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The effect of high glucose on NO and O_2^- through endothelial GTPCH1 and NADPH oxidase

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Abstract

Although endothelial dysfunction deteriorates diabetic angiopathy, the mechanisms are obscure. We revealed that high glucose augmented eNOS through stimulation of eNOS mRNA in cultured BAECs. NO was decreased and O_2^- was increased simultaneously. NOS inhibitor, inhibited O_2^- release, so did NADPH oxidase inhibitor. The effects were synergistic. Both intracellular BH_4 level and GTPCH1 activity were decreased by high glucose, in line with decrease of GTPCH1 mRNA. HMG-CoA reductase inhibitor, atorvastatin increased GTPCH1 mRNA and activity, and BH_4 level. Conclusively, high glucose leads to eNOS dysfunction by inhibiting BH_4 synthesis and atorvastatin stimulate BH_4 synthesis directly, and it may work as atherogenic process.
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Keywords: eNOS dysfunction; Superoxide anion; L–NAME; Apocynin; Aminoguanidine; BH_4; GTPCH1 activity; HMG-CoA reductase inhibiters

Introduction

The acceleration of atherosclerosis in diabetes mellitus results in higher risks of cardiovascular events. Growing clues showed that an impairment of diabetic endothelial function exhibited crucial

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roles. Endothelial NOS plays an important role in vascular endothelium functions by producing NO, an important anti-atherosclerotic agent. Recent studies also showed that eNOS has a dual effect on atherosclerosis (Robert et al., 1998). eNOS itself could be an important source of endothelial superoxide production in hypercholesterolemia (Kirkwood et al., 1995). In diabetic vessels of human, the endothelium was found to be an additional net source of superoxide production because of eNOS dysfunction (Tomas et al., 2002). On the other hands, study of insulin-resistant rat aorta revealed a decreased aortic BH_4 contents as well as increased BH2 (7,8-dihydrobiopterin) levels, when compared with normal and non-insulin-resistant diabetic groups (Shinozaki et al., 1999). They reported that insulin resistance is the pathogenic factor of eNOS dysfunction and BH_4 deficiency. Other study showed that the balance between reduced and oxidized BH_4 is a key redox switch controlling superoxide formation from eNOS (Vasquez-Vivar et al., 2002). Exogenous administration of BH_4 leads to an acute amelioration of endothelium-dependant relaxation in DM rats (Pieper, 1997). Intravenous administration of sepiaptrin, which is an ancestor of BH_4, could improve the endothelial-dependent vasodilatation of diabetic patients clinically (Heitzer et al., 2000). There is little evidence that shows the relationship among high glucose, eNOS dysfunction and BH_4. Hyperglycemia is an independent risk factor for ischemic heart disease proved by clinical studies such as UKPDS. The current study is aimed to reveal the mechanisms of eNOS dysfunction leading by high glucose in an in vivo model.

Materials and methods

Cells

BAECs were isolated from fetal calf as described previously (Hayashi et al., 1995a) and cultured in DMEM with 10% (v/v) of CS, 100 u/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine. Cells were allowed to the confluent of 80%, and then stimulated with different concentration of D-glucose (5.5, 12.5, 25 and 50 mM) as well as other reagents in DMEM with 2% CS and phenol red free for 24 hours. Mannitol was used as control to rule out the effect of osmotic pressure.

Measurement of NOx (nitrite and nitrate)

Measurement of NOx (nitrite and nitrate) in supernatant was performed as described in previous study (Yamada and Nabeshima, 1997). Briefly, the supernatant were taken for the measurement of NOx by HPLC (ENO10, Eicom Co, Kyoto, Japan), where nitrate was converted to nitrite in an in-line copper coated cadmium reduction column (NO–RED), and then nitrite was detected based on Griess reaction.

Western blot analysis of eNOS protein

Determination of eNOS protein expression were performed as described in our previous study (Hayashi et al., 1995b). Protein concentration was determined by Dc protein assay kit (Bio-Rad, CA). 15 μg protein was loaded. Primary anti-eNOS monoclonal antibody (Anti-mouse IgG1

monoclonal antibody, Transduction Laboratories, CA) was incubated in the ratio of 1:2000, overnight. HRP-linked anti-mouse IgG antibody (Cell signaling) was used as second a antibody. Bands of eNOS protein were developed in dark on the film (Fuji Medical X-ray Film, Japan). Band densities were analyzed densitometrically by the National Institutes of Health IMAGE program.

