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High glucose inhibits expression of inducible and constitutive nitric oxide synthase in bovine aortic endothelial cells

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KEY WORDS nitric-oxide synthase; glucose; lipopolysaccharides; Western blotting; vascular endothelium; cultured cells

ABSTRACT

AIM: To investigate the effects of high glucose on the expression of nitric-oxide synthase (NOS) in cultured bovine aortic endothelial cells (BAEC). **METHODS:** BAEC were cultured and passaged in normal glucose (NG) 5.5 mmol·L^{-1}, high glucose (HG) 25 mmol·L^{-1}, or high osmolarity (glucose 5.5 mmol·L^{-1} + mannitol 19.5 mmol·L^{-1}, Mann-BAEC), lipopolysaccharides (LPS)-induced nitric oxide (NO) production was assessed by Griess reaction. The expression of inducible NOS (iNOS) and constitutive NOS (ecNOS) was determined by Western blot. **RESULTS:** At a concentration range from 0.5 to 2 mg·L^{-1}, LPS stimulated NO production in NG-BAEC in a concentration-dependent manner. NO production reached the peak level at LPS 1 mg·L^{-1}. HG inhibited NO production, when compared with NG- and Mann-BAEC (nitrite μmol·L^{-1}: HG-BAEC 43 ± 8, *vs* NG-BAEC 71 ± 11, Mann-BAEC 70 ± 9, $n = 4$ experiments, $P < 0.01$). iNOS expression was decreased by 39.9 % and 39.3 %, and ecNOS by 28 % and 24 % respectively in HG-BAEC, when compared with NG- or Mann-BAEC. However, no marked difference was observed in the LPS-induced NO production and the expression of iNOS and ecNOS between NG- and Mann-BAEC. **CONCLUSIONS:** Inhibition of BAEC NO production by HG was mainly due to a decreased expression of NOS protein.

INTRODUCTION

Diabetes is associated with accelerated atherosclerosis and an increased prevalence of cardiovascular disease[1]. Loss of the modulatory role of the endothelium could be implicated in the pathogenesis of diabetic vascular complications[2]. NO, a very important endogenous modulator of endothelium function, is synthesized by nitric oxide synthase (NOSs) from *L*-arginine. In blood vessels, two distinct NOS isoforms have been identified. The constitutive NOS (ecNOS) in endothelial cells is constitutively present and regulates vascular tone and blood pressure[3]. Induction of inducible NOS (iNOS) in endothelial cells and vascular smooth muscle cells occurs in response to various cytokines and bacterial endotoxin, which may have an adaptive role in the vascular response to injury[4].

Diabetes associated atherosclerotic disease is characterized by impaired endothelium-dependent relaxation and excessive proliferation of vascular smooth muscle cells, which have been considered to be strictly associated with reduced bioavailability of NO[2]. A pool of evidence has demonstrated that hyperglycemia can decrease production of NO and accelerate the inactivation of NO[5]. Decreased expression of iNOS has been shown in cultured vascular smooth muscle cells exposed to high glucose[6]. Recently, two reports are available about altered expression of ecNOS in cultured endothelial cells after exposure to elevated glucose, but the results are inconsistent[7,8]. In the present study, we studied the effect of high glucose on the expression of iNOS induced by lipopolysaccharides (LPS) and ecNOS in cultured bovine aortic endothelial cells (BAEC).

MATERIALS AND METHODS

Reagents LPS was obtained from Sigma Chemical Co. iNOS and ecNOS polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Bostern Bioengineering Co, Wuhan. Phototope-HRP Western Detection System was purchased from New England Biolabs Inc. Nitrite detection kit was purchased from Jinmei Biotechnology Inc,

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Cell culture Bovine aortic endothelial cells (BAEC) were cultured in phenol-free M1640 medium with 20 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 kU·L^{-1}, and streptomycin 100 mg·L^{-1}. After confluence, BAEC were randomly assigned to three groups and subcultured in three different conditions at a ratio of 1:2 with 0.1 % trypsin: normal glucose (5.5 mmol·L^{-1}, NG-BAEC), high glucose (25 mmol·L^{-1}, HG-BAEC), and high osmolarity (glucose 5.5 mmol·L^{-1} + mannitol 19.5 mmol·L^{-1}, Mann-BAEC). Experiments were performed in passage 4 – 10 BAEC.

Measurement of NO production[9] BAEC were seeded in 24-well culture plates and allowed to grow to confluence, then were stimulated with various concentrations of LPS. Culture supernatants were collected after 48 h. This time point was chosen based on our pre-experiments. The accumulation of nitrite, a stable end product of NO formation, was used as a relative measurement of NO production. The detecting method was according to the protocol enclosed in the NO detection kit. Briefly, 100 μL of supernatant were incubated with 100 μL of Griess reagent (1 % sulfanilic acid, 0.1 % naphtylethylenediamine dihydrochloride, and 2.5 % phophoric acid) at room temperature for 10 min, absorbance was determined at 570 nm with an ELISA reader. NO levels were expressed as nitrite concentration and were determined with reference to a standard curve of sodium nitrite.

