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Reduction of High Glucose-Induced Nitric Oxide Synthase Expression in Human Vascular Endothelial Cells by Ascorbic Acid and α-Tocopherol

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ABSTRACT

Hyperglycemia is generally regarded as one of the major causes of vascular complications in diabetic patients. It has been shown that high-glucose concentration increases nitric oxide synthase (NOS) expression and NO generation in cultured human aortic endothelial cells. The epidemiological studies have demonstrated that there is an inverse relation between vitamin use and cardiovascular disease in diabetes patients. This study tested whether α-tocopherol and ascorbic acid can alter high glucose-induced expression of NOS in vascular endothelial cells. Human umbilical vascular endothelial cells (HUVECs) were treated with α-tocopherol and ascorbic acid, and stimulated by high glucose. Expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) was measured by real-time PCR (RT-PCR). Ascorbic acid (100 µM) and α-tocopherol (200 µM) significantly inhibited glucose (25 mM)-induced expression of eNOS mRNA and iNOS mRNA. Ascorbic acid and α-tocopherol are also able to reduce concentration of NOS in HUVECs. Additionally, there is a significant positive relation between expression of eNOS and iNOS mRNA with concentration of NOS. These results suggest that ascorbic acid and α-tocopherol can inhibit high glucose-induced production of NOS proteins by downregulation of its gene expression. Therefore, important role of ascorbic acid and α-tocopherol in the treatment of vascular dysfunction associated with diabetes disease may be via this molecular mechanism. Of course, further studies on regulation of these vitamins to signaling pathways are necessary.

Key words: Glucose, HUVECs, nitric oxide synthase, α-tocopherol, ascorbic acid

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INTRODUCTION

Diabetes mellitus is a frequent and increasing public health problem. Diabetes has a prevalence of 2 to 5 percent in most Western countries, and it is rapidly increasing in Asiatic countries due to changes in dietary habits during the last years (1). Hyperglycemia is generally regarded as one of the major causes of vascular complications in diabetic patients. The endothelium is not a single-cell lining covering the internal surface of blood vessels but in fact plays a crucial role in regulating vascular tone and structure. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of profibrinolytic and prothrombotic activity (2). Endothelial dysfunction has received increasing attention as a potential contributor to the pathogenesis of vascular disease in diabetes mellitus.

It has been reported that high extra-cellular glucose induces reactive oxygen species (ROS) production (2). Several studies have demonstrated that nitric oxide (NO) is abnormal in patients with type 2 diabetes (3). A multitude of experimental arguments have led to the concept that NO is not only involved in the control of vasomotor tone but also in vascular homeostasis function. NO is an endothelium-derived relaxing factor that produced through the conversion of arginin to citrulline by endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) in vascular endothelial cells (4). The promoter region of eNOS gene contains tentative regulatory sequences, including cAMP, phorbol esters, and sterol-responsive elements (5). It has been shown that high-glucose concentration increases eNOS expression and NO generation in cultured human aortic endothelial cells (6). Increasing of NO production rates, often coupled with accelerated NO removal through poorly understood pathways, leads to impaired NO signaling and secondary generation of toxic NO-derived species (7). The reaction of NO with superoxide anion (O_2^-), yielding peroxynitrite ($ONOO^-$), accounts for a major part of the accelerated NO removal (7). Thus this reaction can have been an important role in enhanced rates of NO consumption as endothelium-derived relaxing factor.

In vitro studies have shown that various antioxidants, including vitamin E (α-tocopherol) and vitamin C (ascorbic acid), can prevent hyperglycemia-induced endothelial injury of dysfunction (8),(9). In addition, the epidemiological studies have demonstrated acute administration of vitamins improves endothelium-dependent vasodilatation (9), and there is an inverse relation between vitamin E and cardiovascular disease in diabetes patients (10).

