

Insulin Administration in the Mild Hyperglycaemia Changes Expression of Proinflammatory Adhesion Molecules on Human Aortic Endothelial Cells

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ABSTRACT

An overexpression of cell adhesion molecules (CAMs) on the surface of endothelial cells is one of the first steps in a high glucose-mediated endothelial dysfunction in diabetic patients. The effect of insulin administration in the condition of elevated glucose concentration on the E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) expression on human aortic endothelial cells (HAEC) was investigated. Cells were cultured for 4h in a medium supplemented with homocysteine (7 μ M) and different concentration of glucose (5.5, 8.0, 12.0 and 16.5 mM respectively) with or without insulin (1 mIU/mL) addition. Expression of CAMs was analysed by flow-cytometry using monoclonal antibodies. Controls were CAMs expression in the medium with a corresponding glucose concentration. Obtained results show that short-term exposure of HAECs to moderate high glucose concentrations results in increased expression of E-selectin (2-fold), VCAM-1 (3-fold) and ICAM-1 (47%). At the same time, HAEC grown with 12 mM glucose expressed lesser E-selectin and, more ICAM-1 (for 64%) and VCAM-1 (41%) molecules. 16.5 mM glucose decreased expression of all investigated adhesion molecules. Addition of insulin was not changed expression of CAMs in a medium with 5.5 mM glucose. In conditions of elevated glucose concentration (12 mM), addition of insulin significantly dropped E-selectin (27%) and increased VCAM-1 (23%) expression. In conclusion, moderate elevated glucose concentration increased expression of cell adhesion molecules on HAEC. Insulin administration in the mild hyperglycaemia reduces an expression of the proinflammatory adhesion molecule E-selectin which could contribute in deceleration of macrovascular complications development in diabetic patients.

Key words: cell adhesion molecules, mild hyperglycaemia, insulin, human aortic endothelial cells

Introduction

Insulin is a powerful anabolic hormone in a wide use as an anti-diabetic drug for the treatment of diabetes mellitus type 1 and type 2¹⁻⁴. Results of studies show that the risk of developing of cardiovascular disease as well as risk of cardiovascular events, such as myocardial infarction and stroke, in the type 2 diabetic patients is being two to four times higher than in healthy

subjects⁵⁻⁸. A majority of studies, performed on human beings, has shown that hyperinsulinemia results in an increase of cardio-vascular disease in patient with type 2 diabetes^{9,10}. An association of hyperinsulinemia with increased coronary events in several epidemiological studies led to the hypothesis that insulin may be atherogenic¹¹⁻¹³. On the other hand, it has been shown that in-

sulin has a potent vasodilatory effect *in vivo* on the arterial and the venous system^{14,15}, but the mechanism of its action in the vascular wall is still unclear.

Specific molecular interactions of cell adhesion molecules (CAMs) are exceptionally important in a whole variety of processes such as embryogenesis, organogenesis and neural system development, vascular and epithelial homeostasis, haemostasis, immune response and inflammation^{16,17}. Expression of E- and P-selectin, vascular adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) on the surface of endothelial cells mediates the interaction between blood cells and endothelium. The loss of CAMs adhesive capability and their redundant expression may lead to leukocyte adhesion deficiency and atherosclerotic cardiovascular diseases^{18–22}.

Significant changes in the expression of soluble cell adhesion molecules (sE-selectin, sICAM-1 and sVCAM-1) are found in the serum of patients with type 2 diabetes^{23–25}. Although cell adhesion molecules expression on macrovascular endothelial cells may correlate with their soluble levels, this is not clearly established. Ryysy and Yki-Järvinen²⁶ have reported that decrease in soluble E-selectin is apparently dependent on improvement in glycaemia, which suggests that insulin effects could be indirect through better control of glucose.

As the cell surface expression of CAMs on macrovascular endothelial cells can only be studied *in vitro*, HAEC and HUVEC cell culture represents optimal model for such studies. Numbers of studies shown that human umbilical vein and human aortic endothelial cells, cultivated in elevated glucose concentration, increase CAMs expression^{27,28} which additionally rise if cells are stimulated with tumour necrosis factor- α (TNF- α)^{25,29,30}. Studies of Aljada et al. showed that insulin increases endothelial NOS (e-NOS) expression in HAEC, exerts an anti-inflammatory effect at the endothelial cell level by reducing the expression of intracellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and the pro-inflammatory transcription factor, nuclear factor- κ B^{31,32}.

