CROATICA CHEMICA ACTA
CCACAA, ISSN 0011-1643, e-ISSN 1334-417X
Croat. Chem. Acta **85** (2) (2012) 185–191.
http://dx.doi.org/10.5562/cca1810

Original Scientific Article

An Overexpression of Icam-1 in Mild Hyperhomocysteinemia and Hyperglycemia − A Study of Antidiabetics Administration Effect†

Tatjana Bačun,[a,b,*] Tatjana Belovari,[a] Aleksandar Včev,[a,b] Ivan Mihaljević,[a,c] Toni Hanich,[a] Vladimir Fijačko,[b] and Ljubica Glavaš-Obrovac[a,b]

[a]*J. J. Strossmayer University of Osijek, School of Medicine, University Hospital Centre Osijek, Huttlerova 4, HR-31000 Osijek, Croatia*
[b]*J. J. Strossmayer University of Osijek, School of Medicine, Internal Medicine Clinic, Huttlerova 4, HR-31000 Osijek, Croatia*
[c]*J. J. Strossmayer University of Osijek, School of Medicine, Clinical Institute of Nuclear Medicine and Radiation Protection, Huttlerova 4, HR-31000 Osijek, Croatia*

RECEIVED DECEMBER 9, 2010; REVISED DECEMBER 9, 2011; ACCEPTED JANUARY 26, 2012

Abstract. Elevated plasma homocysteine is connected to atherosclerosis and increased cerebrovascular and ischemic heart disease especially in the patients with type 2 diabetes. Effects of metformin (10 μg mL^{-1}), insulin (1 mUI mL^{-1}), and their combination administration in condition of mild hyperhomocysteinemia (30 μmol dm^{-3}) and hyperglycemia (12 mmol dm^{-3}) on human aortic endothelial cells (HAEC) was investigated. HAEC were cultured 4 h in a medium with homocysteine (7 and 30 μmol dm^{-3}) and glucose (5.5 and 12.0 mmol dm^{-3}). E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) expressions were analysed by flow-cytometry. Controls were CAMs expression on HAEC in medium with physiological concentration of homocysteine and glucose. HAEC, incubated additionally with 30 μmol dm^{-3} homocysteine, increased expression of ICAM-1 for 65 % ($p = 0.07$) and decreased of E-selectin for 30 % ($p = 0.07$) and VCAM-1 for 13 % (0.06). In condition of mild hyperhomocysteinemia and hyperglycemia there was a statistically significant increase of ICAM-1 expression (for 22 %, $p = 0.04$), while administration of metformin and insulin did not statistically significantly influence adhesion molecules expression. Observed significant overexpression of ICAM-1, leads us to conclude that combination of mild hyperhomocisteinemia and hyperglycemia could have significant influence on development of inflammation and atherosclerosis in diabetic patients.(doi: 10.5562/cca1810)

Keywords: Cell adhesion molecules, human aortic endothelial cells, mild hyperhomocysteinemia, mild hyperglycemia, metformin, insulin

INTRODUCTION

Homocysteine (Hcy) is sulfhydryl amino acid metabolite of dietary methionine. Elevated plasma Hcy is one of independent cardiovascular risk factors. It is connected to accelerated atherosclerosis and increased cerebrovascular and ischemic heart disease.[1] Further, more often Hcy levels are elevated modestly in the general population. These are strong predictors of existent and future development of vascular pathologies.[2]

Magnified plasma level of Hcy seems to contribute to cardiovascular disease, in part, by inducing endothelial cell (EC) dysfunction. *In vivo*, moderately elevated Hcy causes EC damage and exacerbates hypertension-related atherosclerosis,[3] and also impairs flow-mediated arterial dilation.[4] Additionally, elevated plasma Hcy level is an independent predictor of coronary heart disease events in patients with type 2 diabetes mellitus.[5] *In vitro*, Hcy increases leukocyte adhesion to cultured endothelial cells,[6] and upregulates vascular cell adhesion molecule-1 expression in cultured HAEC and enhances monocyte adhesion.[7]

One of the key initial events in the development of atherosclerosis is the adhesion of monocytes to endothelial cells, with subsequent transmigration into the vascular intimae. Leukocyte and vascular cell adhesion molecules (CAMs) such as selectins, integrins, E-selectin, vascular adhesion molecule-1 (VCAM-1), and intercel-