Although a lot of studies have been done for protective role of antioxidants, especially vitamins, in the pathophysiology of diabetes and cardiovascular disease (9), (10), (11), (12), (13), (14), (15). but the literature data concerning the molecular mechanism of α-tocopherol and ascorbic acid effect on the production of NO are limited. We have previously shown that α-tocopherol and ascorbic acid are able to decrease LDL glycation as dose dependent in a high-glucose *in vitro* model (16). Therefore, our objective in this study is to evaluate of the effect of α-tocopherol and ascorbic acid on concentration of NOS, and eNOS and iNOS expression in high glucose-exposed human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials

Human umbilical vascular endothelial cells (HUVECs) were obtained from National Cell Bank Iran (NCBI), Pasteur Institute of Iran (Tehran, Iran). They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mg/ml heparin (Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal bovine serum (Gibco BRL). The cultures were maintained at 37^{o}C in an atmosphere of 95% air and 5% CO_2. Medium was refreshed every three days. HUVECs of passages 3-5 were used for experiments. α-tocopherol, and ascorbic acid were purchased from Sigma (St. Louis, Mo, U.S.A), Dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany).

Methods

Cell treatment and cell viability. In this study, HUVECs were treated with a medium containing 5.5 mM glucose (control) or 25 mM glucose (high glucose) for 24 hours (17), (18) in the presence or absence 50, 100, or 200 µM of ascorbic acid or α-tocopherol. The incubation medium was then removed for analysis on NOS expression and NOS concentration, as described below. Cells viability were measured by adding 15 µl of 2 mg/ml MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium) to cells in 96-well plates and incubating for 24 hour at 37^{o}C. Then the medium was removed, 250 µl DMSO was added to each well and the cells were incubated at 37^{o}C for 4 hours. Absorption at 550 nm was read using a microplate reader (19).

Quantitative real-time PCR (RT-PCR) analysis. Total RNA was purified from HUVECs using the Qiagen RNA extraction kit (Germany). The RNA pellet was resuspended in RNasa-free water and its concentration was determinate in a Lambda 18 Spectrometer (Perkin Elmer, Germany) at 260 nm. RNA (5 µl) with concentration of 1 µg was mixed with 2 µl of dNTP, 2 µl of 10× reverse transcriptase (RT) buffer, 2 µl of random hexamer primer (Cinnagen, Iran), 1 µl of ominiscript reverse transcriptase, and 8 µl of RNase-free water. The RT mixture was incubated at 37^{o}C for 60 minutes (method was done according to Qiagen cDNA kit, Germany made). The resulting cDNA was amplified by RT-PCR (ABI, USA) with the conditions of 95^{o}C for 15 minutes (activation enzyme) and 45 cycles of 94^{o}C for 15 seconds (denaturation), 55^{o}C for 30 seconds (annealing), and 72^{o}C for 34 seconds (extension). The primers were purchased from Qiagen Company (Germany). The primers for eNOS were 5'- GTG GCT GTC TGC ATG GAC CT-3' (upstream) and 5'- CCA CGA TGG TGA CTT TGG CT-3' (downstream), for iNOS were 5'-CGCCAAGAACGTGTTCACCA-3' (upstream) and 5'-AGC AGG CAC ACG CCA TGA T-3' (downstream), and for β-actin, used as internal control, were 5'- AGC CTC GCC TTT GCCGA-3' (upstream) and 5'-CTG GTG CCT GGG GCG-3' (downstream). The PCR products were confirmed using melting-curve analysis for each gene. For comparison of transcript amounts between samples, a standard curve of cycle thresholds for serial

dilutions of a cDNA sample was stablished and then used to calculate relative levels of each gene [(20). All RT-PCR assays were performed in triplicate.

Assay of nitric oxide synthase (NOS) concentration. The concentration of NOS in cell culture was measured by enzyme-linked immunosorbent assay with commercial ELISA kits from Abnova Corporation (Taiwan). The cells that were used to measurement of NOS concentration were washed twice with Dulbecco's PBS, and cells were lysed with 400 µl of lysis buffer (25 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 mg/l aprotinin, 10 mg/l leupeptin, 1 mM EDTA, 50 mM NaF, and 1% Triton-X-100). All samples were assayed in triplicate and expressed as pg/ml.

