

## Effects of Hyperglycemia and Metformin on Expression of Adhesion Molecules on Human Aortic Endothelial Cells

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Expression of adhesion molecules on the endothelial cell surface as a response to elevated glucose concentration is considered as a main event in the development of atherosclerosis. The influence of high glucose concentration and metformin, a wide used anti-diabetic drug, on the expression of E-selectin, vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on human aortic endothelial cells (HAECs) was investigated. HAECs were cultured 4 h in a medium with 5.5, 8.0, 12.0, and 16.5 mmol dm<sup>-3</sup> glucose with or without metformin addition. The expression of cell adhesion molecules (CAM) was measured by flow-cytometry. Compared to CAM expression in the medium with 5.5 mmol dm<sup>-3</sup> glucose, glucose concentration of 12.0 mmol dm<sup>-3</sup> increased expression of E-selectin (62 %), VCAM-1 (four fold) and ICAM-1 (81 %). Metformin administration in the medium with 12.0 mmol dm<sup>-3</sup> glucose additionally enhanced E-selectin and VCAM-1 expression compared to effects of corresponding glucose concentration. ICAM-1 expression was only significantly increased in the medium with metformin and 8.0 mmol dm<sup>-3</sup> glucose. In conclusion, metformin in condition with elevated glucose concentration additionally increased expression of CAM on HAECs which could contribute to development of macrovascular complications in diabetic patients.

### Keywords

cell adhesion molecules  
endothelial cells  
atherosclerosis  
diabetes mellitus  
metformin

## INTRODUCTION

Cell adhesion molecules (CAM) are proteins located on the surface of endothelial and smooth muscle cells, leukocyte, platelets and intercellular matrix which mediate cell-cell and cell-matrix interactions. Their specific molecular interactions play a crucial role in a whole variety of processes such as hemostasis, vascular and epithelial homeostasis, immune response, inflammation, embryogenesis, organogenesis and neural system development.<sup>1,2</sup>

The loss of their adhesive capability (leukocyte adhesion deficiency, LAD) as well as their overexpression (atherosclerosis) may lead to different diseases.<sup>3,4</sup> 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, as an answer to the pathophysiological stimulants, mediates the interaction between blood cells and endothelium, which is considered as a main event in the development of the atherosclerosis.<sup>5,6</sup>

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Endothelial dysfunction plays a key role in the pathogenesis of atherosclerotic cardiovascular diseases.<sup>7,8</sup> Diabetes is also associated with accelerated atherosclerosis, and type 2 diabetic patients in particular have increased risk of cardiovascular events such as myocardial infarction and stroke.<sup>9,10</sup> Cardiovascular disease is recognized as the major determining factor of morbidity and mortality in type 2 diabetic patients, and the risk of its developing in these subjects being two to four times higher than in non-diabetic subjects.<sup>11</sup> In serum of patients with type 2 diabetes are significant elevation of soluble adhesion molecules (sE-selectin, sICAM-1 and sVCAM-1).<sup>12–14</sup>

Endothelial cells cultivated in medium with elevated glucose concentration increased CAM expression. Exposure of human umbilical vein endothelial cells *in vitro* to elevated glucose concentration induces expression of ICAM-1<sup>15</sup> also E-selectin, ICAM-1, and VCAM-1. When cells were stimulated with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) additionally CAM expression was observed.<sup>16</sup> Proinflammatory cytokine TNF- $\alpha$  stimulates VCAM-1 expression in HAEC at the concentration of 0.1 ng mL<sup>-1</sup>. Upon stimulation with TNF- $\alpha$ , ICAM-1 and VCAM-1 expression was up-regulated in human aortic (HAEC) and venous (*vena cava*) endothelial cells in a dose dependent manner, and in both, CAM levels peaking after 4 h.<sup>17,18</sup> It should be noted that untreated HAECs constitutively expressed ICAM-1 on their surface but neither expressed E- or P-selectin, VCAM-1 nor shed soluble adhesion molecules.<sup>19</sup> HAEC exposed to low shear flow induced activation of the transcriptional regulator nuclear factor- $\kappa$ B (NF- $\kappa$ B), expression of VCAM-1, and endothelial cell monocyte adhesion.<sup>20</sup> An increased expression of endothelial cell's adhesion molecules in response to glucose has also been correlated with an alteration of nitric oxide (NO) synthesis<sup>21</sup> and endothelium-induced vasodilatation.<sup>22</sup>