† Presented at the *10th Congress of the Croatian Society of Biochemistry and Molecular Biology* held in Opatija, Croatia, September 15–18, 2010.
* Author to whom correspondence should be addressed. (E-mail: *tatjana.bacun@os.t-com.hr*)

lular adhesion molecule-1 (ICAM-1) play critical roles in the adhesion of monocytes to endothelial cells. The expression of E-selectin, VCAM-1 and ICAM-1 is relatively low in normal vascular cells and it is upregulated in response to various stimuli.[8,9]

Metformin (N^1,N^1 –dimethylbiguanide) is widely used for the treatment of type 2 diabetes mellitus, especially if this disease is accompanied by obesity and insulin resistance.[10–13] However, its administration is related to decrease of all-cause and cardiovascular risk of mortality.[14,15] Such improvement in cardiovascular outcomes of metformin administration appears not to be connected only to glycemic control, but also to specific vasculoprotective effects of this medicine.[14,16,17] In addition to its glucose-lowering effects, metformin seems to exert beneficial effects on several cardiovascular risk factors. These risk factors include dyslipidemia, activities of plasminogen activator inhibitor 1, C-reactive protein, fibrinogen, and insulin resistance and hyperinsulinemia.[10,13,18–20] A mechanism of its action in the vascular wall is still unclear. It has been reported that metformin inhibits monocyte adhesion on human endothelial cells induced by advanced glycation endproducts (AGE), expression of endothelial cell adhesion molecules and monocyte differentiation into macrophages and foam cell formation.[21]

Insulin is a powerful anabolic hormone widely used as an anti-diabetic drug for treatment of type 1 and type 2 diabetes mellitus.[22] An association of hyper-insulinemia with increased coronary events in several epidemiological studies has led to the hypothesis that insulin may be atherogenic.[23,24] On the other hand, studies of Aljada *et al.* have shown 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 intercellular adhesion molecule–1, monocyte chemoattractant protein–1 (MCP-1), and the pro-inflammatory transcription factor, nuclear factor-κB.[25,26]

Although many studies have shown a correlation between hyperhomocysteinemia, hyperglycemia and cardiovascular disease, it remains unclear whether drug controlled hyperhomocysteinemia and hyperglycemia could reduce incidence or progression of macrovascular diseases by diminishing expression of CAMs. Therefore, the aim of this study was to investigate effects of elevated homocysteine and glucose concentration, as well as metformin and insulin treatment on the E-selectin, ICAM-1, and VCAM-1 expression on human aortic endothelial cells *in vitro*.

EXPERIMENTAL

Cell Culture

Human aortic endothelial cells – HAEC (Cascade Biologics™ , San Diego, CA) were grown 4 h in Medium 200 (Cascade Biologics™) with low serum growth supplement-LSGS (Cascade Biologics™), 0.1 ng mL^{-1} tumor necrosis factor-alpha (TNF-α; Sigma, St. Louis, Missouri, USA), homocysteine (DL-Homocysteine, Sigma Biochemicals, Dreisenhoffen, Germany) in range of concentration (7 and 30 μmol dm^{-3}), and glucose (Merck, Darmstadt, Germany) in a range of concentration (5.5 and 12.0 mmol dm^{-3}). HAEC were cultivated at 37 °C and 5 % CO_2 and passaged once or twice per week. For experiment purpose, HAEC at passages 5−10 were used.

Drug Treatment

Metformin (1, 1-dimethylbiguanide hydrochloride) was 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^{-1}). HAEC cells were treated 4 hour with metformin at concentration of 10 μg mL^{-1} representing relevant therapeutic plasma concentration in diabetic patients.

Insulin (Actrapid Penfil 100 UI/mL, 3.5 mg mL^{-1}) was kindly supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). Used concentration of 1 mUI mL^{-1} is in the range of therapeutic plasma concentrations in diabetic patients.

The influence of metformin and insulin and their combinations on CAMs expression was analyzed in conditions of mild hyperglycemia (12 mmol dm^{-3}) and mild hyperhomocysteinemia (30 μmol dm^{-3}). Cells grown in corresponding glucose and homocysteine concentrations and in physiological glucose and homocysteine concentration (5.5 mmol dm^{-3} and 7 μmol dm^{-3}, respectively) were used as controls.

Measurement of Adhesion Molecules Expression by Flow-cytometry

HAECs were incubated with appropriate experimental compounds for 4 hours, washed twice with PBS, and 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. Percentage of positive cells were analyzed on a flow-cytometer (FACSCalibur, BD), after washing with PBS. A software system CELLQuest was used for data acquisition and analysis. Minimum of $5x10^3$ cells were analyzed and results were 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 $p < 0.05$ when applied Kruskal-Wallis or Mann-Whitney test. Results are expressed as the mean ± SEM (standard error mean).