Statistical analysis

All data are shown as mean ± SD. Treatment effects were analyzed using one-way analysis of variance (ANOVA). The level of significance was $p < 0.05$.

RESULTS

To verify inducement of high glucose on nitric oxide synthase (NOS), HUVECs were stimulated by 25 mM glucose concentration for 24 hours. High glucose increased the expression of eNOS mRNA and iNOS mRNA in comparison with low glucose (5.5 mM) approximately 92% and 78%, respectively (Figure 1). For determination of the best concentration of vitamins, we assayed the effect different concentrations (50 -200 µM) of ascorbic acid and α-tocopherol on glucose (25 mM)-induced eNOS and iNOS expression for 24 hours. Ascorbic acid inhibited glucose-induced eNOS and iNOS mRNA expression in a significant dose-dependent manner ($p < 0.05$) when the concentration was limited between 50 and 100 µM, whereas α-tocopherol showed a significant inhibition of glucose-induced eNOS and iNOS mRNA expression as dose dependent in concentrations of 50 to 200 µM (Figure 2A,C). According to this study 100 µM concentration of ascorbic acid is able to reduce eNOS and iNOS mRNA expression approximately 74% and 52%, respectively, but 200 µM concentration of α-tocopherol is able to reduce eNOS and iNOS mRNA expression approximately 59% and 49%, respectively (Figure 2B,D).

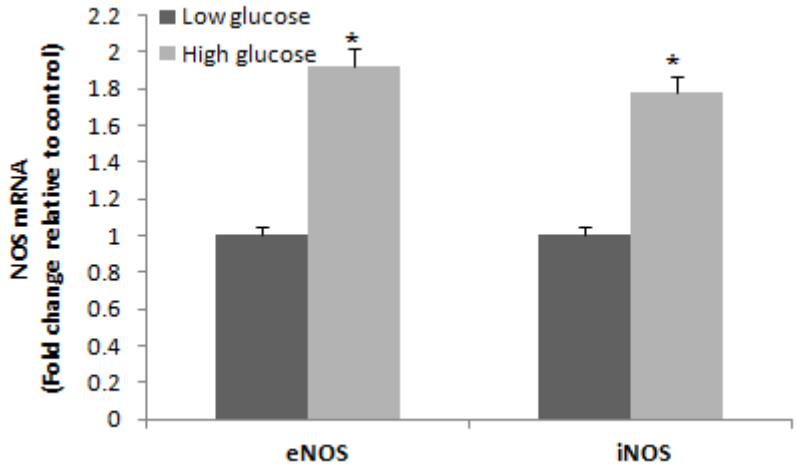

Figure 1. The effect of 5.5 mM glucose concentration, as basal concentration, (black bar) and 25 mM glucose concentration (grey bar) on eNOS and iNOS expression assessed by RT-PCR in HUVECs after 24 hours incubation (37°C, 5% CO_2)

*Data are represented as the mean±SD of triplicate determination. *p < 0.05 compared with control (black bar).*

We investigated NOS concentration of HUVECs treated with 5.5 mM (low glucose) and 25 mM (high glucose) glucose (Figure 3). As shown in this Figure, NOS concentration in presence of high glucose as significant ($p < 0.05$), increase by 86% in comparison to the control (low glucose concentration). To assess whether ascorbic acid and α-tocopherol can inhibit high glucose-induced concentration of NOS, we examined NOS protein concentration by ELISA analysis. Treatment of cells (HUVECs) with ascorbic acid or α-tocopherol reduced the concentration of NOS induced by high glucose in a significant dose dependent manner ($p < 0.05$) (Figure 4A). This study showed that concentration of 100 µM of ascorbic acid and 200 µM of α-tocopherol are able to decrease concentration of NOS protein by 73% and 63%, respectively (Figure 4B).

