THE EFFECT OF HIGH GLUCOSE CONCENTRATION
ON NITRIC OXIDE BIODISPONIBILITY

LAURA TOMA and ANCA SIMA

Institute of Cellular Biology and Pathology “Nicolae Simionescu”, Lipoproteins and Atherosclerosis Department,
8, B.P Hasdeu Street, PO-Box 35-14, Bucharest 050568, Romania, e-mail: anca.sima@icbp.ro

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A key event in the pathology of diabetes is the decreased biodisponibility of the nitric oxide (NO). The aim of the study was to evaluate the mechanisms by which high glucose concentrations determine a reduced biodisponibility of NO. We used human endothelial cells EAhy 926 exposed to a high (4.5‰) glucose concentration for 24h and 72h. Results showed a decrease of NOx level in the culture medium, accompanied by a decrease of the gene expression of the endothelial NO synthase and of the scavenger receptor BI, as compared with EAhy926 cells incubated with normal glucose (1‰) concentration. The intracellular oxygen species were slightly increased. The antioxidant capacity of the cells media incubated with 4.5‰ glucose was decreased. In conclusion, high glucose concentrations determine a decreased biodisponibility of NO, by decreasing its synthesis and possibly by interacting with the reactive oxygen species generated by this pro-diabetic condition.

Key words: High glucose; Nitric oxide; SR-BI; Endothelium.

INTRODUCTION

Diabetes mellitus is now considered as a worldwide epidemic. Its atherosclerotic complications are the most important cause for the morbidity and mortality manifested by diabetic persons1.

There is growing evidence that endothelial dysfunction is a critical component of diabetes2–4. Endothelial dysfunction is characterized by an impairment of the endothelium-dependent vascular tone, namely the reduction of the bioavailability of vasodilators, in particular that of the nitric oxide (NO), in parallel with the increased endothelium-derived contracting factors.

In the endothelial cell, NO is generated by NO synthase (eNOS), which converts L-arginine to L-citrulline5. Beside L-arginine, other substrates used by eNOS are molecular oxygen and NADPH6. The availability of NO in vivo is regulated by a combination of its synthesis and breakdown. A reduced synthesis of NO might happen due to the decreased gene and protein expression of eNOS. A decrease of the eNOS enzymatic activity may be also responsible for a reduced NO synthesis. The secreted NO can also interact with intracellular reactive oxygen species, forming peroxynitrites – very powerful pro-oxidant molecules7.

One of the activators of eNOS has been demonstrated to be the human scavenger receptor class B type I (SR-BI)8, a cell surface glycoprotein which is expressed in various mammalian tissues and cells, including the cells component of the arterial wall (endothelial cells, smooth muscle cells, macrophages). The main role of SR-BI is to participate in both ends of the reverse cholesterol transport pathway: the efflux of free cholesterol from peripheral cells to HDL and the delivery of cholesteryl esters and free cholesterol from HDL to the liver, for secretion into the bile8.
The aim of this study was to determine the effect of high glucose concentrations on endothelial cells, to identify the mechanisms responsible for the decreased NO bioavailability and the SR-BI gene expression.

MATERIALS AND METHODS

Endothelial Cells Culture

A human umbilical vein endothelial cells line, EAhy926, was used. Cells were grown in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 1% glucose and 10% fetal calf serum (FCS). At confluence, the cells were incubated with DMEM without phenol red and ferrous nitrate, supplemented with 2% FCS and 1% glucose (control cells) or 4.5% glucose for 24h and 72h.

NOx assay

NO production was determined by measuring the levels of nitrites in the cell culture medium using the Griess reaction, as described 9.

Gene expression of eNOS and SR-BI

RNA was isolated from the cells using the GenElute Mammalian Total RNA Kit from Sigma and 1 µg of the total RNA was reverse transcribed using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich Co., St Louis., MO, USA), according to the manufacturer instructions. After the reverse transcription, the complementary DNA was subjected to amplification using specific primers for 28S (as internal standard), eNOS and SR-BI (using a MJ Research Machine, Opticon soft). The specific primers used were: 28S sense: 5’ AAA CTC Tgg Agg TCC gT-3’ and antisense 5’-CTT ACC AAA AgT ggC CCA CTA-3’ eNOS: sense 5’-GACGCTACGAGGAGTGGAAG-3’ and antisense 5’-TAGGTCTTGGGTTGTCAGG-3’ and human SR-BI: sense: 5’ GTGTCCTTCCTCGAGTACC-3’ and antisense 5’ GAACACCGTGAGGCCCGAG-3’. The amplification products were detected in a Real-time PCR, by continuously monitoring the SYBr green fluorescence with high affinity for double stranded DNA. The quantification of the PCR products was performed by the “Fit Point Method”. The cDNA was amplified through 35 cycles, with the annealing temperature at 58.5˚C, followed by a melting curve program (from 55˚C to 94˚C, with compound read at every 1˚C), and finally a cooling step at 30˚C.