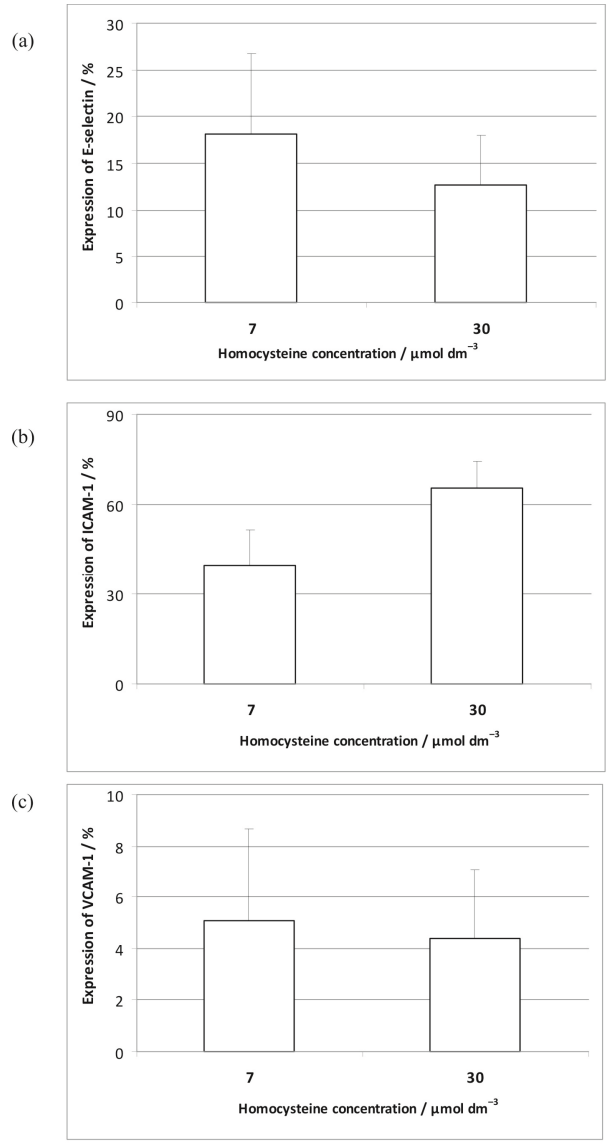

Figure 1. The effect of mild hyperhomocysteinemia on the expression of adhesion molecules: E-selectin (a), ICAM-1 (b) and VCAM-1 (c) on HAEC. The expression of E-selectin, ICAM-1 and VCAM-1 on HAEC grown for 4 hours in condition of mild hyperhomocysteinemia (30 μmol dm^{-3}) was monitored by flow cytometry. Homocysteine concentration of 7 μmol dm^{-3} was used as a control. Glucose concentration in all experiments was 5.5 mmol dm^{-3}. Data represent the mean ± SEM (standard error mean) of three independent experiments.