RT–PCR analysis of eNOS and GTPCH1 mRNA

Total RNA was isolated from BAECs with TRIZOL reagent according to the manufacture's protocol (GIBCO BRL, Life Technologies). eNOS mRNA were analyzed by reactions with RNA PCR kit (One step RNA PCR Kit, Takara, Japan) as described in our previous study (Kano et al., 1999). The programmed cycles for eNOS RT PCR were as follows: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 30 seconds. Bands were visualized on dual intensity transluminator. RT–PCR of GTPCH1 mRNA were carried on such a programmed cycles: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 1 minutes. Sequence of bovine GTPCH1 primer is as follows: sense: 5′ CCGCCTACTCGTCCATCCTGA 3′, antisense: 3′ACCTCGCATTACCATACACAT 5′.

Measurement of intracellular superoxide by FACS

At the end of treatment period, cells were washed with PBS, 2 μl of 5 mM DCFH-DA was added and then incubated in 37 °C for 30 minutes. Cells were detached with trypsin, and centrifuged at 4 °C, 15 000 rpm for 5 minutes. Cell suspensions in PBS were transferred into 5 ml polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson lab ware, Becton Dickinson and company, France). And they were kept on ice for immediate measurement by FACS (Fluorescence-activated cell sorter, BD Biosciences).

Determination of intracellular BH4 level and GTPCH1 activity

Cells were harvested with trypsin and pelleted by centrifugation and frozen at −80 °C. BH_4 measurements were performed by HPLC procedure described by Fukushima and Nixon (Consrtino et al., 1997). Intracellular BH_4 levels were expressed in terms of pmoles per mg protein of the cell pellet. GTPCH1 activity was assayed based on the quantification of D-erythro-neopterin by HPLC after conversion of enzymatically formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythroneopterin by sequential reaction of iodine oxidation and dephosphorylation.

Statistics

Data were reported as mean ± SD, and represent three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann-Whitney *U* test. Significant differences were accepted when $P < 0.05$.

Results

Effects of high glucose on eNOS protein and mRNA expression

After exposure to high glucose for 24 hours, eNOS proteins were increased significantly, and in accordance with it, expression of eNOS mRNA were also enhanced (Fig. 1A, B). As mannitol treatment did not affect the expression of eNOS protein or eNOS mRNA, these results attributed to high glucose itself, not to osmolality.

Effects of high glucose on NOx produced by eNOS

After stimulated by high glucose (12.5 mM, 25 mM) for 24 hours, NOx production was significantly decreased compared with control (5.5 mM), but there were no significant different between the two high glucose groups (12.5 mM, 25 mM) (Fig. 2A). As mannitol treatment did not affect the concentration of NOx, the effect attributed to high glucose itself, not to osmotic pressure.