Metformin is an anti-diabetic drug in a wide use for the treatment of diabetes mellitus type 2, especially when this disease accompanies obesity and insulin resistance. Its mechanism of action is based on the reduction of hepatic glucose production and with the increase of liver and peripheral tissues sensitivity to insulin.<sup>23,24</sup> Furthermore, administration of this drug has been associated with decreased all-cause and cardiovascular risk of mortality.<sup>25,26</sup> Such improvement in the cardiovascular outcomes with metformin administration seems not to be related to glycemic control only but also to specific vasculoprotective effects of this drug.<sup>24</sup> Besides its glucose-lowering effects, metformin appears to have exerted of beneficial effects on several cardiovascular risk factors including dislipidemia, activities of plasminogen activator inhibitor 1, C-reactive protein, fibrinogen, and insulin resistance and hyperinsulinemia.<sup>27,28</sup> Quote metformin has also been reported to decreased oxidative stress.<sup>29</sup> A mechanism of its action in the vascular wall is still un-

clear. It has been reported that metformin inhibited monocyte adhesion on human endothelial cells induced by advanced glycation end-products (AGE), expression of endothelial cell adhesion molecules and monocyte differentiation into macrophages and foam cell formation.<sup>30</sup>

Although many studies have shown a correlation between hyperglycemia and cardiovascular disease, it remains unclear whether glycemic control by antihyperglycemic drugs could help in reduction of incidence or progression of macrovascular diseases in patients with type 2 diabetes. Effects of metformin on CAM were studied extensively, but this was based on the measurements of the soluble CAM in patients serum. Our knowledge about the effects of metformin on CAM *in vitro* is still very limited. For these reasons, the aim of our study was to investigate effects of elevated glucose concentration and treatment of metformin on E-selectin, VCAM-1, and ICAM-1 expression on human aortic endothelial cells *in vitro*.

## EXPERIMENTAL

### *Cell Culture*

Human aortic endothelial cells (HAECs) were obtained from Cascade Biologics<sup>TM</sup> (San Diego, CA). They were grown to sub-confluence in Medium 200 (Cascade Biologics<sup>TM</sup>) with low serum growth supplement (LSGS) (Cascade Biologics<sup>TM</sup>). The cultures were maintained at 37 °C and 5 % CO<sub>2</sub> and passaged once or twice per week. For all experiments, endothelial cell cultures were used at passages 5–10.

For experimental purpose HAECs were cultured 4 h in a Medium 200 with LSGS, 7  $\mu$ mol L<sup>-1</sup> homocysteine (DL-Homocysteine, 2-Amino-4-mercaptobutyric acid; Sigma Biochemicals, Dreisenhoffen, Germany), 0.1 ng mL<sup>-1</sup> tumor necrosis factor-alpha (TNF- $\alpha$ ; Sigma, St. Louis, Missouri, USA) and glucose (*Glucosum monohydricum*, D(+); Merck, Darmstadt, Germany) in a range of concentration (5.5, 8.0, 12.0 and 16.5 mmol dm<sup>-3</sup>). HAECs cultured in the medium with a physiological glucose concentration (5.5 mmol dm<sup>-3</sup>) were used as control for each experiment, and for experiments with metformin, as control were used cells grown in the medium with the corresponding (8.0, 12.0, 16.5 mmol dm<sup>-3</sup>) glucose concentration.

### *Drug Treatment*

Metformin (1,1-dimethylbiguanide hydrochloride) was kindly supplied by Sigma Biochemicals (Dreisenhofen, Germany). The drug was dissolved in a phosphate buffered saline (PBS) and stored at 4 °C as a stock solution (10 mg mL<sup>-1</sup>). In our experiments metformin concentration of 10  $\mu$ g mL<sup>-1</sup> was used, which is in the range of therapeutic plasma concentrations in diabetic patients. The influence of metformin on CAMs expression was analysed in combination with each glucose concentration.