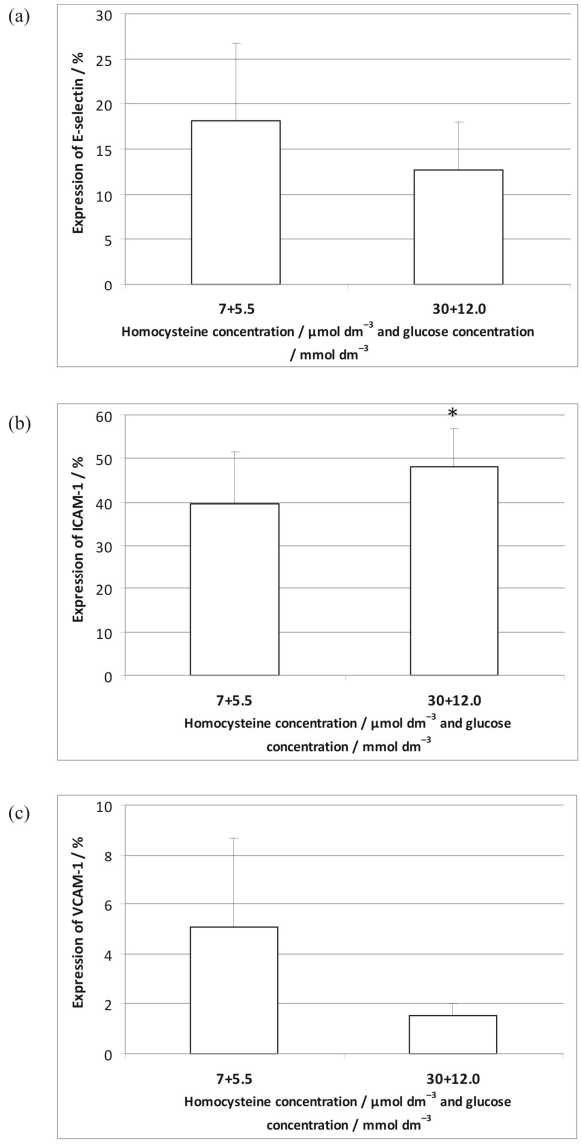

Figure 2. Effect of mild hyperglycemia and mild hyperhomocysteinemia on expression of adhesion molecules E-selectin (a), ICAM-1 (b) and VCAM-1 (c) on HAEC. The expression of adhesion molecules was measured by flow-cytometry upon 4 hours of incubation with homocysteine (30 μmol dm^{-3}) and glucose (12.0 mmol dm^{-3}Homocysteine concentrations of 7 μmol dm^{-3} and glucose 5.5 mmol dm^{-3} were used as controls. Data represent the mean ± SEM (standard error mean) of three independent experiments.

* $p = 0.039$, for homocysteine 7 μmol dm^{-3} and glucose 5.5 mmol dm^{-3} vs. homocysteine 30 μmol dm^{-3} and glucose 12.0 mmol dm^{-3} (Student-N-K test was used to elevate the differences in the control vs. mild hyperhomocysteinemia and mild hyperglycemia group).

RESULTS

The expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1 on HAEC cultivated in medium with increased Hcy concentration (30 μmol dm^{-3}) is shown in Figure 1. Mild hyperhomocysteinemia increased the expression of ICAM-1 for 65.4 %, (p = 0.07), while the expression of E-selectin, (for 30 %, p = 0.07) and VCAM-1 (for 13 %, p = 0.06) was decreased in comparison to their expressions on the HAEC cells in control medium.

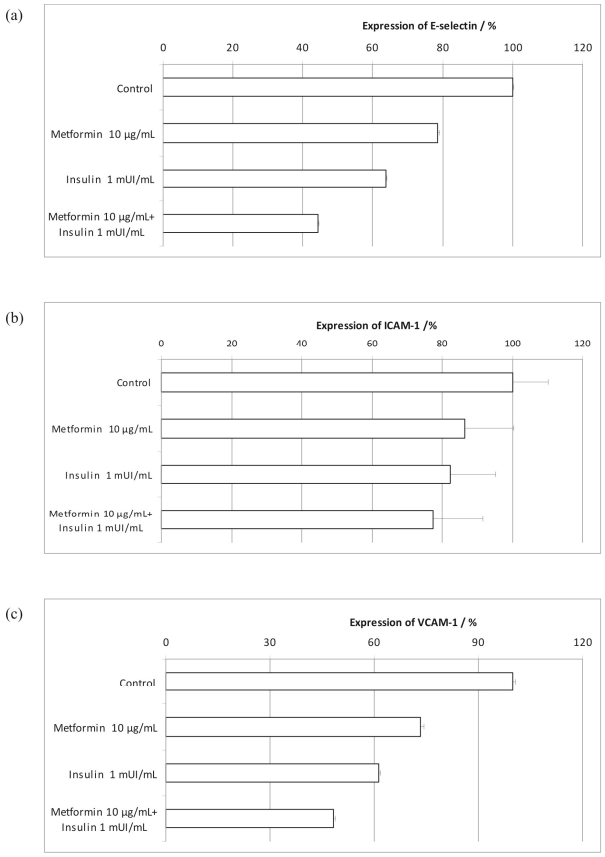

Figure 3. Effect of metformin (10 μg mL^{-1}) and insulin (1 mUI mL^{-1}) administration and their combinations on expression of adhesion molecules E-selectin (a), ICAM-1 (b) and VCAM-1 (c) on HAEC in conditions of mild hyperglycemia (12.0 mmol dm^{-3}) and mild hyperhomocysteinemia (30 μmol dm^{-3}). The expression of adhesion molecules was measured by flow-cytometry After 4 hours of incubation with metformin, insulin and their combinations in mild hyperhomocysteinemia and mild hyperglycemia,. Homocysteine concentrations of 30 μmol dm^{-3} and glucose 12.0 μmol dm^{-3} were used as controls. Measured values of the expression in medium control were taken as 100 %. Data represent the mean ± SEM (standard error mean) of three independent experiments.