Although several studies have shown that insulin has a potential to reduce inflammatory response, it remains unclear whether treatment by insulin could help in the reduction of incidence or progression of macrovascular diseases by diminishing expression of CAMs. Therefore, the aim of the present study was to investigate effects of elevated glucose concentration and insulin administration in condition of increased glucose on E-selectin, ICAM-1 and VCAM-1 expression on human aortic endothelial cells *in vitro*.

Materials and Methods

Cell culture

Human aortic endothelial cells – HAEC (Cascade BiologicsTM, San Diego, CA) were grown in Medium 200 (Cascade BiologicsTM) with low serum growth supple-

ment – LSGS (Cascade BiologicsTM), 7 μ mol/L homocysteine (DL-Homocysteine, Sigma Biochemicals, Dreischoffen, Germany), 0.1 ng/mL tumor necrosis factor- α (TNF- α ; Sigma, St. Louis, Missouri, USA) and glucose (Merck, Darmstadt, Germany) in a range of concentration (5.5, 8.0, 12.0 and 16.5 mmol/L). HAEC were cultivated at 37°C and 5% CO₂ and passaged once or twice per week. For experiment purpose, HAEC at passages 5–10 were used.

Drug treatment

Insulin (Actrapid Penfil 100 UI/mL, 3.5 mg/mL) was kindly supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). Used concentration of 1 mUI/mL is in the range of therapeutic plasma concentrations in diabetic patients. The influence of insulin on CAMs expression was analyzed in combination with each glucose concentration. Cells grown in corresponding (8.0, 12.0, 16.5 mmol/L) glucose concentrations and in physiological glucose concentration (5.5 mmol/L) were used as controls.

Measurement of adhesion molecules expression by flow-cytometry

After incubation with different concentration of glucose and insulin the HAECs were washed twice with PBS, incubated with fluorescent label-marked monoclonal antibodies (R-phycoerythrin, R-PE, conjugated mouse anti-human monoclonal antibody) anti E-selectin, anti-ICAM-1 and anti-VCAM-1 (B.D. Pharmigen, San Diego) for 20 min at 4°C. After washing with PBS, percent of positive cells (mean fluorescence intensity – MFI wasn't analyzed) were analyzed on a flow-cytometer (FACS Calibur, BD). A software system CELLQuest was used for data acquisition and analysis. Data for minimum of 5x10³ cells were collected and results expressed as a percentage of fluorescent cells for each marker.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Differences were considered to be statistically significant at $p < 0.05$. Results are expressed as the mean \pm SEM (standard error mean).

Results

Human aortic endothelial cells incubated in a medium with physiological concentration of glucose (5.5 mmol/L) showed detectable (around 18.12%) expression of E-selectin. The presence of moderate higher glucose concentration (8.0 mmol/L) enlarged E-selectin expression (compared to control, by 2-fold), but this did not reach statistical significance. Interestingly, incubation of HAECs in a medium with higher glucose concentration (12.0 and 16.5 mmol/L), expression of E-selectin decreased (compared to control, by 23% and by 88%, respectively). The expression of ICAM-1 on HAECs at 5.5 mmol/L glucose was detectable (around 39.64%). Higher concentrations of glucose (8.0 and 12.0 mmol/L) showed

mild changes in ICAM-1 expression (increased for 47% and for 64%, respectively), whereas incubation in 16.5 mmol/L glucose resulted in a substantial decrease (12%). As presented in Figure 1, 5.06% of HAECs expressed VCAM-1 after incubation in medium with 5.5 mmol/L glucose. Incubation of cells with higher glucose concentrations (8.0 mmol/L and 12.0 mmol/L) enlarged VCAM-1 expression (compared to control, by 3-fold and by 41%, respectively). In contrast, 16.5 mmol/L glucose resulted in decreased VCAM-1 expression (43%).

