

Microvascular Reactivity and Endothelial Function in Type 2 Diabetic Patients with Hyperlipidemia Treated with Simvastatin: 3-year Follow-up

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Abstract: Aim of this study was to evaluate microvascular reactivity (MVR) by laser Doppler flowmetry in Type 2 diabetes mellitus (T2DM) with hyperlipidemia during three years of simvastatin treatment. Additionally, markers of endothelium and fibrinolysis were evaluated. Twenty patients with T2DM and hyperlipidemia were treated with 20 mg of simvastatin daily for 3 months, treatment was then interrupted for 3 months (wash-out) and again started and maintained continually up to total of 36 months of follow-up. Maximal perfusion (max), velocity of perfusion increase (max/t) and percent increase of perfusion compared to baseline (%) was measured during post-occlusive reactive hyperemia (PORH) and thermal hyperemia (TH). VCAM-1, ICAM-1, E-selectin and P-selectin were used as markers of endothelium, tissue plasminogen activator (tPA) and its inhibitor (PAI-1) as markers of fibrinolysis. Baseline MVR in diabetic patients was comparable to controls. MVR decreased at months 3, 12, and 36 compared to baseline (PORHmax 26 ± 12 , 35 ± 17 , 26 ± 11 vs. 56 ± 30 PU, $p < 0.05$, THmax 67 ± 19 , 81 ± 37 , 58 ± 24 vs. 134 ± 70 PU, $p < 0.01$, PORHmax/t 2.0 ± 1.4 , 2.8 ± 1.7 , 1.9 ± 1.3 vs. 7.7 ± 7.4 PU/s, $p < 0.05$, THmax/t 1.1 ± 0.6 , 1.0 ± 0.4 , 0.7 ± 0.4 vs. 1.5 ± 0.7 PU/s, $p < 0.05$,

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respectively). MVR in diabetic patients approached baseline values following 3 months of the wash-out period (PORHmax 40 ± 18 PU, NS; THmax 101 ± 38 PU, NS). In T2DM, VCAM-1 and E-selectin concentrations were higher at baseline (533 ± 170 vs. 365 ± 56 $\mu\text{g/l}$, $p < 0.01$ and 66 ± 27 vs. 40 ± 12 $\mu\text{g/l}$, $p < 0.01$, respectively). VCAM-1 and ICAM-1 increased at months 3 and 36, concentrations of selectins declined. Fibrinolysis was impaired in T2DM at baseline compared to controls (tPA 6.3 ± 1.9 vs. 4.9 ± 2.0 $\mu\text{g/l}$, $p < 0.05$; PAI-1 92 ± 23 vs. 132 ± 76 $\mu\text{g/l}$, $p < 0.001$). PAI-1 increased at months 3 and 36 (109 ± 37 and 132 ± 76 $\mu\text{g/l}$, $p < 0.05$). No relationship was found between MVR and lipid parameters, endothelial activity, fibrinolysis or diabetes control. A reversible MVR decrease was observed in T2DM following simvastatin treatment together with changes in endothelial activity and fibrinolysis. Mechanisms of observed changes and their significance in the vascular impairment are not clear and further research is needed.

Introduction

Hypercholesterolemia is an important independent cardiovascular risk factor [1]. Lipid-lowering therapy significantly reduces cardiovascular morbidity and mortality and treatment with statins proved its efficacy in large clinical trials also in patients with Type 2 diabetes [2–4]. Hyperlipidemia is associated with endothelial dysfunction [5] and impaired vascular reactivity [6] in diabetic patients and significant changes in biochemical markers of endothelial dysfunction are found in these subjects [7–9]. Improved reactivity of large vessels was repeatedly described in different studies after treatment with statins [10–12]. However, despite profound lipid lowering, neutral effect of high dose of atorvastatin on serotonin-induced and sodium nitroprusside-induced vasodilation was observed in Type 2 diabetic patients using venous occlusion plethysmography [13].

It is not clear whether treatment with statins can modify the risk of development of microvascular diabetic complications. The effect of statin treatment on microvascular reactivity in non-diabetic hypercholesterolemic subjects has been either positive [14, 15] or neutral [16]. It has been suggested that treatment with cerivastatin decreases microalbuminuria [17] in Type 2 diabetes, however, no changes were observed after cerivastatin in non-hyperlipidemic Type 2 diabetic patients [18].

Mechanisms by which statins influence endothelial function are not clear yet and are not limited only to cholesterol-lowering. Direct effect of statins on endothelium includes changes in nitric oxide and prostaglandin synthesis and release in the vessel wall [19–22]. Statins may also have direct effect via endothelium independent mechanisms. These include inhibition of inflammation, decrease of oxidative stress or pro-thrombogenic response, and many other effects [23, 24]. Many of these effects are mediated by inhibition of isoprenoids [25, 26].