TABLE I. Effect of glucose concentration on expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 in HAECs<sup>(a)</sup>

[Glucose] / mmol dm <sup>-3</sup> :	Expression of adhesion molecules / %			
	5.5	8.0	12.0	16.5
Adhesion molecule				
E-selectin	0.24 ± 0.10	0.29 ± 0.16	0.39 ± 0.28	0.18 ± 0.09
VCAM-1	0.23 ± 0.15	0.08 ± 0.03	0.94 ± 0.82	0.15 ± 0.06
ICAM-1	17.19 ± 4.40	12.57 ± 3.05	31.1 ± 11.29	7.68 ± 0.34

<sup>(a)</sup>After 4-hour incubation with various glucose concentrations the expression of E-selectin, VCAM-1 and ICAM-1 was measured by flow-cytometry. Data represent the mean ± standard error mean of four independent experiments.

*Measurement of Adhesion Molecules Expression by Flow-cytometry*

After incubation with different concentrations of glucose and metformin 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-ICAM-1, anti-VCAM-1 and anti E-selectin (B.D. Pharmigen, San Diego) for 20 min at 4 °C. After washing with PBS, positive cells (mean fluorescence intensity, MFI, wasn't analysed) were analysed on a flow-cytometer (FACSCalibur, BD). A software system CELLQuest was used for data acquisition and analysis. Data for minimum of 5 x 10<sup>3</sup> cells were collected and results expressed as the fraction 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 significant at *p*<0.05. Results are expressed as the mean ± standard error mean.

**RESULTS**

Effects of glucose on expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 in HAECs are shown in Table I. Human aortic endothelial cells incubated in the medium with physiological concentration of glucose (5.5 mmol dm<sup>-3</sup>) showed detectable but slight (around 0.2 %) expression of E-selectin. The presence of higher glucose amount (8.0 mmol dm<sup>-3</sup> and 12.0 mmol dm<sup>-3</sup>) enlarged E-selectin expression (compared to the control by 20.8 % and 62.5 %, respectively), but this did not reach statistical significance. Interestingly, incubation of HAECs in the medium with 16.5 mmol dm<sup>-3</sup> glucose decreased expression of E-selectin by 25 %. The expression of VCAM-1 on HAECs at 5.5 mmol dm<sup>-3</sup> glucose was modest (around 0.2 %). Notably, higher concentrations of glucose (8.0 and 16.5 mmol dm<sup>-3</sup>) showed complex effects with moderate changes in VCAM-1 expression (decreased for 65 % and 34.8 %, respectively), whereas incubation in the medium with 12.0 mmol dm<sup>-3</sup>

glucose resulted in a substantial increase (four fold). As presented in Table I, 17 % of HAECs expressed ICAM-1 after incubation in the medium with 5.5 mmol dm<sup>-3</sup> glucose. Incubation of cells with 8.0 mmol dm<sup>-3</sup> glucose showed modest effects on ICAM-1 expression. However, ICAM-1 expression was increased in the medium with 12.0 mmol dm<sup>-3</sup> glucose (for 81 %), but was decreased with 16.5 mmol dm<sup>-3</sup> glucose (for 2.2 fold) in respect to 5.5 mmol dm<sup>-3</sup> glucose. Although all three CAM, analysed in this study, showed increasing trend of expression on HAECs in the presence of 12.0 mmol dm<sup>-3</sup> glucose and this was not statistically significant probably due to the relatively high variability of CAM expression in our cell culture conditions.

