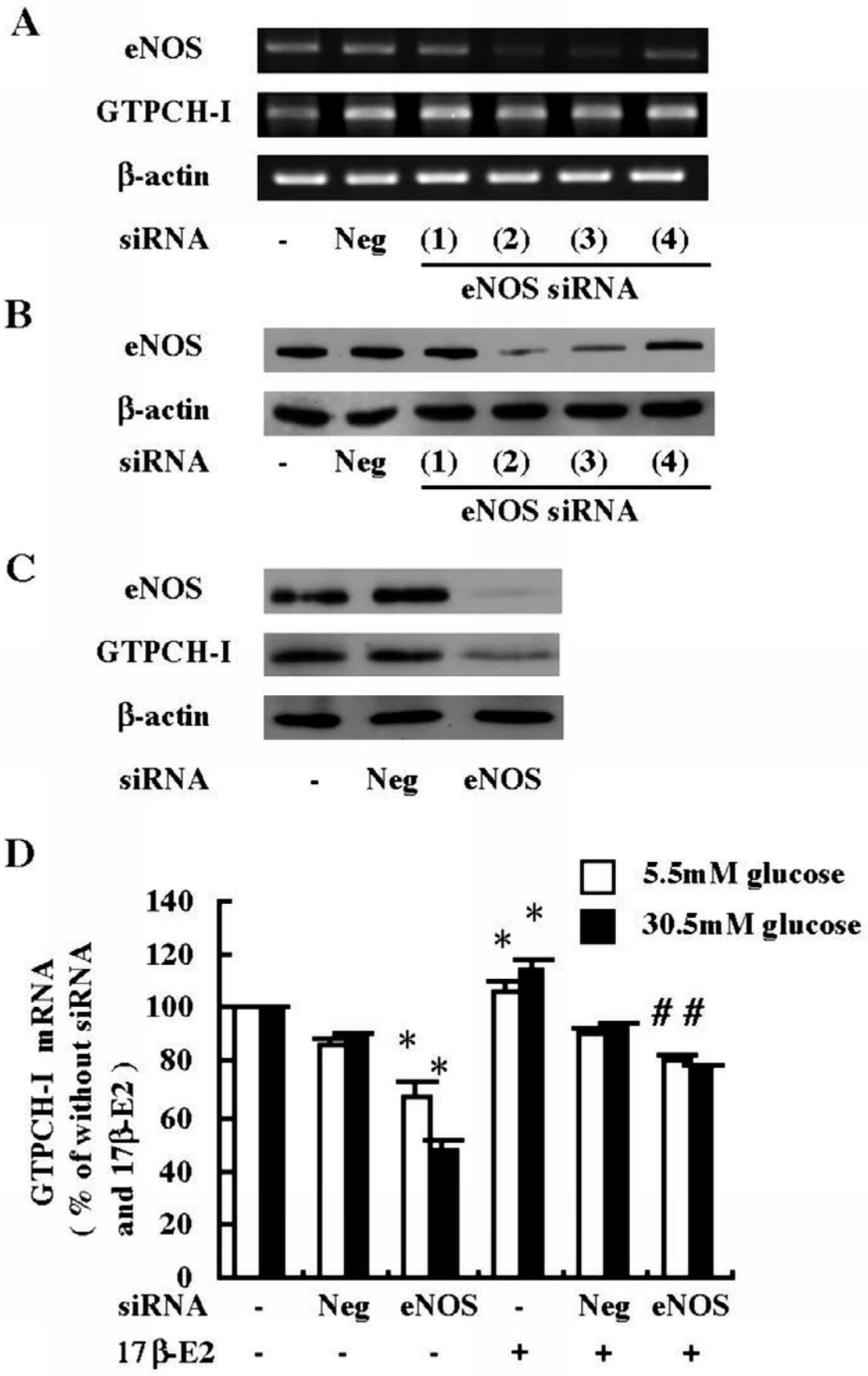

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Figure 6