Moreover, we have investigated the effects of insulin on the CAM expression at HAECs incubated in medium with various concentrations of glucose. Insulin was added at 1 mIU/mL to mimic the concentrations used in diabetic patients. The data for CAM expression in the absence of insulin are used as a control. As shown in Figure 2, the addition of insulin was not changed CAMs expression on HAECs incubated in 5.5 mmol/L glucose. Interestingly, at moderate higher glucose levels, 8.0 mmol/L, the addition of insulin decreased (29%) E-selectin expression. Addition of insulin to the medium with mild hyperglycaemia (12.0 mmol/L) resulted in significantly ($p=0.037$) decreased of E-selectin expression (27%). At 16.5 mmol/L glucose, expression was moderate increased (9%) (Figure 2a). The effect of insulin on ICAM-1 expression were modest (Figure 2b). The addition of insulin resulted in a reduction in VCAM-1 expression on HAECs incubated at 8.0 mmol/L glucose (33%) and increased expression at 12.0 mmol/L and 16.5 mmol/L glucose (compared to control, by 23% and by 78%, respectively).

Discussion and Conclusion

In the present investigation we used an *in vitro* model to study effects of elevated glucose concentration and in-

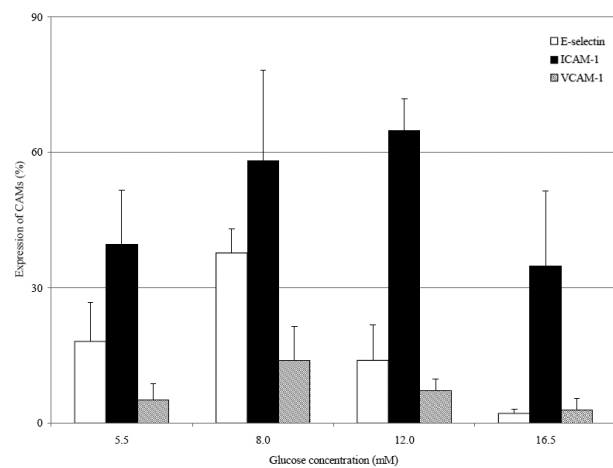


Fig. 1. Effect of high glucose concentrations on cell adhesion molecules expression on HAEC. Glucose concentration of 5.5 mmol/L was used as a control. After 4h incubation with various glucose concentrations (5.5, 8.0, 12.0, and 16.5 mmol/L, respectively) the expression of E-selectin, ICAM-1 and VCAM-1 was measured by flow-cytometry. Data represent the mean±SEM (standard error mean) of four independent experiments.

sulin administration on cell adhesion molecules (CAMs) expression on the surface of human aortic endothelial cells (HAEC). As shown in Figure 1, HAEC incubated in a medium with physiological concentration of glucose (5.5 mmol/L), express investigated adhesion molecules, E-selectin (18.12%), ICAM-1 (39.64%), and VCAM-1 (5.06%) in detectable amounts. Our results show similarity to the data reported by Kanda et al.²⁵ and Maurus et al.³³ although in our study the CAMs expression was relatively modest. The cause could be due short time of cells' exposition to elevated glucose (4 hours) and relatively low concentration of added TNF- α . Namely, we observed that without the addition of TNF- α and at 0.01 ng/mL of TNF- α in a physiological glucose concentration and in conditions of hyperglycaemia, exposed cells did not increase expression of adhesion molecules (data not shown). To arouse the CAMs expression we used the lowest effective concentration of TNF- α (0.1 ng/mL) in order