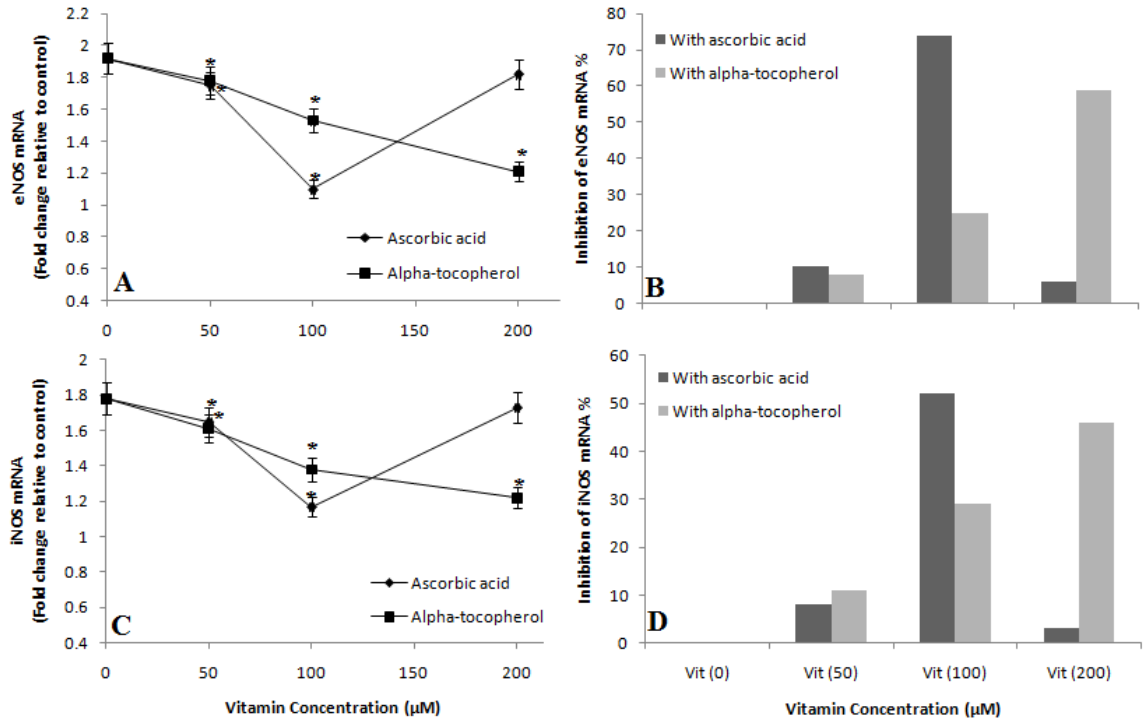

Figure 2. The effect of 50, 100, and 200 µM concentrations of ascorbic acid (♦) and α-tocopherol (■) on eNOS (A), and iNOS (C) expression in presence high glucose concentration (25 mM); The comparison of inhibition percent of eNOS (B), and iNOS (D) expression in presence 25 mM glucose concentration and different concentrations (50-200 µM) of ascorbic acid and α-tocopherol. HUVECs treatment with vitamins at 37ºC with air of 5% CO_2 for 24 hours

*Data are represented as the mean±SD of triplicate determination. *p < 0.05 compared with control (in the absence of vitamins).*