Reactive oxygen species quantification

After exposing EAhy926 cells to the increased glucose concentration, the cells were washed twice with PBS and incubated with 10 µM 2-7-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Co., St Louis., MO, USA), for 30 minutes at 37˚C. Than, the cells were washed again twice with PBS and were harvested by scraping in 1 ml PBS and lysed by sonication. The cell lysate was centrifuged at 2.000xg, 10 min and the fluorescence of the supernatant was quantified in a TECAN spectrofluorimeter. The excitation/emission wavelengths for DCFH-DA were 485/530 nm.

Reactive nitrogen species measurement

The formation of the nitrogen species was detected using the cell-permeant oxidant-sensitive nonfluorescent probe, dihydrorhodamine 123 (DHR) (Sigma-Aldrich Co., St Louis, MO, USA). Oxidation of DHR by two electrons yields the highly fluorescent product rhodamine 123. The determination of the nitrogen species followed the same protocol as that for the reactive oxygen species determination, except that the DHR was used as fluorochrome. The excitation/emission wavelengths for DHR were 505/530.

Protein assay

Protein concentration of each sample was measured using the BCA reactive.

Total peroxyl radical-trapping potential

The antioxidant capacity of the cells medium was determined by measuring the total peroxyl radical-trapping potential (TRAP) by a modified protocol from Valkonen et al.10. The method employs AAPH [2,2’-diazobis (2-amidinopropane) dihydrochloride] (Sigma-Aldrich) as oxidant agent, DCFH-DA (2,7-dichlorofluorescein-diacetate) (Sigma-Aldrich) as oxidant, DCFH-DA and Trolox (Sigma-Aldrich) as internal standard.

Statistical analysis

All the statistical calculations were done using the One Way ANOVA Test, and the results are given as means ± SD.

RESULTS

Evaluation of NOx in the culture medium

Incubation of EAhy926 cells with 4.5% glucose concentration for 24h and 72h lead to significantly decreased NOx levels in the medium (20%–40%) (Fig. 1). The levels of NO were statistically lower after 72h incubation of the endothelial cells with the high glucose concentration, compared to the value after 24h incubation. The results are presented as histograms in which the NOx of probes (4.5% glucose) are normalized to the control values (1% glucose).
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Modulation of the gene expression for eNOS and SR-BI

In order to study the mechanisms by which the nitric oxide levels are modulated in this experimental model, we evaluated the eNOS and SR-BI gene expression.

The decrease of NOx levels that was observed upon incubation of EAhy926 cells with increased glucose concentrations was accompanied by an important decrease in the eNOS gene expression. A 30% to 70% decrease in eNOS gene expression was determined in the cells incubated in 4.5‰ versus 1‰ glucose concentration. The eNOS gene expression was lower after 72h incubation of the endothelial cells with the high glucose concentration, compared to the value obtained after 24h incubation (Fig. 2). The results are presented as histograms in which the gene expressions of eNOS and SR-BI in the probes (4.5‰ glucose) are normalized to the control values (1‰ glucose).

Incubation of EAhy926 cells with increased glucose concentrations determined an important decrease in the SR-BI gene expression. Results of the experiments showed that incubation of EAhy926 cells with 4.5‰ glucose for 24h and 72h induced a decrease between 20% and 50% of the SR-BI gene expression. After 72 h, the SR-BI gene expression tended to increase, as compared to 24h (Fig. 3).

Accumulation of the intracellular oxygen and nitrogen reactive species

Incubation of the EAhy926 endothelial cells with 4.5‰ glucose concentration for 24 h or 72 h determined a slight increase of intracellular oxygen reactive species (Fig. 4). The results are presented as histograms in which the ROS level in the probes (4.5‰ glucose) are normalized to the control values (1‰ glucose).

Fig. 1. NOx levels in the culture medium of the EAhy926 cells incubated with 1‰ or 4.5‰ glucose concentrations, for 24h and 72h.

Fig. 2. Quantification of eNOS gene expression in EAhy926 cells incubated in 1‰ or 4.5‰ glucose concentration, for 24h and 72h.