Preparation of lysates For iNOS detection, cells were seeded into 12-well culture plates with NG-, HG-, or Mann-M199 containing 20 % FBS. After confluence, all the culture media were changed to the NG-M199 with 20 % FBS, and an indicated concentration of LPS was added. The treating time was chosen to be 48 h, since a peak expression of iNOS was obtained at this time point in our experimental conditions. After being washed with ice-cold PBS thrice, cells were lysed with 60 μL of ice-cold lysis buffer containing: NaCl 50, Na_3VO_4 2, phenylmethylsulfonyl fluoride 0.5, and HEPES 10 mmol·L^{-1} at pH 7.4, along with 0.01 % Triton X-100 and leupeptin 10 mg·L^{-1} was added. The lysates were obtained by centrifugation at 18 000 × g at 4 ℃ for 15 min. Total cell protein was determined by the dye method[10]. For ecNOS detection, after confluence in 12-well culture plates with NG-, HG- or Mann-M199 containing 20 % FCS, protein samples of three cell species were extracted as above.

Western blot A 1/4 volume SDS sample buffer containing Tris-HCl 0.33 mol·L^{-1}, 10 % SDS (wt/vol), 40 % glycerol (vol/vol), and 20 % dithiothreitol (vol/vol) containing 0.4 % bromophenol blue was added to cell lysates. After being boiled for 5 min, the extracted protein 10 μg was electrophoresed on 8 % SDS-polyacrylamide gel (SDS-PAGE). Then the protein was transferred to nitrocellulose membrane, which was then blocked for 1 h at room temperature with 5 % BSA in PBST (Na_2HPO_4 80, NaH_2PO_4 20, NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated at 25 ℃ with the primary antibodies against iNOS or ecNOS at a 1:500 dilution for at least 4 h, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1:1000 dilution. Immunoreactive signals were visualized by the Phototope Western Detection System. Bands of iNOS and ecNOS were quantitatively determined by thin-layer chromatography with Shimadzu Dual-Wavelength Chromato-Scanner (Japan, Model CS-930).

RESULTS

NO production At concentrations of 0.5 to 2 mg·L^{-1}, LPS stimulated nitrite production in NG-BAEC in a concentration-dependent manner after 48-h incubation. Nitrite reached the peak level at LPS 1 mg·L^{-1} (Tab 1A).

Tab 1. A: Concentration-dependent effect of LPS on nitrite production in NG-BAEC. B: Effect of LPS-stimulated nitrite production in NG-, HG-, and Mann-BAEC. *n* = 4 experiments. $\bar{x} \pm s$, average of duplicates contributes one determination. $^aP > 0.05$, $^cP < 0.01$ *vs* NG-BAEC, $^fP < 0.01$ *vs* Mann-BAEC.

A:

LPS/ mg·L^{-1}	Nitrite concentration/ μmol·L^{-1}
0	7.3±2.1
0.25	20±4
0.5	30±7
1.0	71±11
2.0	71±9

B:

LPS/ mg·L^{-1}	BAEC nitrite concentration/μmol·L^{-1}		
	NG	HG	Mann
1.0	71±11	43±8^{cf}	70±9^{a}

Therefore, we chose LPS 1 mg·L^{-1} for all further experiments. NO production was markedly inhibited in HG-BAEC, when compared with NG- and Mann-BAEC (nitrite μmol·L^{-1}: HG-BAEC 43 ± 8, *vs* NG-BAEC 71 ± 11, Mann-BAEC 70 ± 9, n = 4 experiments, P < 0.01). But no difference was observed between NG- and Mann-BAEC (Tab 1B).

iNOS expression The expression of iNOS, stimulated by incubation with LPS 1 mg·L^{-1} for 48 h, was inhibited in HG-BAEC, when compared with NG- and Mann-BAEC. Densitometric analysis showed 39.9 % and 39.3 % decrease in iNOS protein expression when compared with NG- and Mann- BAEC, respectively. However, no difference was observed in the expression of iNOS between NG- and Mann-BAEC (Fig 1A, Tab 2).

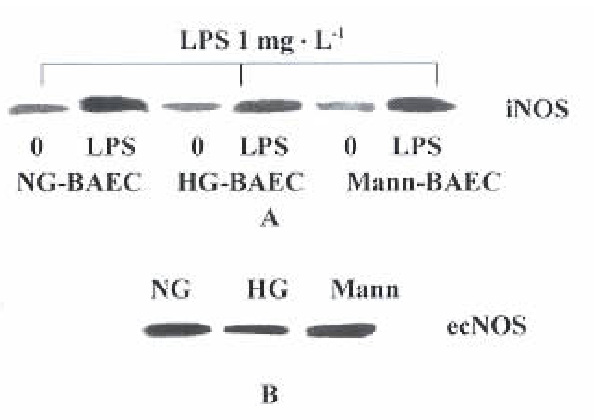

Fig 1. Effect of high glucose on LPS-induced iNOS and ecNOS protein expression by Western blotting in BAEC. A: iNOS, B: ecNOS.