VCAM-1, ICAM-1, E-selectin and E-selectin were used as markers of endothelial function. These cytoadhesive molecules reflect the activity of endothelium and can

also participate in development of vascular complications [27]. Their increase has been described in Type 2 diabetic patients [28, 29]. PAI-1 and tPA were selected for assessment of fibrinolysis. PAI-1 is a multifunctional protein that acts as a physiologic regulator of fibrinolysis and cell migration [30]. Impaired fibrinolysis can be observed in Type 2 diabetes, too [31, 32]. Laser Doppler flowmetry is an easy and non-invasive method for testing the skin microvascular reactivity and can also be used for clinical monitoring of drug effects on microvascular bed [33, 34, 15].

The aim of this study was to compare the microvascular reactivity in Type 2 diabetes with hyperlipidemia before and after short-term and long-term treatment with simvastatin. The total period of follow-up was 36 months. Three months wash-out period following three initial months of treatment was designed to test the reversibility of any observed effects. Detected changes in microvascular reactivity and endothelial function or fibrinolysis can contribute to the understanding of vascular impairment in Type 2 diabetic patients with hyperlipidemia.

Patients and Methods

Twenty Type 2 diabetic patients (10 men, 10 women) were selected for the study. All of them were non-smokers with hyperlipidemia. Their characteristics are shown in Table 1. Four of them had a history of coronary heart disease. Concomitant medication consisted predominantly of beta-adrenergic blockers (n=12), angiotensin-converting enzyme inhibitors (n=7), calcium channel blockers (n=6), diuretics (n=6), and acetylsalicylic acid (n=11). Diabetes was treated with metformin (n=14), sulphonylurea (n=5) and insulin (n=6). Antidiabetic drugs were combined in 5 patients. Therapy and doses of drugs were not changed during the experiment except of insulin therapy as well as the diet and exercise recommendation. Insulin doses were increased in 4 cases to improve diabetes control. All patients completed the visit at month 12, 17 patients completed the 36 months follow-up. All available data from all visits were included in statistical evaluation. Control group consisted of 20 healthy persons (8 men, 12 women)

Table 1 – Baseline characteristics of patients and control group, and results of BMI, HbA_{1c}, systolic and diastolic blood pressure during the follow-up. Data are mean ± SD

	Type 2 diabetic patients (n=20)					Controls (n=20)
	baseline	simvastatin 3 months	after wash-out	simvastatin 12 months	simvastatin 36 months	
Age (years)	57 ± 10	–	–	–	–	52 ± 7
BMI (kg/m ²)	29.7 ± 3.8	29.2 ± 3.5	29.6 ± 3.6	29.8 ± 3.5	30.1 ± 3.6	28.1 ± 3.5
HbA _{1c} (%)	9.0 ± 2.1	9.0 ± 1.9	9.0 ± 1.8	8.9 ± 2.3	9.3 ± 2.4	4.9 ± 0.4
sBP (mm Hg)	141 ± 19	145 ± 17	143 ± 21	142 ± 18	144 ± 20	135 ± 10
dBp (mm Hg)	83 ± 11	85 ± 5	85 ± 5	84 ± 8	85 ± 10	81 ± 8

used for comparison of baseline values. The study was approved by local Ethics committee and informed consent was obtained for all subjects.

Simvastatin was administered to the patients in one daily dose of 20 mg taken in the evening (Simgal[®], Merck). The treatment was discontinued after three months from baseline and again started after three months of wash-out period. Then the treatment continued without interruptions for next 31 months up to total 36 months of follow-up. MVR was measured at baseline, after 3 months of simvastatin treatment, then after 3 months of wash-out period and then at months 12 and 36. Blood samples were taken between 7:00 and 8:00 after an overnight fast at baseline and after three months of treatment, 3 months of wash-out and at months 12 and 36. VCAM-1, ICAM-1, E- and P-selectins, tPA, and PAI-1 were not measured at month 12.

