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Effect of High Glucose Concentrations on Expression of ELAM-1, VCAM-1 and ICAM-1 in HUVEC with and without Cytokine Activation

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Summary

Diabetes mellitus is associated with an increased prevalence of endothelial dysfunction and development of atherosclerotic vascular diseases. We demonstrate here that hyperglycemia results in the expression of adhesion molecules on endothelial cells *in vitro*. Incubation of human umbilical vein endothelial cells (HUVEC) in a culture medium with 11.0 mM, 16.5 mM and 22.0 mM glucose concentrations induced the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1). This effect was detectable after 24 h incubation of HUVEC with a high glucose concentration. The effect of high glucose concentration on TNF-α induced expression of ELAM-1, VCAM-1 and ICAM-1 was negligible, if at all. These results show that even a short-term exposure of endothelial cells (ECs) to high glucose concentration leads to their activation associated with increased expression of adhesion molecules such as ELAM-1, VCAM-1 and ICAM-1.

Key words

Adhesion molecules • Atherosclerosis • Endothelial cells • Diabetes mellitus • High glucose

Introduction

Prevalence of the atherosclerotic vascular disease is markedly increased among individuals with diabetes mellitus (Kannel and McGee 1979, Jang *et al.* 1998). Recent evidence has suggested that endothelial dysfunction, a proposed risk factor for atherosclerosis, plays a key role in the pathogenesis of diabetic atherosclerotic cardiovascular disease (Haffner *et al.* 1998).

Leukocyte adhesion to arterial endothelial cells is thought to be an important step in the development of atherosclerosis (Carter and Grant 1997). Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the endothelial-leukocyte adhesion molecule-1 (ELAM-1) play an essential role in this step (Ross 1999). Although the exact mechanisms of diabetes-induced endothelial dysfunction are unknown, pathological elevation of glucose levels has been shown to induce various effects at the cell level either directly (Tesfamariam *et al.* 1990) or through the formation of intermediate products such as advanced glycation end-product (AGE) adducts (Esposito *et al.* 1989). It has been reported that exposure of the vascular endothelium to elevated glucose concentration induces expression of

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ICAM-1 *in vitro* (Baumgartner-Parzer *et al.* 1995, Takami *et al.* 1998). Furthermore, Otsuki *et al.* (1997) and Matsumoto *et al.* (2000) reported that serum concentrations of soluble adhesion molecules (sICAM-1, sVCAM-1, sELAM-1) are elevated in patients with type 2 diabetes. An increased expression of endothelial cell adhesion molecules in response to glucose has also been correlated with an alteration of nitric oxide (NO) synthesis (Sobrevia and Mann 1997) and endothelium-induced vasodilatation (Williams *et al.* 1998).

In this study, we have investigated the effects of elevated glucose concentrations on the expression of adhesion molecules (ELAM-1, VCAM-1 and ICAM-1) on basal and cytokine (TNF-α) stimulated human umbilical vein endothelial cells (HUVEC).

Methods

Cell cultures

Human umbilical cords were obtained from normal placentas, excised after birth (kindly provided by the Department of Obstetrics and Gynecology of the University Hospital Královské Vinohrady, Praha) and placed in a sterile container filled with a transfer buffer. HUVEC were isolated and cultured by a modified method according to Jaffe *et al.* (1973) and Marin *et al.* (2001). Briefly, primary endothelial cells (ECs) were harvested from umbilical cord veins treated with 0.2 % collagenase (Gibco, UK) and incubated for 25 min at 37 ºC and 5 % CO_2. After incubation, the collagenase solution containing HUVEC was flushed from the cord by perfusion with 20 ml of Medium 199 (M 199, BioWhittaker, USA). The cells were centrifuged for 5 min at 1500 rpm, the medium was discarded, and resuspended in 5 ml of fresh culture medium. The cell suspension cultured on gelatin-coated tissue culture flasks (Greiner GmbH) in M 199 containing 20 % fetal bovine serum (FCS, Gibco, BRL, Paisley, UK), 20 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor (both from Gibco, BRL, Paisley, UK), penicillin 100 U/ml + streptomycin 0.1 mg/l (Sevapharma, Czech Republic), gentamycin 25 µg/ml (Gibco, UK) and amphotericin 2.5 mg/ml (Gibco, UK). The cultures were maintained at 37 °C and 5 % CO_2 and passaged once or twice a week by treating confluent monolayer with 0.125 % trypsin, washing the cell suspension with phosphate buffered saline (PBS) and resuspending the cells in doubled volume of fresh medium. Isolated ECs were identified by indirect immunofluorescence using monoclonal antibody anti-CD31, Human Endothelial Cell (Dako, Denmark). For all experiments, endothelial cell cultures were used at passages 1-4.