Furthermore, we have investigated the effects of metformin on the CAM expression at HAECs incubated in media with various concentrations of glucose. To mimic the concentration used in diabetic patients, 10 µg mL<sup>-1</sup> of metformin was added. The data for CAM expression in the absence of metformin are included in Figure 1 in order to illustrate possible interaction between glucose and metformin effects. As shown in Figure 1a, the addition of metformin has only gentle effects on E-selectin expression on HAECs incubated in the medium with 5.5 mmol dm<sup>-3</sup> glucose. Interestingly, at higher glucose concentrations, 8.0 mmol dm<sup>-3</sup> and 12.0 mmol dm<sup>-3</sup>, the addition of metformin increased E-selectin expression (5 fold and 2 fold, respectively), while at 16.5 mmol dm<sup>-3</sup> glucose, the expression was diminished. The effect of metformin on VCAM-1 expression was modest (Figure 1b) and followed the pattern observed with the glucose effects: an ascending trend up to the presence of 12.0 mmol dm<sup>-3</sup> glucose, and decrease at 16.5 mmol dm<sup>-3</sup> glucose. The addition of metformin resulted in a significant reduction (*p* = 0.017) in ICAM-1 expression on HAECs incubated at 5.5 mmol dm<sup>-3</sup> glucose (Figure 1c). In contrast, the addition of metformin to the medium with 8.0 mmol dm<sup>-3</sup> glucose resulted in significantly (*p* = 0.02) augmentation of ICAM-1 expression (Figure 1c). The effects of metformin on ICAM-1 expression in the presence of 12.0 and 16.5 mmol dm<sup>-3</sup> glucose were minor.

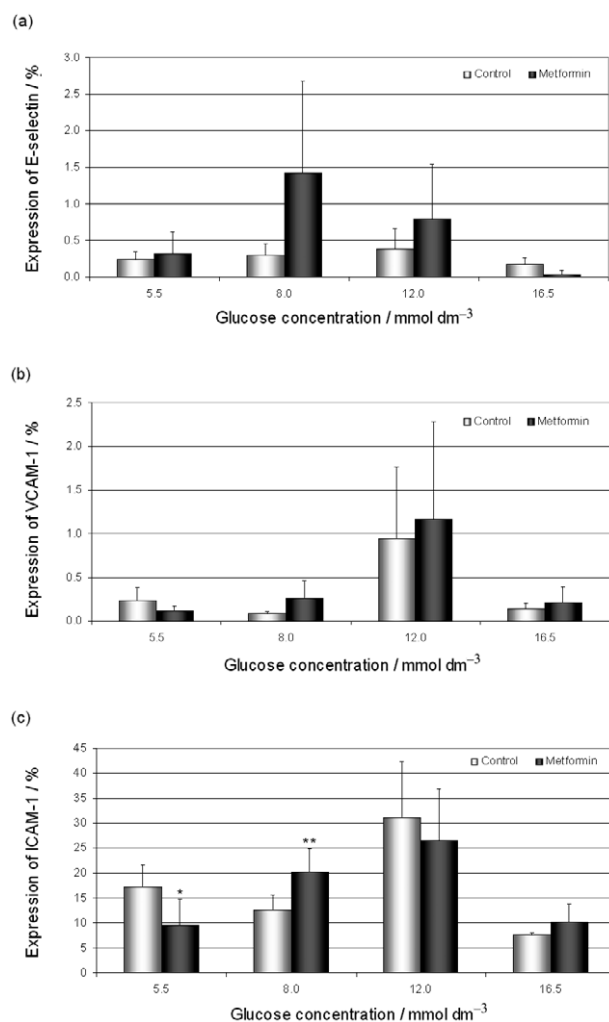


Figure 1. Effect of glucose and metformin ( $10 \mu\text{g mL}^{-1}$ ) on expression of adhesion molecules E-selectin (a), VCAM-1 (b) and ICAM-1 (c) in HAECs. After 4-hour incubation the expression of adhesion molecules was measured by flow-cytometry. Data represent the mean of three independent experiments. (\*)  $p = 0.017$  for  $5.5 \text{ mmol dm}^{-3}$  glucose vs.  $5.5 \text{ mmol dm}^{-3}$  glucose with metformin, (\*\*)  $p = 0.02$  for  $8.0 \text{ mmol dm}^{-3}$  glucose vs.  $8.0 \text{ mmol dm}^{-3}$  glucose with metformin.