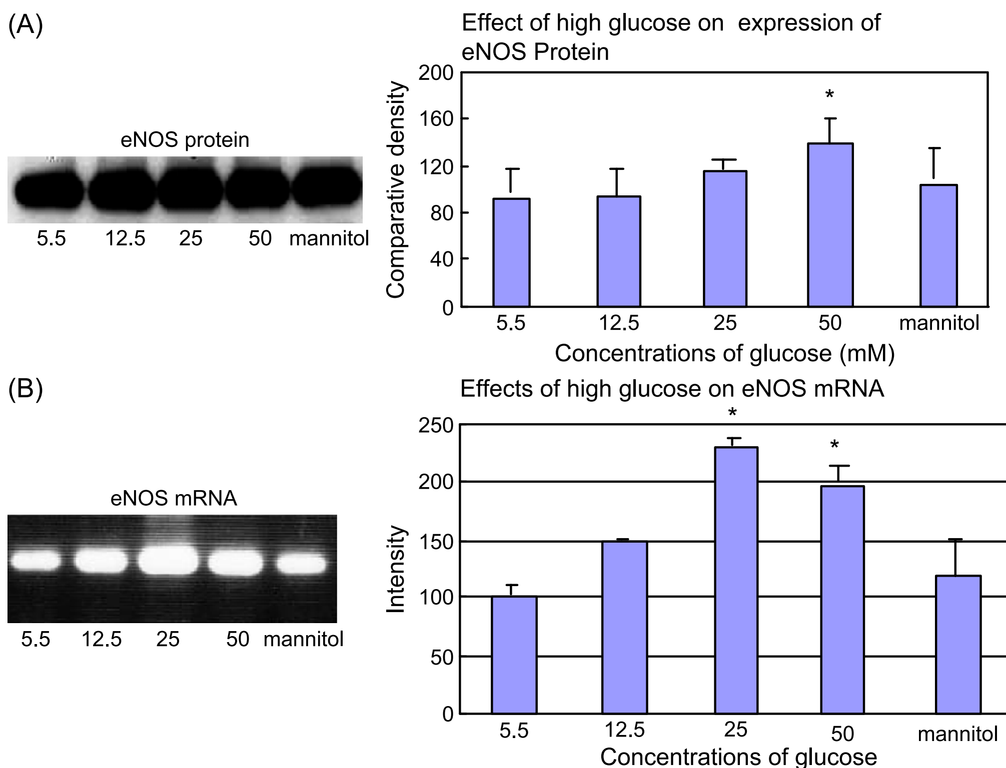

Fig. 1. The effects of high glucose on eNOS expression. Western blot and RT–PCR analysis of eNOS protein **(A)** and mRNA expression **(B)** after 24 hours exposure to different concentrations of glucose. Data represents the mean ± SEM of three separated experiments. The effects of high glucose on NOx production **(A)** *P < 0.05 vs control.

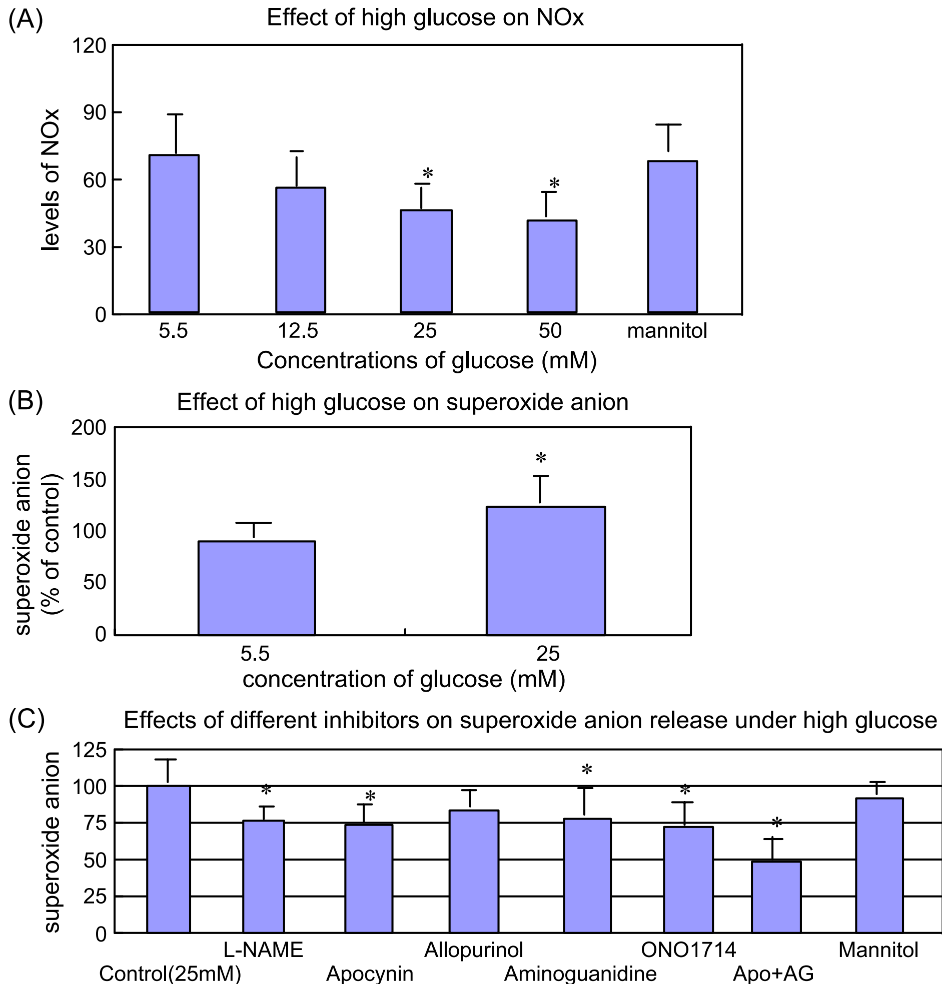

Fig. 2. The effects of high glucose on NOx production **(A)**, superoxide anion **(B)**. Effects of different inhibitors on superoxide anion under the stimulation of high glucose **(C)**. Data represents the mean ± SEM. *P < 0.05 vs control.