Incubation of HUVEC with various concentrations of glucose

Three to five days old confluent cultures of HUVEC were cultivated in culture medium containing 11.0 mM, 16.5 mM, or 22.0 mM glucose concentrations, respectively. The cells cultivated in a culture medium with a physiological glucose concentration (5.5 mM) were used as controls. After 24 h incubation with glucose, a portion of ECs cultures were treated with 10 ng/ml TNF-α (Sigma, Aldrich, USA). At 4 h (ELAM-1), 8 h (VCAM-1) and 12 h (ICAM-1) after TNF-α induction, samples from both TNF-α treated and untreated cultures were taken for measuring of adhesion molecule expression by flow-cytometry.

Measurement of adhesion molecules expression by flow-cytometry

The ECs cultures were washed with PBS, detached using ice-cold PBS and 0.005 % EDTA, thoroughly resuspended and incubated with fluorescent label-marked monoclonal antibodies anti-ICAM-1, anti-VCAM-1 (both from B.D. Pharmingen, San Diego) and anti-ELAM-1 (Bender, Vienna, Austria) for 15 min at 37 °C. A minimum of 5 x 10^3 cells were analyzed by fluorescence-activated flow-cytometry (FACS) on ORTHO-Diagnostic systems FACS-Cytoronabsolute (Johnson & Johnson).

Statistical analysis

The results were evaluated using methods of variation analysis (ANOVA test). The data are given as mean values ± S.D. $P \leq 0.05$ value was considered statistically significant.

Results

The effect of high glucose concentrations on spontaneous and TNF-α induced expression of ELAM-1, VCAM-1 and ICAM-1 in cultured HUVEC is shown in Table 1. Cultivation of HUVEC for 24 h in a high glucose concentration stimulated an expression of all adhesion molecules studied (ELAM-1, VCAM-1 and ICAM-1). From the glucose concentrations tested, 16.5 mM glucose had the most pronounced effect on adhesion molecules expression. At this concentration, the expression of ELAM-1 was 6-fold increased above the baseline level found in the control culture ($p=0.006$).

VCAM-1 was increased 7-fold (p=0.003) and ICAM-1 two-fold (p=0.05), respectively. However, even a smaller elevation of glucose concentration (11.0 mM) stimulated the expression of ELAM-1 and VCAM-1 significantly. At very high glucose concentration (22.0 mM), the rate of adhesion molecule induction was not as pronounced as at 11.0 mM and 16.5 mM concentrations. This may be partially due to toxicity of this glucose concentration for HUVEC.

As was expected, TNF-α *per se* stimulated significantly the expression of ELAM-1, VCAM-1 and ICAM-1. The effect of high glucose on TNF-α induced expression of these adhesion molecules was negligible, if any (Figs 1-3).

Table 1. The expression of ELAM-1, VCAM-1 and ICAM-1 in cultured HUVEC following the exposure to 5.5 mM (control), 11.0 mM, 16.5 mM and 22.0 mM glucose with and without the induction by TNF-α.

