UltraRapid Communication

Regulation of Endothelial Nitric Oxide Synthase Expression by Albumin-Derived Advanced Glycosylation End Products

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Abstract—We examined whether albumin-derived advanced glycosylation end products (AGEs) downregulate the expression of endothelial nitric oxide synthase (NOS). Significant reductions in NOS activity and cGMP levels in bovine aortic endothelial cells were observed when exposed to different concentrations of albumin-derived AGEs. Western and Northern blot analyses showed significant decreases at the protein and transcript levels. Both reductions became evident after 24 hours of exposure. Nuclear run-on assays showed that AGE-BSA did not modify the transcription rate of the NOS III gene; however, AGE-BSA treatment markedly reduced the half-life of NOS III mRNA. In addition, AGE-treated endothelial cells displayed significant reduction on their antiplatelet properties. These results indicate that NOS expression is reduced by AGEs by increasing the rate of mRNA degradation and may be relevant to the impairment of some endothelial functions observed in diabetes and aging. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;86:e50-e54.)

Key Words: advanced glycosylation end products ■ nitric oxide synthase ■ diabetes

Endothelial cell–generated nitric oxide (NO) accounts for the biological activity of endothelium-derived relaxing factor. NO is derived from the guanidino nitrogen atoms(s) of the amino acid L-arginine through a reaction catalyzed by NO synthase. In endothelial cells, NO synthase (NOS III) has been characterized as a constitutively expressed calcium- and calmodulin-dependent enzyme. However, the level of NOS III expression can be altered by a growing number of different stimuli. Tumor necrosis factor-α and lipopolysaccharide decrease expression of NOS III. In contrast, estrogens and shear stress can increase NOS III expression. The effect of hypoxia is less clear; some authors have reported either an increase or decrease in NOS III mRNA.

Vascular diseases account for the majority of the clinical complications of diabetes mellitus. Although the molecular basis of the mechanisms involved for the development of vascular disease in diabetes mellitus is poorly understood, several lines of evidence demonstrate impairment in endothelium-dependent relaxation of diabetic blood vessels.

Reducing sugar such as glucose can react nonenzymatically with the amino groups of proteins to form complex structures called advanced glycosylation end products (AGEs). In normal aging processes and in some pathological conditions such as diabetes, the excessive accumulation of AGEs could lead to tissue dysfunction. Recently, AGEs have been shown to quench NO and, therefore, may play a role in the defective endothelium-dependent vasodilatation in experimental diabetes.

To our knowledge, the effect of AGEs on NOS III expression is unknown. We have undertaken the present study to test the hypothesis that AGEs reduce the expression level of NOS III. We show a significant decrease in the amount of NOS III protein and mRNA in AGE-exposed bovine endothelial cells.

Materials and Methods

Cell Cultures

Bovine aortic endothelial cells (BAECs) were prepared from aortas obtained from a slaughterhouse. Their endothelial phenotype was verified by demonstrating acetylated LDL uptake and the expression of von Willebrand factor and NOS III by immunofluorescence.

Preparation of AGE-Modified Albumin

AGE-BSA was prepared as described. Such treatment has been shown to be sufficient for the accumulation of large amounts of AGE on albumin. We verified the presence of AGE structures in our preparation of AGE-BSA by a direct ELISA using a specific antiserum to a common AGE immunological epitope found both in vitro and in vivo as described. We ruled out the effect of early glycosylation products by incubating AGE-BSA with 200-fold molar excess of sodium borohydride.

NOS Catalytic Activity

BAECs exposed to different concentrations of AGE-BSA for 48 hours were harvested, sonicated, and centrifuged at 1000g for 5 minutes. NOS activity in total cell lysates (100 to 150 µg of total proteins) was measured by the conversion of [14C]arginine to [14C]citrulline.
Determination of cGMP

BAECs (10^6 cells) were challenged with 0.5 mmol/L isobutyl-1-methylxanthine at the time of AGE-BSA addition. The content of cGMP was measured using a specific binding kit according to the recommendations of the supplier (Amersham).

Western Blot Analysis

Total protein (20 μg) was electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to a nylon membrane. The membrane was blocked with 5% skim milk, incubated with a monoclonal antibody against NOS III (Transduction Labs), washed, incubated with sheep anti-mouse Ig conjugated with horseradish peroxidase, and developed with a chemiluminescent reporter system (ECL, Amersham).

Northern Blot Analysis

Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method. Fifteen micrograms was separated by electrophoresis on a 1% agarose-MOPS gel with 2.2 mol/L formaldehyde and transferred to a nylon membrane by capillary action. Membranes were hybridized with a cDNA probe specific for bovine NOS III labeled with [α-32P] dCTP. After overnight hybridization, blots were washed and exposed to an x-ray film (Hyperfilm, Amersham) for 48 hours. Human GAPDH was used to probe the GAPDH message. Quantification of autoradiographic results was performed with a laser densitometer and ImageMaster software.

In Vitro Elongation of Nascent RNA

(Run-on Assay)

Nuclei from 10^9 BAECs were prepared and in vitro transcription with [32P] UTP was performed essentially as described. cDNAs for NOS III and β-tubulin were used as probes. pBlueScript I plasmid DNA was used as a control.