Effects of mild hyperglicemia (12 mmol dm^{-3}) and mild hyperhomocysteinemia (30 μmol dm^{-3}) on expression of adhesion molecules are shown in Figure 2. The expression of ICAM-1 was statistically significant increased for 22 % (p = 0.04), while the expression of E-selectin was reduced for 29.7 % (p = 0.32) and VCAM-1 was decreased by 69 % (p = 0.22) on HAEC incubated in this medium compared to expression in control medium.

Metformin and insulin were added separately and as a combination into the medium with 12 mmol dm^{-3} glucose (mild hyperglycemia) and 30 μmol dm^{-3} homocysteine (mild hyperhomocysteinemia) (Figure 3). Flow-cytometry showed that addition of metformin (10 μg mL^{-1}) decreased E-selectin expression by 22 %, and that addition of insulin (1 mUI mL^{-1}) decreases E-selectin expression by 36 % (p = 0.08). Addition of metformin and insulin combination decreased E-selectin expression by 56 % (p = 0.06) Metformin and insulin had the similar effects on other two investigated adhesion molecules. The expression of ICAM-1 and VCAM-1 was decreased by addition of metformin (14 % and 27 %, respectively), insulin (18 % and 39 %, respectively) and combination of metformin and insulin (23 % and 52 %; p = 0.11, respectively). The greatest decrease of adhesion molecules expression (E-selectin by 56 %, ICAM-1 by 23 %, and VCAM-1 by 52 %) was noticed when metformin 10 μg mL^{-1} and insulin 1 mUI mL^{-1} were used (Figure 3).

DISCUSSION

The role of homocysteine (Hcy) as an independent cardiovascular risk factor has been recognised,[2,27–29] but its role in pathogenesis is not completely clear. Numerous *in vivo* studies showed that hyperhomocysteinemia provokes endothelial dysfunction increased by oxidative stress[30,31] and decreases discharge of NO causing increase of vasodilatation.[32–34] Furthermore, elevated Hcy in plasma is connected to increased lipid peroxidation and production of reactive oxygen species (ROS),[35–37] which testify its thrombogenic activity and eventually disorder in the coagulation system.[38–41] Silverman *et al.* showed that elevated Hcy in culture of human aortic endothelial cells after 24 h of incubation increases expression of VCAM-1 and adhesion of monocytes to the endothelial cells surface. In addition, the function of endothelial cells depends on endothelial cyclooxygenase activity and elevated Hcy influencing activity of endothelium-derived nitrogenous oxide and reactive oxygen species as well.[7] High Hcy concentration stimulates proliferation and migration of smooth muscular cells and collagen synthesis with thickening of tunica intima and media.[35,37,42–45] All of the above mentioned indicates that high concentration of plasma Hcy has both, atherogenic and thrombogenic effect.

Our experiments showed that exposure of HAEC to slightly elevated Hcy concentration (30 μmol dm^{-3}) for 4 hours resulting with increased ICAM-1 expression by 65 % (p = 0.07). In the same experimental condition expression of E-selectin and VCAM-1 was decreased in comparison to control (Figure 1). This could be explained by chronology of CAMs expression in adhesion cascade.[46] E-selectin increases first, and subsequently ICAM-1 and VCAM-1 expression could be observed. Based on this fact, in different experimental systems shorter of longer time of exposure and expression of other molecules in adhesion cascade would be detected.[46–48] Although statistically insignificant observed

result is physiologically very important since ICAM-1 expression is involved in the firm adhesion of monocytes to endothelium and beginning of inflammatory and atherogenic events. Besides, results of our study showed that short time exposure to mild elevated Hcy concentration and mild hyperglycemia caused modest but statistically significant (p = 0.039) increase of ICAM-1 expression. Although changes in expression of other molecules did not reach statistically significant levels those suggests that exposure of HAEC to different risk factors activate endothelial cells.