Tab 2. Densitometric quantifications of iNOS and ecNOS protein. n = 4 experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* NG-BAEC. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* Mann-BAEC. $^iP < 0.01$ *vs* Control.

LPS mg·L^{-1}		10^{-3} × Absolute peak area/mm^2 NG-BAEC	HG-BAEC	Mann-BAEC
iNOS	0	80 ± 17	78 ± 16^{ad}	89 ± 15^{a}
	1.0	218 ± 17^{i}	131 ± 22^{cfi}	216 ± 23^{ai}
ecNOS		204 ± 27	147 ± 23^{ce}	191 ± 27^{a}

ecNOS expression The expression of ecNOS was reduced by 28 % and 24 % in HG-BAEC, when compared with NG- and Mann-BAEC, respectively. No difference was observed in ecNOS expression between NG- and Mann-BAEC (Fig 1B, Tab 2).

DISCUSSION

We have demonstrated that LPS-induced NO production decreased in BAEC after exposure to elevated glucose. This reduction was due to decreased expression of iNOS protein. In order to simulate chronic hyperglycemia more closely, BAEC were subcultured in high glucose for at least 4 passages rather than adding high glucose acutely. The altered NO production and iNOS expression of BAEC in high glucose were not due to changes in osmolarity since cells cultured in high mannitol showed similar NO production and iNOS expression when compared to cells in normal glucose. Similar results have recently been obtained in the studies with rat vascular smooth muscle cells[6,11]. It is well known that inhibition of vascular smooth muscle cell proliferation is one of the important anti-atherosclerotic properties of NO[12]. iNOS activity has been demonstrated in atherosclerotic lesion[13]. High glucose-induced inhibition of iNOS in vascular smooth muscle cells has been considered to be associated with accelerated development of atherosclerosis in diabetes[6]. In endothelial cells, NO synthesis is focused on ecNOS and its function is involved in endothelium-dependent vasorelaxation. However, being a small gas molecule, NO derived from iNOS in endothelial cells can theoretically reach subendothelial space to inhibit the smooth muscle cell proliferation. Thus, our results further suggest that hyperglycemia may be responsible for the excessive vascular smooth muscle cell proliferation due to its inhibition of NO availability from endothelium.

There is substantial evidence that vasodilation mediated by endothelium-derived NO is impaired in animal models of diabetes and in diabetic patients[2]. Previous studies have demonstrated impaired endothelium-dependent relaxation in isolated blood vessels after exposure to elevated glucose[14, 15]. In this study, we observed a decreased expression of ecNOS in BAEC cultured in high glucose, which is consistent with Chakravarthy's study with retinal vascular endothelial cells[8]. However, a contrasting result was reported by Cosentino in human aortic endothelial cells[7], their study demonstrating that prolonged exposure to high glucose increases ecNOS gene expression, protein expression, and NO release. These controversial results may result from different cell types or different experimental conditions. A more prolonged exposure to elevated glucose was carried out in our experiments.

In summary, this study demonstrates that elevated glucose inhibited induction of iNOS as well as NO release

and decreased the expression of ecNOS in bovine aortic endothelial cells. These findings may explain the impaired endothelial function and excessive vascular smooth muscle cell proliferation, which play important roles, in the development of diabetic vascular disease.

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高糖抑制牛主动脉内皮细胞诱导型和结构型一氧化氮合酶的表达

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关键词 一氧化氮合酶；葡萄糖；脂多糖类；蛋白质印迹；血管内皮；培养的细胞

目的：研究高糖对新生小牛主动脉内皮细胞(BAEC)一氧化氮合酶(NOS)表达的影响. **方法**：BAEC 培养并传代于含正常葡萄糖(5.5 mmol·L^{-1}，NG-BAEC)，高糖(25 mmol·L^{-1}，HG-BAEC)或高渗(葡萄糖 5.5 + 甘露醇 19.5 mmol·L^{-1}，Mann-BAEC)的无酚红 M1640 培养基. Griess 反应检测脂多糖(LPS)诱导的一氧化氮(NO)产生. Western blot 法检测结构型 NOS(ecNOS)及诱导型 NOS(iNOS)表达. **结果**：LPS (0.25 – 2 mg·L^{-1})剂量依赖性刺激 BAEC 产生 NO，并在 LPS 1 mg·L^{-1}达峰值. 高糖显著抑制 LPS 诱导的 NO 产生(亚硝酸盐 μmol·L^{-1}：HG-BAEC 43 ± 8，*vs* NG-BAEC 71 ± 11，Mann-BAEC 70 ± 9，$n = 4$，$P < 0.01$). 同样，与 NG-和 Mann-BAEC 相比，HG-BAEC iNOS 表达下降 39.9 %和 39.3 %，ecNOS 表达下降 28 %和 24 %，而 NG-与 Mann-BAEC 之间，LPS 诱导的 NO 产量和 iNOS 和 ecNOS 的表达无差别. **结论**：高糖抑制 BAEC NO 的释放，与 NOS 的低表达有关.

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