Fig. 3. Quantification of SR-B1 gene expression in EAhy926 cells incubated in 1‰ or 4.5‰ glucose concentrations for 24h and 72h.

Fig. 4. Quantification of reactive oxygen species (ROS) in EAhy926 cells incubated with 1‰ or 4.5‰ glucose concentrations, for 24h and 72h.
The reactive nitrogen species (RNS) levels did not vary statistically significant after incubation of EAhy926 cells with 4.5‰ glucose under our experimental conditions (Fig. 5). The results are presented as histograms in which intracellular RNS in the probes (4.5‰ glucose) are normalized to the control values (1‰ glucose).

![Fig. 5. Quantification of reactive nitrogen species (RNS) in EAhy926 cells incubated with 1‰ or 4.5‰ glucose concentrations for 24h and 72h.](image)

Measurement of the antioxidant capacity of the cells media indicated a decrease of TRAP values after incubation in 4.5‰ glucose (Fig. 6). The decrease continues throughout the entire time of the experiment, the lowest level being attained after 72 hours of incubation with 4.5‰ glucose concentration. The results are presented as histograms in which TRAP from the media of the probes (4.5‰ glucose) are normalized to the control values (1‰ glucose).

![Fig. 6. Histogram with the antioxidant capacity of the cell media incubated with 1‰ or 4.5‰ glucose concentrations for 24h and 72h.](image)

**DISCUSSIONS**

Data from the literature are controversial regarding the NOx production and the eNOS gene expression in cells cultured in media with high glucose concentrations. There are reports that demonstrate a decline in NOx levels \(^{11,12}\) and mRNA of eNOS in different cell types in culture \(^{11–14}\) and also in animal models \(^{15}\). It was also demonstrated that high glucose concentrations inhibit insulin-stimulated NOx production in human aortic endothelial cells \(^{16}\). There are also articles that demonstrate that NOx levels and eNOS gene expression are not altered in high glucose conditions \(^{17–19}\). The data reported here indicate that NOx levels in the human endothelial EAhy926 cells medium are decreased following 24h to 72h exposure to 4.5‰ glucose concentration. The eNOS mRNA expression is also decreased in EAhy926 cells incubated with 4.5‰ glucose, in correlation with the decreased levels of NOx of the cells media. Beside its role in modulating the endothelium-dependent vasodilatation, NO is also a powerful antioxidant \(^{20,21}\) and is capable to inhibit platelet adherence and aggregation (NO inhibits activator platelets factor-PAF) \(^{22}\), leukocytes adhesion and infiltration (by inhibiting VCAM-1, MCP-1, RANTES) \(^{23,24}\), and proliferation of smooth muscle cells. All these processes are implicated in the progression of atherosclerosis.

The measured antioxidant capacity of the endothelial cells medium indicated a decrease in TRAP levels and an increase in intracellular ROS levels in the cells incubated with 4.5‰ glucose. Because of the increase of ROS, we were expecting to observe also an increase in peroxynitrites levels in the cells incubated in high glucose concentration, but this did not happen. An explanation might be the severe decrease of NOx levels measured in the culture media of the endothelial cells incubated with high glucose concentration, in parallel with the small increase in ROS.

Scavenger receptor SR-BI may protect against atherosclerosis by participating in the reverse cholesterol transport. SR-BI-associated atheroprotection may also be related to its modulation of the NO production in the endothelium. Uittenbogaard and colleagues showed that HDL prevented the inhibition of eNOS activity induced by the oxidized LDL, while antibodies directed against SR-BI impaired this effect \(^{25}\). Using SR-BI and eNOS transfected cells, Yuhanna *et al.* showed that SR-BI mediates the activation of eNOS by...
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HDL. There are no data in the literature regarding the effect of high glucose on SR-BI gene expression, although it is known that this receptor is implicated in the uptake of advance glycation end-products proteins that are formed in diabetes. In the present study, we observed a small decrease in SR-BI gene expression after incubation of the endothelial cells in medium containing high glucose concentration. Because SR-BI is an activator of eNOS, a decrease of the expression of SR-BI may be another explanation of the decreased NO levels that we observed.

The presented data suggest that in endothelial cells incubated with high glucose concentrations the NO bioavailability is decreased, either by the decreased NO secretion, or by the reaction of NO with the reactive oxygen species secreted in the medium. The impaired NO synthesis might be caused by two possible mechanisms: the decreased gene expression of eNOS, and/or that of the eNOS activator, SR-BI.

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