Slightly elevated level of plasma Hcy could be found in the general populations, which magnify risk for different cardiovascular diseases.[2,49,50] A role of homocysteine in the development of cardiovascular diseases in hyperglycemia is not clear yet. There are very few data resulting from *in vitro* investigation, and so far there are no available data about effects of combined interaction of hyperhomocysteinemia and hyperglycemia on expression of adhesion molecules *in vitro.* Our results (Figure 2) show that mild hyperglycemia and mild hyperhomocysteinemia after short-term HAEC stimulation (for 4 hours) lead to increased ICAM-1 expression, while high concentrations of glucose alone, did not exert such effect.[51] Since increased ICAM-1 expression is early key event in atherogenesis, our results address the pro-inflammatory and pro-atherogenic effect of this combination. Furthermore, it is noted that atherosclerosis develops faster in patients, who have more than one risk factor, no matter how noticeable it is.[52] It is also observed that macrovascular complications develop faster in type 2 diabetic patients if they have additional risk factors of atherosclerosis, for example hypertriglyceridemia, hypertension, smoking, or more of them at the same time.[52,53] Direct relationship between high Hcy values in plasma and risks for cardiovascular diseases in type 2 diabetics was also described.[5] In addition, *in vivo* studies point to plasmatic Hcy increase as an independent cardiovascular risk factor connected to atherosclerosis and ischemic cardiovascular disease.[27–29] Results of our study support *in vivo* investigations[49] demonstrating that apparently healthy men who have risk factors of atherogenesis (age, smoking, diabetes, systolic pressure, family anamnesis of coronary disease, serum concentration of homocysteine and fibrinogen), have increased sICAM-1 and therefore, sICAM-1 should be tested as a possible marker of pre-clinical atherosclerosis.

In this study, the effects of two risk factors on endothelial cell activation in controlled conditions are described for the first time. Observed changes on cellular level shed a light on clinically observed fact that development of atherosclerosis in patients with mild hyperglycemia and mild hyperhomocysteinemia is faster than in patients with only one risk factor. The question arises whether the usage of ordinary drug treatments decreases the expression of adhesion molecules and what is the most efficient drug treatment in those conditions. So far, there are no published data about effect of metformin and insulin on the expression of adhesion molecules in conditions of mild hyperglycemia and mild hyperhomocysteinemia.

Furthermore, we showed that addition of drug (metformin 10 μg mL^{-1}, insulin 1 mUI mL^{-1}, metformin 10 μg mL^{-1} and insulin 1 mUI mL^{-1}) in medium with 12 mmol dm^{-3} glucose and 30 μmol dm^{-3} Hcy decreases the expression of all three tested adhesion molecules. The highest reduction of CAMs expression was observed after treatment with combination of metformin (10 μg mL^{-1}) and insulin (1 mUI mL^{-1}). Observed effect approves this often used drug combination. In a contrast to previously observed decrease of E-selectin after insulin administration in mild hyperglycemia,[51] perceived decrease was not statistically significant. There is a question of prevention of macrovascular complications in those patients and the influence of the usage of new therapeutically approaches.

Results of this investigation showed that the administration of metformin, insulin and their combinations do not significantly change the expression of adhesion molecules in conditions of mild hyperglycemia and mild hyperhomocysteinemia. It could be possible that the use of tested drugs in those conditions, without regulation of both risk factors glycemia and hyperhomocysteinemia is not sufficient to prevent the development of macrovascular complications of diabetes. It should be investigated whether the use of acetylsalicylic acid, statins, folic acid or their combinations leads to the decrease of expression of adhesion molecules and deactivation of endothelial cells in mentioned conditions. Perhaps, the solution for the prevention of macrovascular complications in the diabetic disease should be a search for new drug treatments, which among other things could decrease the activation of endothelial cells and have anti-inflammatory effect.[54,55] Despite the possibilities of advanced medicine, special attention should be given to primary prevention of diabetes through balanced diet, physical activity, and weight reduction.

CONCLUSION

Mild homocysteine concentration (30 μmol dm^{-3}) during short time exposure (4 hours) resulted in increased expression of ICAM-1. This is the precondition for adhesion of monocytes on endothelial cells and beginning of inflammatory and atherogenic process.

Mild hyperglycemia and mild hyperhomocysteinemia significantly increased expression of ICAM-1, already after short-term stimulation (4 hours). Expression of E-selectin and VCAM-1 was decreased. The

results showed the importance of combined interaction of hyperglycemia and hyperhomo-cysteinemia on the expression of adhesion molecules, and on their pro-inflammatory and pro-atherogenic effect.

The addition of medicaments (metformin 10 μg mL^{-1}, insulin 1 mUI mL^{-1}, and combination of metformin 10 μg mL^{-1} and insulin 1 mUI mL^{-1}) in medium with mild hyperglycemia and mild hyperhomocysteinemia decreased the expression of all three adhesion molecules. The greatest decrease, although not statistically significant, occurred after the addition of metformin 10 μg mL^{-1} and insulin 1 mUI mL^{-1}, which supports this often used drug treatment. This therapeutic combination results in a modest decrease in expression of adhesion molecules on endothelial cells. However, due to the other risk factors that may not be affected, this therapy alone seems unlikely to be sufficient to prevent the development of macrovascular complications in diabetic patients. *In vivo* studies should be done to test whether observed moderate changes in adhesion molecules expression have physiological and pathophysiological significance in living organism.