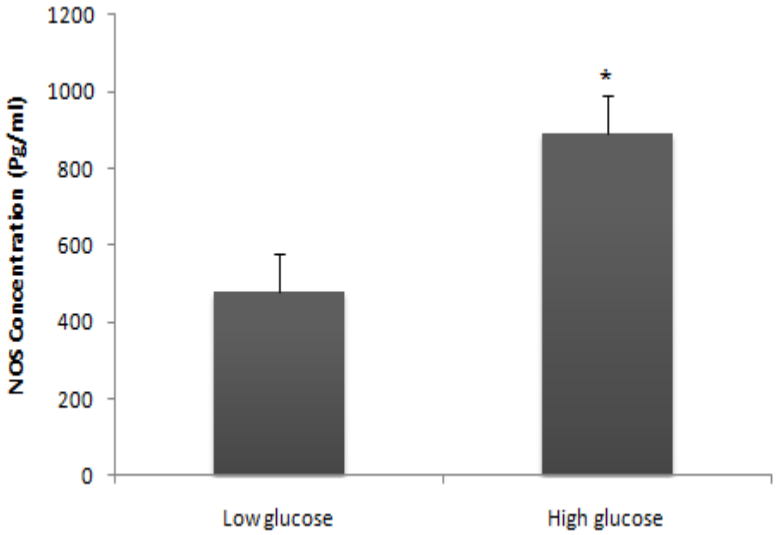

Figure 3. Effect of low glucose (5.5 mM), as basal concentration, and high glucose (25 mM) on concentration of NOS in HUVECs after 24 hours incubation (37ºC, 5% CO_2)

*Data are represented as the mean±SD of triplicate determination. *p < 0.05 compared with control (in the presence of low glucose).*

Finally, according to linear (Pearson) analysis, correlation between eNOS and iNOS mRNA expression and NOS concentration in high glucose-induced HUVECs treated with ascorbic acid and/or α-tocopherol have been shown in Figure 5. This Figure, shows a significant positive correlation between concentration of NOS and expression of eNOS mRNA in presence ascorbic acid (r = 0.99; $p < 0.007$) (Figure 5A) and/or α-tocopherol (r = 0.99; $p < 0.007$) (Figure 5B). Additionally, a significant positive relation was also found between NOS concentration and iNOS mRNA expression with ascorbic acid (r = 0.98; $p < 0.02$) (Figure 5A) and/or α-tocopherol (r = 0.99; $p < 0.007$) (Figure 5B).

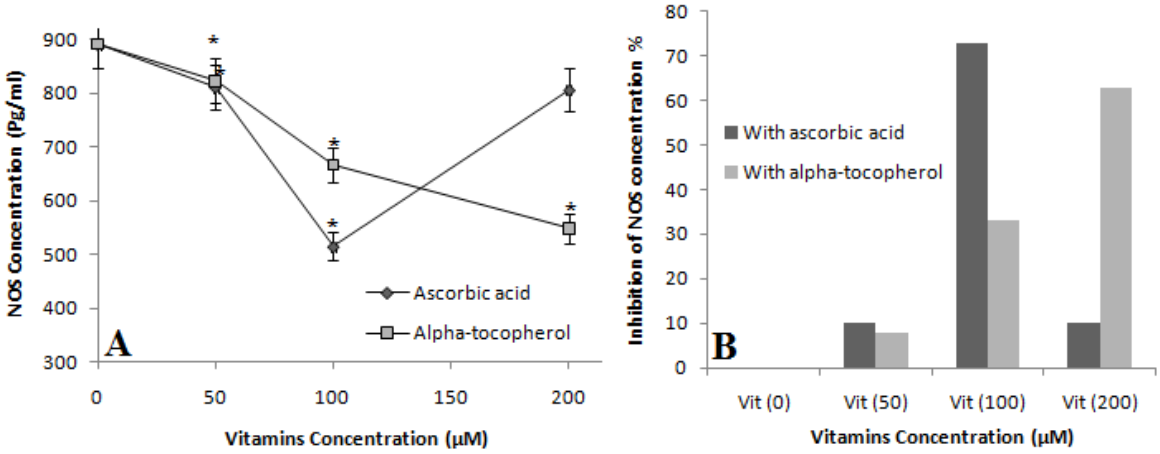

Figure 4. (A) The effect of 50, 100, and 200 µM concentrations of ascorbic acid (♦) and α-tocopherol (■) on HUVECs nitric oxide synthase (NOS) concentration in presence high glucose concentration (25 mM); (B) The comparison of inhibition percent of NOS protein concentration in presence high level of glucose (25 mM) and different concentrations (50-200 µM) of ascorbic acid and α-tocopherol. HUVECs treatment with vitamins at 37°C with air of 5% CO_2 for 24 hours

*Data are represented as the mean±SD of triplicate determination. *p < 0.05 compared with control (in the absence of vitamins)*