Effects of high glucose on intracellular superoxide anion and possible route of superoxide production

The intracellular superoxide anion was largely increased by the stimulation of high glucose (25 mM), compared with control (5.5 mM), after 24 hours exposure (Fig. 2B). And the stimulatory effects of high glucose could be abolished by L–NAME(100 μM) and apocynin(10 μM), respectively (Fig. 2C). However, the effect of allopurinol (10 μM), aminoguanidine(10 μM), or ONO 1714 was relatively limited, and mannitol did not affected superoxide production (Fig. 2C).

Effects of high glucose on intracellular BH4 levels and GTPCH1 activities

As showed in Fig. 3A and B, both of intracellular BH_4 levels and GTPCH1 activities were decreased significantly by high glucose exposure.

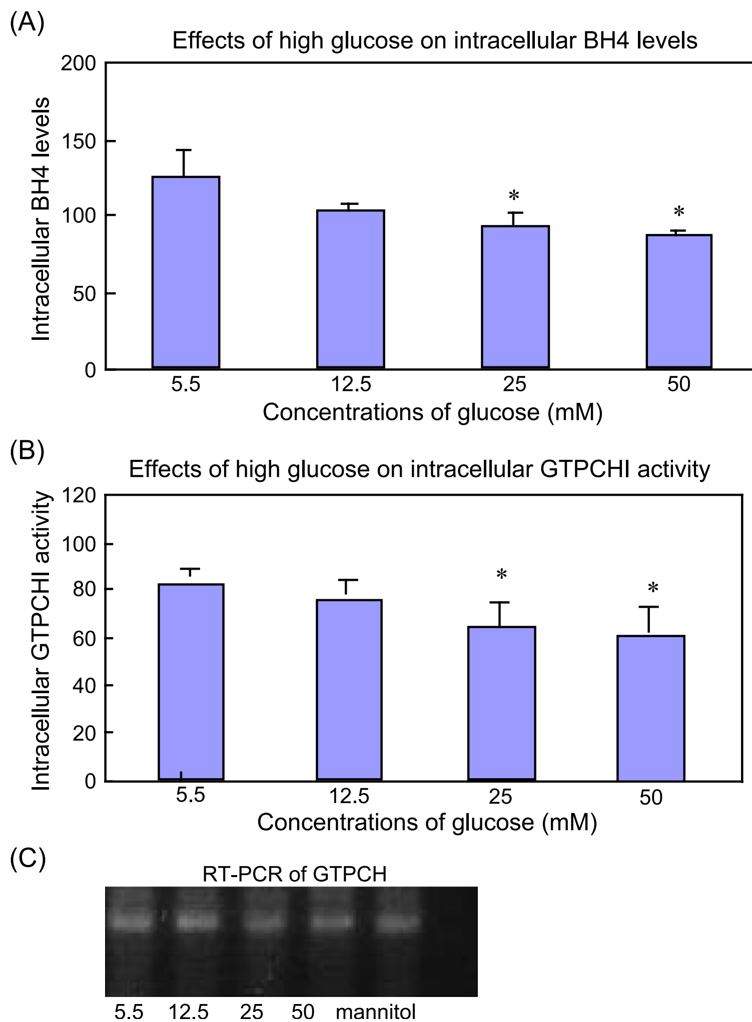

Fig. 3. The effects of high glucose on intracellular BH_4 levels **(A)**, GTPCH1 activities **(B)** and GTPCH1 mRNA expression **(C)**.

Effects of high glucose on expression of GTPCH1 mRNA

As revealed in Fig. 3C, in accordance with the inhibition of intracellular GTPCH1 activities, the expression of GTPCH1 mRNA abundance was also decreased by exposure to high glucose. It tended to correlate with GTPCH1 protein and activities (data not shown).

Effect of HMG-CoA reductase inhibitor on intracellular GTPCH1 activity and BH4 level

Atrovastatin exhibited a stimulatory effect on intracellular BH_4 accumulation (Fig. 4A) and GTPCH1 activities (Fig. 4C) and it was shown in a time- and concentration–dependent manner (part of data not shown).