		% of positive cells						
Adhesion molecules		**5.5 mM glucose (control)**	**11.0 mM glucose**	**P value vs control**	**16.5 mM glucose**	**P value vs control**	**22.0 mM glucose**	**P value vs control**
ELAM	TNF-α (–)	6.5±3.8	30.8±7.7	p=0.003	42.1±9.5	p=0.006	22.5±7.8	p=0.007
	TNF-α (+)	72±1.9	74±2.5		81±3.5		71±4.6	
VCAM-1	TNF-α (–)	4.0±3.3	21.4±8.5	p=0.006	28±10	p=0.003	16.1±6.1	p<0.01
	TNF-α (+)	46±8.2	62±5.2		79±2.1		53±9.8	
ICAM-1	TNF-α (–)	12.4±5.1	18.8±7.3	p=NS	25.2±10.2	p<0.05	16.8±5.8	p=NS
	TNF-α (+)	87±9.3	83±1.5		88±1.5		83 ± 6.2	

Values are mean ± S.D. of four separate experiments; TNF-α (–) expression of adhesion molecules without induction by TNF-α, TNF-α (+) expression of adhesion molecules after induction by TNF-α.

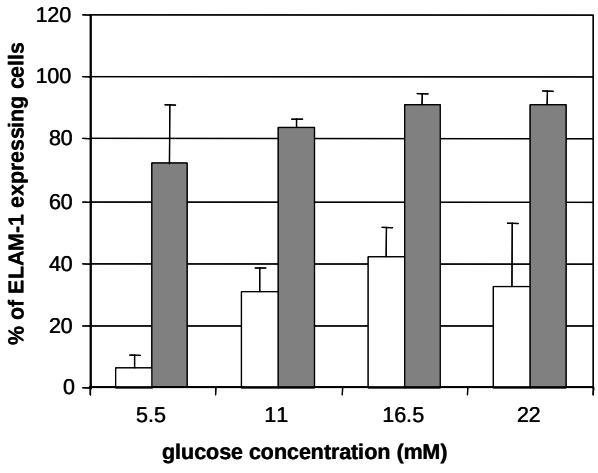

Fig 1. Effect of high glucose concentrations on expression of ELAM-1 in HUVEC with (full bars) and without (open bars) induction by TNF-α. Endothelial cell monolayer were treated for 24 h with various glucose concentrations in Medium 199 supplemented with 20 % FCS and the cell surface expression of ELAM-1 was measured by flow-cytometry.

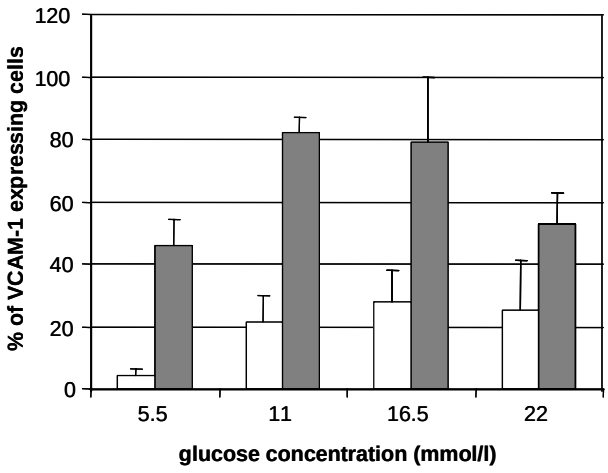

Fig 2. Effect of high glucose concentrations on expression of VCAM-1 in HUVEC with (full bars) and without (open bars) induction by TNF-α. After 24 h incubation with various glucose concentrations expression of VCAM-1 was measured by flow-cytometry.

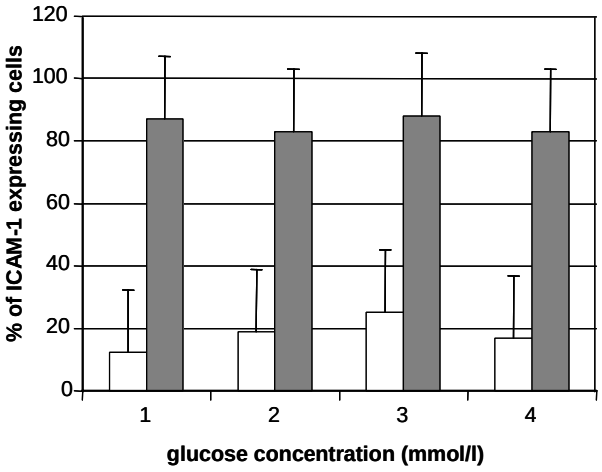

Fig 3. Effect of high glucose concentrations on expression of ICAM-1 in HUVEC with (full bars) and without (open bars) induction by TNF-α. After 24 h incubation with various glucose concentrations expression of VCAM-1 was measured by flow-cytometry.