REFERENCES

1. G. J. Hankey and J. W. Eikelboom, *Lancet* **354** (1999) 407−413.
2. C. J. Boushey, S. A. Beresford, G. S. Omenn, and A. G. Motulsky, *JAMA* **274** (1995) 1049−1057.
3. D. Matthias, C. H. Becker, R. Riezler, and P. H. Kindling, *Atherosclerosis* **122** (1996) 201−216.
4. J. C. Chambers, O. A. Obeid, and J. S. Kooner, *Arterioscler. Thromb. Vasc. Biol.* **19** (1999) 2922−2927.
5. M. Soinio, J. Marniemi, M. Laakso, S. Lehto, and T. Rönnemaa, *Ann. Intern. Med.* **140** (2004) 94−100.
6. N. P. Dudman, S. E. Temple, X. W. Guo, W. Fu, and M. A. Perry, *Circ. Res*. **84** (1999) 409−416.
7. M. D. Silverman, R.J. Tumuluri, M. Davis, G. Lopez, J. T. Rosenbaum, and P. I. Lelkes, *Arterioscler. Thromb. Vasc. Biol.* **22** (2002) 587−592.
8. D. T. Price and J. Loscalzo, *Am. J. Med.* **107** (1999) 85−97.
9. R. Ross, *N. Engl. J. Med.* **340** (1999) 115−126.
10. D. M. Nathan, J. B. Buse, M. B. Davidson, E. Ferrannini, R. R. Holman, R. Sherwin, and B. Zinman, *Diabetologia* **52** (2009) 17−30.
11. R. Giannarelli, M. Aragona, A. Coppelli, and S. Del Prato, *Diabetes Metab.* **29** (2003) 28−35.
12. M. H. Uehara, N. E. Kohlmann, M.T. Zanella, and S. R. Ferreira, *Diabetes. Obes. Metab.* **3** (2001) 319−325.
13. K. Cusi, A. Consoli, and R. A. DeFronzo, *J. Clin. Endocrinol. Metab.* **81** (1996) 4059−4067.
14. F. Abbasi, J. W. Chu, T. McLaughlin, C. Lamendola, E.T. Leary, and G.M. Reaven, *Metabolism* **53** (2004) 159−164.
15. UK Prospective Diabetes Study (UKPDS) Group, *Lancet* **352** (1998) 854−865.
16. P. Vague, *Diabetes. Metab.* **29** (2003) 5−7.
17. N. F. Wiernsperger, *Diabetes. Technol. Ther.* **2** (2000) 259−272.
18. N. V. Chu, A. P. Kong, D. D. Kim, D. Armstrong, S. Baxi, R. Deutsch, M. Caulfield, S. R. Mudaliar, R. Reitz, R. R. Henry, and P. D. Reaven, *Diabetes Care* **25** (2002) 542−549.
19. D. K. Nagi and J. S. Yudkin, *Diabetes Care* **16** (1993) 621−629.
20. P. J. Grant, *Diabetes Metab.* **17** (1991) 168−173.
21. J. C. Mamputu, N. F Wiernsperger, and G. Renier, *Diabetes Metab.* **29** (2003) 71−76.
22. I. Aganović and Ž. Reiner, *Šećerna bolest*, in: *Interna medicina* (4th ed.) Naklada Ljevak, Zagreb, 2008, pp. 1244−1264.
23. A. Festa, R. Jr D'Agostino, L. Mykkänen, R. P. Tracy, D. J. Zaccaro, C. N. Hales, and S. M. Haffner, *Arterioscler. Thromb. Vasc. Biol.* **19** (1999) 562−568.
24. E. Standl, *Clin. Invest. Med.* **18** (1995) 261−266.
25. A. Aljada, H. Ghanim, R. Saadeh, and P. Dandona, *J. Clin. Endocrinol. Metab.* **86** (2001) 450−453.
26. A. Aljada, R. Saadeh, E. Assian, H. Ghanim, and P. Dandona *J. Clin. Endocrinol. Metab.* **85** (2000) 2572−2575.
27. M. M. Sagheb, M. A. Ostovan, Z. Sohrabi, E. Atabati, G. A. Raisjalai, and J. Roozbeh, *Saudi J. Kidney Dis. Transpl*. **21** (2010) 863−866.
28. M. E. Temple, A. B Luzier, and D. J. Kazierad, *Ann. Pharmacother.* **34** (2000) 57−65.
29. G. J. Hankey and J. W. Eikelboom, *Indian Heart J.* **52** (2000) 18−26.