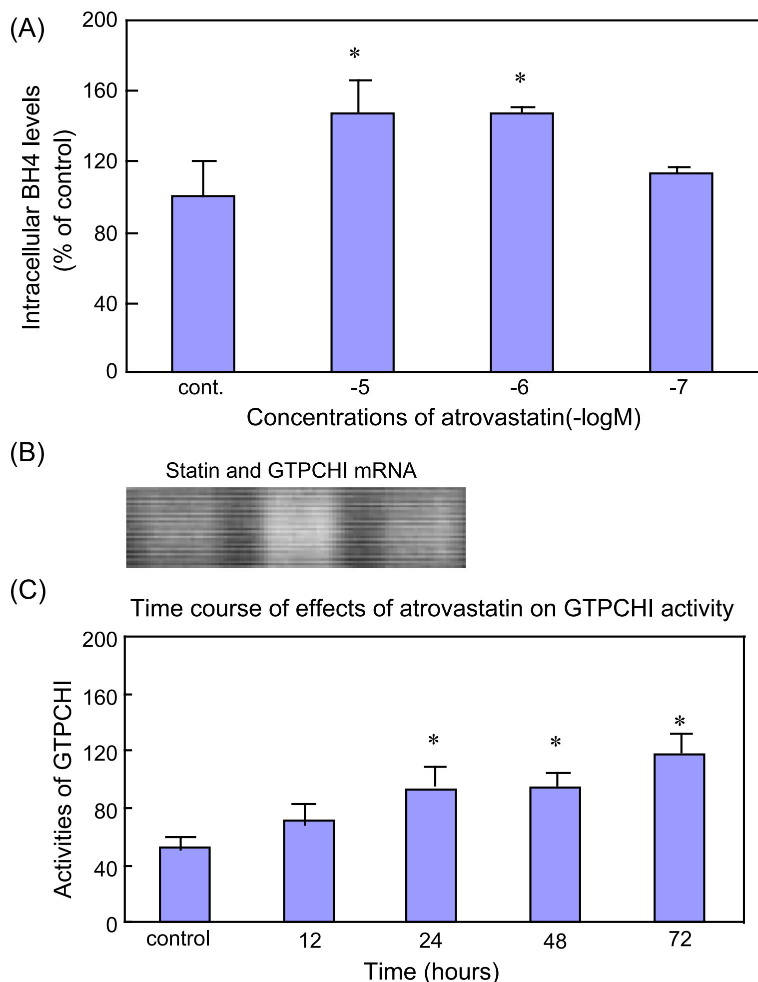

Fig. 4. Effects of different concentrations on atrovastatin on intracellular BH_4 levels **(A)** and GTPCH1 mRNA **(B)**. Time-course of effects of atrovastatin on intracellular GTPCH1 activities **(C)**. Data represents the mean ± SEM of three independent experiments. *$P < 0.05$ vs control.

HMG-CoA reductase inhibiters and expression of GTPCH1 mRNA

In accordance with the stimulatory increase of intracellular GTPCH1 activities and BH_4 accumulations, expression of GTPCH1 mRNA was also augmented by atrovastatin (Fig. 4B).

Discussions

This study demonstrated that in bovine aortic endothelial cells, the expression of eNOS was increased by exposure to high glucose (Fig. 1A). This may be the result of augmentation of eNOS mRNA expression by high glucose (Fig. 1B). Although, the effect of high glucose on eNOS protein

was dose dependent, the maximum level of eNOS mRNA expression was maximum between 25 and 50 mM glucose. We cannot elucidate the mechanism in the difference of effective concentration between protein and mRNA levels. In the preliminary experiment of 72 hours exposure to high glucose, eNOS expression was maximum between 25 and 50 mM glucose. We speculate that the difference of effective glucose concentration between eNOS protein and mRNA is due to the difference of time course. It exhibited conflictions to the reasonable hypothesis that, the eNOS abundance should be decreased by high glucose, which is based upon the clues of impaired endothelium-dependent relaxation in diabetic vessels of both human and animal experiments (Consrtino et al., 1997; Ding et al., 2000; Johnstone et al., 1993; Makimattila et al., 1996; Noyman et al., 2001; Steinberg et al., 1996). Further, measurement of NOx revealed a marked decrease when cells were allow to grow in high glucose (Fig. 2). Two possible explanations could be applied: one comes from the rapid reaction between NO and superoxide, while another one means the possibility if virtually decreased capacity of NO production by eNOS. One possible clue for eNOS dysfunction caused by high glucose gives rise to the hypothesis that increased abundance of eNOS caused by high glucose could not produce NO in proportion to that under normal glucose.