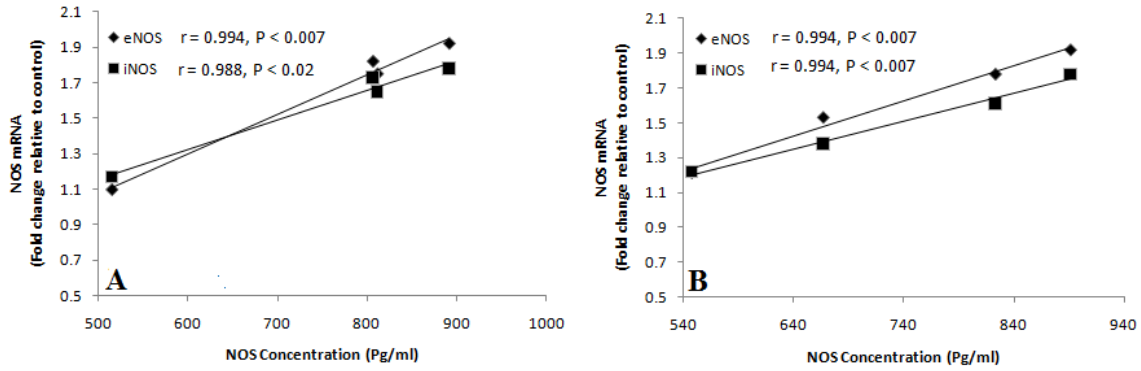

Figure 5. Correlation between expression and concentration of eNOS (♦) and iNOS (■) in presence of high glucose (25 mM) and different concentrations (50 – 200 µM) of ascorbic acid (A) and α-tocopherol (B) in HUVECs

DISCUSSION

Dysfunction of endothelial cells are early manifestation of atherosclerosis that the most important result it is cardiovascular disease (21). Diabetes mellitus is an important risk factor for cardiovascular disease (2),(22). Pathways of nitric oxide (NO) signaling and reactions of lipid oxidation are important in both the maintenance of vascular homeostasis and the progression of vascular disease. Studies biochemical and cell biology have demonestrated that NO with oxidizing lipids could lead to either vascular protection or vascular injury (23), (24). The effect of NO is dependent to amounts it, if NO generated at low levels by nitric oxide synthase (NOS) can terminate propagating lipid radicals, reaction that would be protective. However, if NO generated at elevated levels, it can be converted to prooxidant species that can potentiate injury to vascular cells (24).

In this investigation, we showed that glucose could induce the expression of eNOS and iNOS mRNA in human vascular endothelial cells. Glucose (25 mM) increased the expression of eNOS 1.92-fold and iNOS 1.78-foldin compare with control (5.5 mM glucose). These results are similar to previous studies that were reported by Cosentino et al. (6), Ding et al. (25), Zhu et al. (26), and Srinivasan et al.(27). In addition, according to Tesfamariam et al. report (28) it has been shown that concentration of high glucose is able by activating protein kinase C increase eNOS and iNOS genes expression in endothelial cells. According to results of this study and other investigations, we suggest that hyperglycemia may via increase of eNOS and iNOS gene expression in human vascular endothelial cells, accelerate atherosclerosis in diabetic patients. Several studies demonstrated that NO-derived reactive species, for example, peroxynitrite ($ONOO^-$), nitrogen oxide (NO_2), and nitryl chloride (NO_2Cl) have an important role in

lipid oxidation and endothelial cells dysfunction (29), (30). Peroxynitrite is unique as a lipid oxidant, because it mediates oxidation of unsaturated fatty acids in the absence of transition metal catalysts (29). This NO-derived reactive species is more than two orders of magnitude potent than hydrogen peroxide in catalyzing lipid oxidation *in vitro* (31). Nitrogen oxide will both oxidize and nitrate unsaturated lipids (30), (32). These reactions result in formation of a complex mixture of products including nitrated lipid derivatives and alkylnitrites. The oxidation of nitrite will yield nitryl chloride, which initiates lipid oxidation and can yield on LDL particle similar to that found in foam cells and so may be operative in atherogenesis (33), (34).