30. K. Matsumoto, Y. Sera, Y. Ueki, G. Inukait, E. Niirot, and S. Miyake, *Diabet. Med.* **19** (2002) 822−826.
31. M. Otsuki, K. Hashimoto, Y. Morimoto, T. Kishimoto, and S. Kasayama, *Diabetes* **46** (1997) 2096−2101.
32. T. S. Altannavch, K. Roubalová, P. Kucera, and M. Andel, *Physiol. Res*. **53** (2004) 77−82.
33. H. Omi, N. Okayama, M. Shimizu, M. Okouchi, S. Ito, T. Fukutomi, and M. Itoh, *J. Diabetes Complications*. **16** (2002) 201−208.
34. K. Matsumoto, S. Miyake, M. Yano, Y. Ueki, and Y. Tominaga, *Atherosclerosis* **152** (2000) 415−420.
35. X. D. Ke, A. Foucault-Bertaud, C. Genovesio, F. Dignat-George, E. Lamy, and P. Charpiot, *Mol. Cell. Biochem.* **335** (2010) 203−210.
36. C. Matté, F. M. Stefanello, V. Mackedanz, C. D. Pederzolli, M. L. Lamers, C. S. Dutra-Filho, M. F. Dos Santos, and A. T. Wyse, *Int. J. Dev. Neurosci.* **27** (2009) 337−344.
37. S. Voutilainen, G. Alfthan, K. Nyyssönen, R. Salonen, and J. T. Salonen, *Ann. Med.* **30** (1998) 300−306.
38. M. K. Al-Obaidi, H. Philippou, P. J. Stubbs, A. Adami, R. Amersey, M. M. Noble, and D. A. Lane, *Circulation.* **101** (2000) 372−377.
39. A. Khajuria and D. S. Houston, *Blood* **96** (2000) 966−972.
40. S. R. Lentz and J. E. Sadler, *J. Clin. Invest.* **88** (1991) 1906−1914.
41. G. M. Rodgers and M. T. Conn, *Blood.* **75** (1990) 895−901.
42. C. Jiang, H. Zhang, W. Zhang, W. Kong, Y. Zhu, H. Zhang, Q. Xu, Y. Li, and X.Wang, *Am. J. Physiol. Cell Physiol.* **297** (2009) 1466−1476.
43. A. Majors, L. A. Ehrhart, and E. H. Pezacka, *Arterioscler. Thromb. Vasc. Biol.* **17** (1997) 2074−2081.
44. J. C. Tsai, M. A. Perrella, M. Yoshizumi, C. M. Hsieh, E. Haber, R. Schlegel, and M. E. Lee, *P. Natl. Acad. Sci. USA* **91** (1994) 6369−6373.
45. M. R. Malinow, F. J. Nieto, M. Szklo, L. E. Chambless, and G. Bond, *Circulation.* **87** (1993) 1107−1113.
46. M. P. Bevilacqua and R. M. Nelson, *J. Clin. Invest.* **91** (1993) 379−387.
47. G. Brevetti, V. Schiano, and M. Chiariello, *Vasc. Med.* **11** (2006) 39−47.
48. S. M. Albelda, C. W. Smith, and P.A. Ward, *FASEB J.* **8** (504−512)
49. L. E. Rohde, C. H. Hennekens, and P. M. Ridker, *Arterioscler. Thromb. Vasc. Biol.* **19** (1999) 1595−1599.
50. M. Ciaccio and C. Bellia, *Curr. Clin. Pharmacol.* **5** (2010) 30−36.
51. T. Bacun, L. Glavas-Obrovac, T. Belovari, I. Mihaljević, T. Hanich, V. Feher-Belaj, and Vcev A, Coll Antropol. **34** (2010)

911−915.
52. Ž. Reiner and S. Gamulin, *Poremećaji metabolizma lipida*, in: *Patofiziologija* (5th ed.) Medicinska naklada, Zagreb, 2002, pp. 166−182.
53. S. J. Haffner and H. Cassells, *Am. J. Med.* **115** (2003) 6−11.
54. Y. Hattori, T. Jojima, A. Tomizawa, H. Satoh, S. Hattori, K. Kasai, and T. Hayashi, *Diabetologia* **53** (2010) 2256−2263.
55. R. X. Yang, S. Y. Huang, F. F Yan, X. T. Lu, Y. F. Xing, Y. Liu, Y.F. Liu, and Y. X. Zhao, *Acta Pharmacol. Sin.* **10** (2010) 1395−1400.