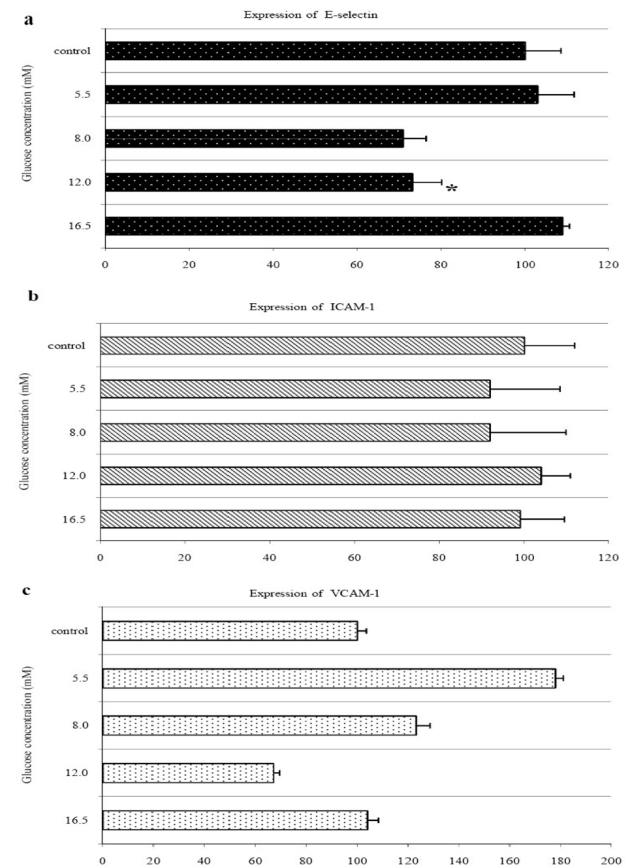


Fig. 2. Effect of high glucose concentrations and insulin (1 mIU/mL) administration on expression of adhesion molecules E-selectin (a), ICAM-1 (b) and VCAM-1 (c) on HAEC. Glucose concentrations of 5.5, 8.0, 12.0, and 16.5 mmol/L, respectively, are used as controls. After 4 h incubation with various glucose concentrations and insulin the expression of adhesion molecules was measured by flow-cytometry. Data represent the mean of three independent experiments. * $p=0.037$, for glucose 12.0 mmol/L vs. glucose 12.0 mmol/L with insulin (Student-N-K test was used to elevate the differences in the control vs. insulin group).

to exclude effects of cytokines' activity on CAMs expression.

The presence of moderately elevated glucose concentration (8.0 mmol/L) enlarged E-selectin expression, compared to control, by 2-fold, but this did not reach statistical significance (Figure 1). In comparison to control, the same glucose concentration caused mild changes in ICAM-1 expression (increased for 47%) and significantly enhanced VCAM-1 expression by 3-fold. Obtained results support the hypothesis that increased expression of CAMs is an early response of endothelial cells to moderate hyperglycaemia¹⁷ and it is possible that CAMs are the most sensitive indicator of early vascular dysfunction²⁶.

As shown in Figure 1, incubation of HAEC in medium with 12.0 mmol/L glucose, resulted in increased ICAM-1 (for 64%) and VCAM-1 (for 41%), whereas expression of E-selectin was slightly decreased (for 12%). The same effect was previously observed in HUVEC cultured in medium with similar glucose concentration²⁹. Glucose in concentration of 16.5 mmol/L diminished expression of E-selectin for 88%, and VCAM-1 for 43%, as already shown in our recent study²⁷. The main cause could be in cytotoxicity of glucose at this concentration. The cytotoxicity of glucose was already described in HUVEC after treatment with 22.0 mmol/L glucose²⁹. However, without performing *in vivo* experiments it is not clear whether the effect of high glucose concentration is direct or indirect.

Results of our study also showed that the induction of adhesion molecules can reflect direct effects of glucose on endothelial cells in culture. Observed induction of expression of all three types of adhesion molecules on HAEC *in vitro* after short-term exposure is in good correspondence to the results of previous studies which showed that hyperglycaemia increases circulating endothelial adhesion molecules in type 2 of diabetic patients^{24,34,35}. Moreover, increased plasma concentrations of these adhesion molecules were associated with insulin concentrations and insulin resistance³⁶.

Furthermore, we have investigated the effects of insulin on the CAMs expression at HAEC incubated in medium with various concentrations of glucose. Insulin was added at 1 mIU/mL to mimic the concentrations used in diabetic patients. The data for CAMs expression in the absence of insulin are used as a control. As shown in Figure 2, the addition of insulin did not change CAMs expression on HAEC incubated in 5.5 mmol/L glucose. This result indicates that insulin does not contribute to the development of inflammation in vascular wall and ultimately

to the development of atherogenesis through expression CAMs on the surface of endothelial cells.

The addition of insulin resulted in decreased CAMs expression at moderately higher glucose levels (8.0 mmol/L). Addition of insulin to the medium with mild hyperglycaemia (12.0 mmol/L) resulted in a significant ($p=0.037$) decrease of E-selectin expression (27%), while expression of ICAM-1 and VCAM-1 was slightly enhanced, for 4% and for 23%, respectively (Figure 2). At 16.5 mmol/L glucose, the effects of insulin on expression of E-selectin and ICAM-1 were modest (Figure 2a and b). The addition of insulin resulted in a reduction in VCAM-1 expression on HAECs incubated at 8.0 mmol/L glucose (33%) and increased expression at 12.0 mmol/L and 16.5 mmol/L glucose (compared to control, by 23% and by 78%, respectively).