Platelet Aggregation

Human-washed platelets were prepared as described. Briefly, 10^8 platelets were incubated in the presence of control and AGE-treated BAECs. Under these experimental conditions, 10^6 control BAECs was the proportion able to inhibit the 50% of platelet aggregation induced by 40 mU/mL thrombin. Control and AGE-BSA–treated BAECs were added 2 minutes before the addition of thrombin.

Statistical Analysis

All values are presented as mean ± SEM of multiple experiments. Comparisons between multiple treatment groups of changes in NOS activity and cGMP levels were made by ANOVA and Newman-Keuls test. Student’s t test (two-tailed) for unpaired data was used to determine statistical significance in the platelet aggregation studies. Differences were considered significant at P<0.05.

Results

AGE-BSA Decreases NOS III Catalytic Activity and cGMP Accumulation

We first evaluated the effects of albumin-derived AGEs on both NOS activity and cGMP levels. As depicted in Figures 1A and 1B, we detected a marked dose-dependent reduction in both NOS activity (from 80% to 36%) and cGMP levels (from 66% to 33%) in BAECs exposed to increasing amounts (0.25 to 10 μmol/L) of AGE-BSA for 48 hours. Unmodified BSA and boiled AGE-BSA did not show any effect. There was no difference in the effect achieved by either AGE-BSA and NaBH4-reduced AGE-BSA, suggesting that the effects on BAEC NOS III expression were not mediated by reversible BSA adducts such as Amadori products.
AGE-BSA Has No Effect on the Transcriptional Rate of the NOS III Gene

To explore the mechanism by which AGE-BSA decreases the steady-state NOS III mRNA levels, nuclear run-on experiments were conducted to ascertain whether AGE-BSA attenuates the transcription of NOS III gene. As shown in Figure 4, AGE-BSA did not affect NOS III gene transcription.

AGE-BSA Changes NOS III mRNA Stability

In view of the preceding results, we next examined the effects of AGE-BSA on the half-life of NOS III transcript. Actinomycin-D–treated BAECs were cultured for 24 hours in the presence or absence of 10 μmol/L of AGE-BSA, before harvesting of total RNA. As can be seen in Figure 5, actinomycin-D did not modify the hybridization signals of NOS III under control conditions (no AGE-BSA treatment) over 24 hours. However, the presence AGE-BSA drastically shortened the half-life to less than 20 hours.

AGE-BSA Decreases Antiplatelet BAEC Function

To further investigate the physiological consequences of NOS III downregulation by AGE-BSA, we evaluated the effects on the antiplatelet properties of BAECs, on the basis of BAEC-derived NO production. As expected, AGE-BSA treatment markedly modified BAEC antiplatelet properties. In our experimental model, 10⁶ BAECs were needed to reduce thrombin-induced platelet aggregation by 50%, whereas the same amount of 10 μmol/L AGE-BSA–treated cells reduced platelet aggregation by only 18%, as depicted in Figure 6. Treatment with heat-inactivated AGE-BSA did not modify the antiplatelet properties of BAECs.
Discussion

Numerous physiological and pathophysiological stimuli are now known to increase or decrease NOS III gene transcription. We have found that exposure of BAECs to albumin-derived AGEs results in marked decreases in the expression of NOS III activity, protein, and transcript levels but does not affect the transcriptional rate of NOS III gene.

The major mechanism involved in downregulation of NOS III expression seems to be due to an enhanced rate in mRNA degradation, as demonstrated for both tumor necrosis factor-α and lipopolysaccharide.1,2 In the present study, we show that an increase in mRNA degradation rather than a decrease in transcription mediates the inhibition of NOS III gene expression.

The decrease of NOS III mRNA by AGE-BSA requires at least 24 hours to become apparent. This long lag time suggests that the reduction of NOS III transcripts by AGE-BSA may involve another factor. Although the identity of newly transcribed gene product(s) required for the observed decrease in NOS III expression seems to be due to an enhanced rate in mRNA degradation rather than a decrease in transcription, which may represent a new insight into the vascular mechanism(s) of NOS III mRNA destabilization induced by AGE-BSA.

It is noteworthy that platelet hyperactivity is a typical feature of the diabetes-associated prothrombotic state.24 The fact that AGE-BSA markedly reduced the antiplatelet activity of endothelial cells, mainly because of the reduction of NOS III expression, may be an additional factor to aggravate vascular complications. Inhibition of endothelial cell–derived NO enhances polymorphonuclear leukocyte adherence and emigration in postcapillary venules as demonstrated by Kubes et al,25 suggesting the role of NO in preventing leukocyte-endothelial cell adhesion. Furthermore, NO decreases cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).26 Recently, aortic tissue AGE accumulation has been associated with a significant increase in expression of VCAM-1 and ICAM-1.27

In addition, vascular smooth muscle cell proliferation is clearly associated with the accelerated vasculopathy observed in diabetes. It has been demonstrated that NO mediates the cytostasis of subendothelial vascular smooth muscle cells,28,29 and AGEs may favor cell proliferation by quenching endothelium-derived NO30 and thus plays a significant role in the onset of proliferative vascular lesions.

In summary, AGE-BSA markedly reduces NOS III expression, which may represent a new insight into the vascular complication observed in diabetes. Whether downregulation of NOS III expression has any effect on impaired endothelium-dependent relaxation must await further investigation. Thus, a better understanding of such a mechanism may lead to new strategies in the prevention of vascular dysfunction.

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References

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