Vaziri et al. (35) demonstrated that antioxidant therapy ameliorated hypertension in rats, as well as, prospective studies have been shown cardiovascular benefits from antioxidant therapy (8), (9), (10). In the present study, ascorbic acid and α-tocopherol were used as protective agents against high glucose-induced NOS expression. It has been shown in several studies that antioxidants reduced vascular oxidative stress (36), (37). Additionally, ascorbic acid increased vasodilatation of forearm resistance arteries in humans with hypercholesterolemia (38), long-term smokers (39), essential hypertension (40), and coronary artery disease (41). Although numerous studies have shown antioxidants play a key role in oxidative stress inhibition. However, the literature concerning the effect vitamins on the expression and concentration of NOS in presence of high glucose is limited. In this investigation, we indicated that treatment of HUVECs with ascorbic acid and α-tocopherol decreased high glucose-induced eNOS and iNOS expression.

A dose-dependent manner was observed when the concentration of ascorbic acid and/or α-tocopherol was limited to the range of 50 to 100 µM or 50 to 200 µM, respectively. According to this study, optimum concentration for ascorbic acid and α-tocopherol obtained 100 µM and 200 µM, respectively. In presence of ascorbic acid (100 µM) high glucose-induced eNOS and iNOS expression were inhibited by 74% (1.92-fold decreased to 1.1-fold) and 52% (1.78-fold decreased to 1.17-fold), respectively. α-tocopherol (200 µM) also inhibited the expression of eNOS and iNOS in presence of high glucose by 59% (1.92-fold decreased to 1.21-fold) and 49% (1.78-fold decreased to 1.22-fold), respectively. According to these results, we suggest that 100 µM ascorbic acid and 200 µM α-tocopherol could restore eNOS and iNOS expression to levels observed in 5.5 mM glucose.

Our results are in agreement with the finding of several studies. For instance, Kang et al. (42) demonstrated that α-lipoic acid is able to decrease eNOS expression in bladder of rats with streptozotocin-induced diabetic. Similarly, Askwith et al.(43) also reported that *in vitro* taurine treatment reduces expression of NOS in high glucose-exposed human Schwann cells. In addition, there are other numerous studies that have been showed antioxidants, for example, melatonin, vitamin C (44), hesperidin (45), and vitamin E (46) could decrease expression of NOS isoforms *in vitro* and/or *in vivo*.

The mechanism of ascorbic acid and α-tocopherol on NOS downregulation is still unknown. However, according to study of Askwith et al. (43) the effect of antioxidants on NOS expression may be mediated by an antioxidant action, as well as, they also showed which this effect could be mediated by carbonyl scavenging or perhaps by restoring Ca^{+2} signaling (43).

In the present study, we also indicated that high glucose (25 mM) increase concentration of NOS protein in HUVECs by 86%, and found that ascorbic acid and α-tocopherol could reduce high glucose-induced NOS concentration in a significant and dose-dependent manner. The effective concentration of ascorbic acid and α-tocopherol that obtained in the present experimental study was 100 µM and 200 µM, respectively. These results confirmed our previous data in this study.

Finally, this investigation showed a positive and significant correlation between NOS protein content with NOS mRNA expression in HUVECs at present high glucose and ascorbic acid and α-tocopherol. According to these results, we suggest that ascorbic acid and α-tocopherol could decrease concentration of high glucose-induced NOS protein in HUVECs via NOS gene downregulation. Therefore, this is may be one of molecular mechanisms that ascorbic acid and α-tocopherol could prevent the increase in atherosclerosis induced by diabetes in human.

In conclusion, the results obtained in the present study show that both ascorbic acid and α-tocopherol reduced high glucose-induced expression of eNOS and iNOS in human endothelial cells. These vitamins appear to play an important role in the regulation of NOS with a mechanism of action which is at least partially independent of an antioxidant effect. These findings suggest molecular mechanism of these vitamins that possible preventive intervention for diabetes-accelerated atherosclerosis. Of course, further studies on regulation of ascorbic acid and α-tocopherol to signaling pathway is necessary.

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