## DISCUSSION

The induction of adhesion molecules on the surface of endothelial cells is one of the first steps in a high glucose-mediated endothelial dysfunction in diabetic patients. In the present study it was demonstrated that short-term exposure of HAECs to high glucose concentrations results in the increased expression of cells surface molecules E-selectin, VCAM-1 and ICAM-1. Such stimulative effect of high glucose concentration on the expression of adhesion molecules has been previously reported. Thus, human umbilical vein endothelial cells exposed for short periods of time to high glucose concentrations increased expression of ICAM-1,<sup>15</sup> ICAM-1, P-selectin and E-se-

lectin<sup>31,32</sup> and E-selectin, VCAM-1 and ICAM-1.<sup>16</sup> Aortic endothelial cells adapted by long-term cultivation at high glucose concentrations displayed expression of VCAM-1 also.<sup>33</sup> However, the results concerning the rate of induction of various types of adhesion molecules have not been consistent and depend on the experimental system used. Induction of expression of all three types of adhesion molecules on HAECs *in vitro* after short-term exposure found in this study, is in good correspondence to the results of previous studies which showed that hyperglycemia increases circulating endothelial adhesion molecules in type 2 of diabetic patients.<sup>13,14,34</sup> Moreover, increased plasma concentrations of these adhesion molecules were associated with insulin concentrations and insulin resistance.<sup>35</sup>

In our study, exposure of endothelial cells to elevated glucose concentration resulted with increased CAM expression. It is observed that 17 % of glucose non stimulated HAECs expressed ICAM-1, while the expression of E-selectin and VCAM-1 was low. Our results showed similarity to the results found by Kanda *et al.* and Maurus *et al.*<sup>18,19</sup> Short-term exposure of endothelial cells to  $8.0 \text{ mmol dm}^{-3}$  glucose increased expression of E-selectin. These data support the hypothesis that increased expression of E-selectin is an early response of endothelial cells to hyperglycemia<sup>2</sup> and it is possible that E-selectin is the most sensitive indicator of early vascular dysfunction.<sup>36</sup> Incubation of HAEC in the medium with  $12.0 \text{ mmol dm}^{-3}$  glucose, showed increase in E-selectin, VCAM-1 and ICAM-1 expression. The same effect was previously observed in HUVECs grown in medium with similar glucose concentration.<sup>16</sup>

Induction of adhesion molecules in HAECs after the treatment with  $16.5 \text{ mmol dm}^{-3}$  glucose was not pronounced as with  $12.0 \text{ mmol dm}^{-3}$  glucose. The main cause could be the cytotoxicity of glucose at that concentration. The cytotoxicity of glucose was already described in HUVECs after treatment with  $22.0 \text{ mmol dm}^{-3}$  glucose.<sup>16</sup> It is well known that high glucose concentration can influence various physiological processes in many cell type.<sup>37</sup> However, from the experiments *in vivo* it is not clear whether the effect of high glucose concentration is direct or indirect. Results of our study showed that the induction of adhesion molecules can reflect direct effects of glucose on endothelial cells in culture. It should be mentioned that measured increases of CAMs expression were modest, but they follow the increased trend. The cause could be relatively low concentration of added TNF- $\alpha$ . This concentration was used to exclude effects of cytokine's activity on CAM expression,<sup>18</sup> which would make it difficult to interpret results. Moreover, exposure of cells to high glucose concentrations for a short time (4 h) can also be a reason for low number of positive cells.

There are several mechanisms by which high glucose concentration can participate in endothelial dysfunc-

tion.<sup>38–40</sup> Some studies described that high glucose amount can activate a signaling pathway mediated by protein kinase C. Their activation results in increased transcription of various genes including those for adhesion molecules.<sup>38–41</sup> Another mechanism, which is important mediator of endothelial dysfunction, involves AGE adducts induced by a long-term exposure of endothelial cells to high glucose concentration.<sup>33</sup> AGE are associated with and may be a causal factor in development of vascular complications in diabetes.<sup>42,43</sup> These products may accelerate vasculopathy in diabetes by triggering monocyte adhesion to the endothelium.<sup>44</sup>