Discussion

We have demonstrated in the present study that short-term exposure of HUVEC to high glucose concentrations results in the enhanced expression of surface ELAM-1, VCAM-1 and ICAM-1. This stimulative effect of high glucose concentration on the expression of adhesion molecules by endothelial cells has been previously reported in various studies performed both *in vivo* and *in vitro*. However, the results concerning the rate of induction of various types of adhesion molecules have not been consistent depending on the experimental system used. Stimulation of ICAM-1 expression by high glucose concentration was observed most often (Baumgartner-Parzer *et al.* 1995, Taki *et al.* 1996, Takami *et al.* 1998, Kado *et al.* 2001). Recently, the activation of VCAM-1 was demonstrated by Esposito *et al.* (2001) in aortic endothelial cells adapted by long-term cultivation at high glucose concentrations. Puente-Navazo *et al.* (2000) who exposed HUVEC for short periods of time to high glucose concentrations, observed activation of ICAM-1 and P-selectin, but not ELAM-1 and VCAM-1.

One of the main findings of this study was that short-term exposure of ECs to high glucose resulted in the induction of all three types of adhesion molecules on HUVEC *in vitro*. These results correspond to the findings of Marfella *et al.* (2000) and Matsumoto *et al.* (2002) who documented that hyperglycemia increases circulating ICAM-1 and VCAM-1 in type 2 diabetic patients. Moreover, increased plasma concentrations of these adhesion molecules were associated with insulin concentrations and insulin resistance (Bluher *et al.* 2002).

It has been documented that high glucose can influence various physiological processes in many cell types (Danne *et al.* 1993, Haneda *et al.* 1997). However, from the experiments *in vivo* it is not clear whether the effect of high glucose concentration is direct or indirect. For example, the indirect effect of oxidative stress and free radical formation is responsible for the induction of adhesion molecule expression.

In our experiments, we have demonstrated that the induction of adhesion molecules can reflect direct effects of glucose on ECs in culture. Three different elevated concentrations of glucose were tested in order to eliminate potential toxic effect of high glucose concentration on HUVEC. At 11.0 mM and 16.5 mM glucose neither cell viability nor proliferation was influenced. At 22.0 mM concentration, cell viability was not influenced, but cell doubling was inhibited (results not shown) There are several mechanisms by which high glucose can participate in endothelial dysfunction (Nagel *et al.* 1994, Ceriello 1997, Cominacini *et al.* 1997). Some studies described that high glucose content can activate a signaling pathway mediated by protein kinase C (PKC) (Inoguchi *et al.* 1994, Williams *et al.* 1997, Koya and King 1998). The activation of PKC results in the increased transcription of various genes including those for adhesion molecules. Upregulation of mRNA for ICAM-1 in the presence of high glucose concentration was documented by Kado *et al.* (2001). This hypothesis is further supported by the findings these authors and Booth *et al.* (2002) that PKC inhibitors (staurosporine, bisindolylmaleimide-1) attenuated stimulatory effect of high glucose concentrations on ICAM-1 expression (Kado *et al.* 2001, Booth *et al.* 2002). Another mechanism involving AGE adducts was reported by Esposito *et al.* (2001) who demonstrated that AGE are important mediators of endothelial dysfunction induced by a long-term exposure of ECs to high glucose.

The induction of adhesion molecules on the surface of ECs is one of the first steps in high glucose-mediated endothelial dysfunction in diabetic patients. Together with other mechanisms, e.g. inhibition of NO synthesis (Takahashi *et al.* 1996) resulting in vasoconstriction and endothelial impairment due to the free radical formation (Kashiwagi *et al.* 1996), it contributes to the development of inflammation in vascular wall and finally to vascular pathological changes.

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