It is now generally agreed that oxidative stress plays a crucial role in the formation and deterioration of atherosclerosis (Tomas et al., 2002; Chen et al., 1995). In order to clarify the dysfunction of eNOS, we also studied the production of superoxide anion after exposure of high glucose by FACS. As shown in Fig. 2, high glucose increased intracellular superoxide anion significantly. In order to identify the sources of superoxide, different kinds of inhibitors which is related to possible pathways of superoxide were applied. It is amazingly to find that L–NAME, which is the specific inhibitor of NOS, exhibited a strong inhibitory effect on superoxide production and restored superoxide anion to almost the same level as control. It means that eNOS becomes an important source of superoxide anion in high glucose. From this point of view, high glucose could lead to dysfunction of eNOS.

Since increased superoxide anion could also be inhibited partially by apocynin, but not by allopurinol and aminogunidine independently, it confirmed that in case of high glucose, NADPH oxidase, but neither xanthine oxidase nor iNOS is not the possible source of superoxide production as well as eNOS. The mechanisms underline these phenomena are still unknown. Evidences from diabetic animal models and human studies showed cofactor of eNOS, tetrahydrobiopterin (BH_4), may be the redox of NO or superoxide production of eNOS (Heitzer et al., 2000; Pieper, 1997). We further focused on the effects of high glucose on intracellular BH_4 level and activity of GTPCH1-the rate-limiting enzyme in the de novo biosynthesis of BH_4, which is the most important pathway under physiological conditions. BH_4 is absolutely required for eNOS activity (Chen et al., 1995; Hattori et al., 2003). By acting as a cofactor of eNOS, evidences showed that it is involved in: 1) stabilization eNOS in its dimeric form, which is pivotal for eNOS to function normally; 2) electron transfer from the reductase domain to oxidase domain; 3) active site integrity. And in some pathological situations, it could even help overcome 'paradoxical deficiency' of L-arginine. As it is showed in Fig. 3, both intracellular BH_4 levels and activities of GTPCH1 were markedly decreased by the exposure to high glucose comparing with control. It has been revealed that BH_4 react with superoxide rapidly, thus decreases BH_4 accumulation in cells. Results of the present study show that in case of high glucose, the deceased activity of GTPCH1 could also be an important reason for the decreased BH_4 levels. So it is reasonable to think that it is the combination of the two possibilities lead to an absolute shortage of intracellular BH_4, and accordingly, the dysfunction of eNOS arises. But it is still difficult to identify which one plays a more important role.

We speculated that the transcriptional regulation of GTPCH1 mRNA is responsible for the decreased GTPCH1 mRNA by high glucose treatment. Preliminary experiment showed that the decreased activity of GTPCH1 associated decreased protein level. Gesierich et al. reported the importance of the complex formation of GTPCH1 with GTPCH1 feedback regulatory protein (GERP) in negative feedback regulation by end product BH_4, and phenylalanine upregulated GTPCH1 mRNA without changing GERP (Gesierich et al., 2003; Hattori et al., 2003). They speculated that the substrate level and transcription of the interacting protein regulation of BH_4 biosynthesis. In the present study, the amount of BH_4 was decreased by high glucose treatment, and the protein also decreased.

HMG-CoA reductase inhibitors are now generally convinced to be a potent antiatherosclerotic agent. Its pleiotropic effects include a direct stimulatory effect on eNOS or iNOS as reported (Gorren et al., 2002; Hayashi et al., 1995a; List et al., 1997). And we have for the first time revealed that HMA-CoA reductase inhibitors could upregulate GTPCH1 mRNA expression, thus stimulate the activity of GTPCH1 as well as intracellular BH_4 levels in cultured endothelial cells, directly. Statin was reported to enhance cytokine-mediated inducible nitric oxide synthesis in smooth muscle cells (Hattori et al., 2002). It has been reported that the effect of statin was abolished by exogenous mevalonate or GTPCH1 inhibitor, GGTI-298. Our data further, give a richer meaning to the pleiority of antiatherosclerotic effects of HMG–OA reductase inhibitor (Laufs et al., 1998; Tsunekawa et al., 2001). Finally, mannitol concentration was adjusted to the osmotic pressure in 50 mM high glucose. Preliminarily, we examined the effect of mannitol on high glucose treatment, and we made sure that it did not significant effect on eNOS protein and mRNA and GTPCH1 mRNA, and BH_4 concentration.

Conclusively, high glucose could lead to the dysfunction of eNOS by inhibiting the synthesis of BH_4 and activating NADPH oxidase. Statin could enhance eNOS activity through stimulating GTPCH1, thus increases BH_4 levels, directly.

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