The majority of studies have shown that administration of metformin results in a decrease of cardio-vascular disease in type 2 diabetes.<sup>26</sup> However, the mechanisms for this effect remain unknown. Metformin-mediated decrease in CAM expression has been implicated as a possible mechanism for such effects.<sup>27</sup> However, this is based on studies that measured levels of soluble CAM in patient's serum rather than their level of expression. Although CAM expression on macrovascular endothelial cells may correlate with their soluble levels, this is not clearly established. Rytsy *et al.*<sup>36</sup> have reported that decrease in soluble E-selectin expression is apparently dependent on improvement in glycemia. This suggests that metformin effects could be indirect through better control of glucose. In contrast, others have shown that metformin effects on soluble VCAM-1 are direct.<sup>27</sup> As the cell surface expression of CAM on macrovascular endothelial cells can only be studied *in vitro*, HAEC culture represents optimal model for such studies. Furthermore, *in vitro* model can eliminate possible indirect effects of metformin. However, short-term experiments *in vitro* have their limitations and the effects of metformin when used long-term *in vivo* could differ from those observed in our study.

In our experimental conditions, addition of therapeutic concentrations of metformin (10 µg mL<sup>-1</sup>) significantly increased expression of ICAM-1 at 8.0 mmol dm<sup>-3</sup> glucose, while at higher glucose concentration metformin decreased ICAM expression. Our results are in good agreement with previous report which demonstrated that metformin does not inhibit high glucose-induced neutrophil adhesion to endothelial cells.<sup>31</sup> Expression of CAM could contribute to the development of inflammation in vascular wall and finally development of atherogenesis. In the study performed with AGE, therapeutic concentrations of metformin interfere with several biological processes implicated in atherogenesis, including decreased expression of E-selectin, VCAM-1 and ICAM-1 on the surface of endothelial cells, monocyte adhesion to endothelial cells, monocyte differentiation into macrophages, and foam cell formation.<sup>30</sup> Discrepancies between these results may be related to differences in the

signal transduction pathways involved in glucose *vs.* AGE-induced leukocyte adhesion.

Results of this study showed that high glucose concentration leads to the activation of endothelial cells which is associated with increased expression of E-selectin, VCAM-1 and ICAM-1 in HAEC. In conclusion, the short-term effects of metformin on CAM expression on HAEC in culture are modest and thus unlikely to provide the mechanisms for metformin's beneficial effects on cardiovascular complications in diabetes. However, we can not exclude a possibility that a long-term administration of metformin may result in significant changes in CAM expression of endothelial cells. Furthermore, some of the metformin's *in vivo* effects could be indirect through better regulation of glycemia.

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## SAŽETAK

### Utjecaj hiperglikemije i metformina na ekspresiju staničnih adhezijskih molekula na humanim aortalnim endotelnim stanicama

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Ekspresija staničnih adhezijskih molekula (engl. *Cell Adhesion Molecules*, CAM) na površini endotelnih stanica, kao odgovor na povišene koncentracije glukoze, smatra se središnjim događajem u nastanku ateroskleroze. Ispitivan je utjecaj povišene koncentracije glukoze i široko primjenjivanoga antidijabetičkog lijeka metformina na ekspresiju E-selektina, adhezijske molekule žilne stijenke-1 (engl. *Vascular Adhesion Molecule*, VCAM-1) te međustanične adhezijske molekule-1 (engl. *Intercellular Adhesion Molecule-1*, ICAM-1) na humanim aortalnim endotelnim stanicama (engl. *Human Aortic Endothelial Cells*, HAEC). HAEC su kultivirane 4 h u mediju s 5,5, 8, 12 i 16,5 mmol dm<sup>-3</sup> glukoze s dodatkom metformina i bez njega. Koncentracija glukoze od 12 mmol dm<sup>-3</sup> povećala je ekspresiju E-selektina (62 %), VCAM-1 (4 puta) i ICAM-1 (81 %) na HAEC. Dodatak metformina u medij s koncentracijom glukoze od 12 mmol dm<sup>-3</sup> dodatno je povećao ekspresiju E-selektina i VCAM-1. Ekspresija ICAM-1 se značajno povećala u mediju s 8 mmol dm<sup>-3</sup> glukoze i metforminom. Može se zaključiti da primjena metformina u uvjetima povišenih koncentracija glukoze dodatno povećava ekspresiju adhezijskih molekula što bi moglo doprinijeti razvoju makrovaskularnih komplikacija u bolesnika oboljelih od šećerne bolesti.