Reduction of expression of E-selectin on HAEC *in vitro* after short-term exposure to glucose, which was found in this study, could contribute to elucidation of the results obtained in clinical studies which showed that insulin administration leads to a sustained decrease in sE-selectin concentration in patients with type 2 diabetes²⁶. In clinical settings it is not clear yet whether the effect of insulin is direct or indirect via the reduction of glycaemia. With the help of *in vitro* model used in this study, it is possible to eliminate indirect effects of insulin. Indeed, we have shown that insulin has direct effect on the expression of proinflammatory adhesion molecule E-selectin. This finding supports the use of sE-selectin as a marker of endothelial activation, induced by hyperglycaemia²⁶.

We demonstrate that the short-term effect of insulin administration in the mild hyperglycaemia condition reduces the expression of the proinflammatory adhesion molecule E-selectin, which could contribute to the slowing down of the development of macrovascular complications in diabetic patients. However, we cannot exclude a possibility that a long-term administration of insulin may result in significant changes in CAMs expression of endothelial cells. Furthermore, some of the insulin's *in vivo* effects could be indirect through better regulation of glycaemia.

Acknowledgements

This study was supported by the grant of Croatian Ministry of Science No 219-0982914-2176. Authors gratefully acknowledge Blaženka Dobrošević, BSc, for her help in samples analysis by flow-cytometry.

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PRIMJENA INZULINA U BLAGOJ HIPERGLIKEMIJI MIJENJA EKSPRESIJU UPALNIH ADHEZIJSKIH MOLEKULA NA HUMANIM AORTALNIM ENDOTELNIM STANICAMA

SAŽETAK

Pojačana ekspresija staničnih adhezijskih molekula (engl. Cell Adhesion Molecules, CAM) na površini endotelnih stanica je jedan od prvih koraka u endotelnoj disfunkciji uzrokovanj povišenom koncentracijom glukoze u dijabetičara. Ispitivan je utjecaj primjene inzulina u uvjetima povišene koncentracije glukoze na ekspresiju E-selektina, adhezijske molekule žilne stijenke-1 (engl. Vascular Adhesion Molecule-1, VCAM-1) te međestanične adhezijske molekule-1 (engl. Intercellular Adhesion Molecule-1, ICAM-1) na humanim aortalnim endotelnim stanicama (engl. Human Aortic Endothelial Cells, HAEC). Stanice su kultivirane 4 h u mediju s dodatkom homocisteina (7 μM) i različitim koncentracijama glukoze (5,5; 8,0; 12,0 i 16,5 mM) s i bez dodatka inzulina (1 mIU/mL). Ekspresija CAMs analizirana je protočnom citometrijom uz primjenu monoklonskih protutijela. Kontrole su bile CAM ekspimirane u mediju s odgovarajućom koncentracijom glukoze. Dobiveni rezultati ukazuju da kratko vrijeme izlaganja HAEC blago povišenoj koncentraciji glukoze dovodi do povećanja ekspresije E-selektina (2 puta), VCAM-1 (3 puta) i ICAM-1 (47%). Istovremeno, HAEC izložene 12 mM glukoze smanjuju ekspresiju E-selektina, a povećavaju ICAM-1 (za 64%) i VCAM-1 (41%). 16,5 mM glukoze smanjuje ekspresiju svih ispitivanih adhezijskih molekula. Dodatak inzulina nije mijenjao ekspresiju CAM u mediju s 5,5 mM glukoze. U uvjetima povišene koncentracije glukoze (12 mM), dodatak inzulina značajno smanjuje E-selektin (27%) i povećava ekspresiju VCAM-1 (23%). Može se zaključiti, blago povišena koncentracija glukoze povećava ekspresiju staničnih adhezijskih molekula na HAEC. Dodatak inzulina u umjerenu hiperglikemiju reducira ekspresiju proupalne adhezijske molekule E-selektin što možda doprinosi usporavanju napredovanja makrovaskularnih komplikacija u dijabetičara.