Skin microvascular reactivity (MVR) was measured by laser Doppler flowmetry using a PeriFlux PF 4001 Master laser instrument and a PeriTemp 4001 Heater thermostatic unit manufactured by Perimed (Sweden). Instrument settings were as follows: time constant 0.02 s, sampling frequency 32 Hz, averaging from 2 samples. Measurements were done at a room temperature of 22 °C in a sitting position, and all subjects rested for at least 30 minutes in order to acclimatize before examination. Post-occlusive reactive hyperemia (PORH) and thermal hyperemia (TH) tests were performed for the assessment of microvascular reactivity. A single thermostatic probe (type 455, 23 mm diameter, fibre separation 0.25 mm) was used for both tests. Optical fibres in this probe are integrated into the heating plate and thus the entire area of tissue under the probe is heated. The probe was fixed with double-stick discs (3M, USA) to the forearm and its temperature was set to 32 °C for the purpose of skin thermal stabilization during PORH. A temperature of 44 °C was used during TH as the thermal stimulus.

Basal perfusion (PORHb) was measured for 2 minutes before the PORH test. The brachial artery was then occluded by a sphygmomanometer cuff inflated to a suprasystolic pressure for 3.5 minutes. The cuff was applied around the arm before the procedure started in order to avoid any extra manipulation with the extremity during the test. PORH was recorded after 3.5 minutes of arterial occlusion. Maximal perfusion during hyperemia was recorded (PORHmax) as well as the time needed for reaching this maximal perfusion (PORHt). The velocity of the perfusion increase (PORHmax/t) was calculated as PORHmax/PORHt . Relative hyperemia (PORH%) was calculated as a percent increase above the baseline ($\text{PORH\%} = (\text{PORHmax/PORHb-1}) * 100$). Thermal hyperemia was measured 10 minutes later than the PORH test, at the same location. The probe temperature was set to 44 °C and parameters THmax, THt, and THmax/t were recorded or calculated similarly as those in the PORH test. TH% was calculated using formula $\text{TH\%} = (\text{THmax/PORHb-1}) * 100$. Perfusion is given in arbitrary perfusion units (PU). Perisoft for DOS 5.10C2 and Perisoft for Windows 2.5 software were used for recording and evaluating perfusion data. Records were blinded and the evaluation

was performed by a single operator. The intra-individual coefficient of variation of the laser Doppler method in 5 healthy subjects measured ten times on ten consecutive days varied from 17 to 24% in TH and 19 to 25% in PORH, depending on the analyzed parameter.

We did not repeat the examination of MVR in control group because it was demonstrated that the effect of time difference in healthy subjects is negligible – about 0.55–0.88 flow units per year, i.e. estimated 0.4–0.7% decrease in both resting and stimulated skin blood flow [35].

Total serum cholesterol (TC), HDL-cholesterol (HDL-C) and triglycerides (TG) were measured by photometric enzymatic method on routine analyzer. LDL-cholesterol (LDL-C) was calculated by Friedeman using equation ($LDL = TC - HDL - TG/2,2$). Fasting blood glucose (FBG) was measured by routine enzymatic method. Glycated hemoglobin HbA_{1C} was analyzed by high-performance liquid chromatography method on Variant II analyzer (BioRad, USA).

Serum E-selectin and P-selectin concentrations were measured by ELISA kits manufactured by RD System Europe Ltd (Abingdon, UK). Fibrinolysis was characterized by plasma concentrations of tissue plasminogen activator tPA and its inhibitor PAI-1 determined by ELISA method using Coalisa tPA and PAI-1 kits (KABI Diagnostics, Sweden). Concentration of these parameters from baseline, month 3 and wash-out were measured in one assay to minimize the effect of variation.

Statistical evaluation was performed by Statistica for Windows 6.0 software. Basic descriptive statistics was calculated for presented parameters. ANOVA, Student's *t*-test or Wilcoxon's test, Mann-Whitney and Kolmogorov-Smirnov tests were used for comparing data between groups. Tests were selected depending on normality of data distribution. Pearson's and Spearman's correlations were used for analysis of relationships between measured parameters. Data are expressed as mean \pm SD if not stated otherwise.

Results

No statistically significant change in the long-term diabetes control assessed by HbA_{1C} was observed during the study. There was a non significant trend to increase in blood pressure and BMI (results in Table 1). Significant decrease in total and LDL-cholesterol was observed in all patients following statin administration (Table 2).

Parameters of skin microvascular reactivity in diabetic patients were non-significantly higher at baseline compared to the control group except for PORHmax/t which was significantly lower in diabetic patients. Following three months of treatment with simvastatin a statistically significant decrease in microvascular reactivity was observed compared to baseline. Simvastatin treatment was then interrupted for three months. MVR restored partially after the three months of the wash-out period. Although it did not reach its baseline values except for PORH%, the differences compared to baseline were not statistically significant.

Treatment with simvastatin was then restarted and maintained up to the month 36. At month 12 the statistically significant decrease in MVR was observed again and persisted up to month 36 in almost all parameters (Table 2).

VCAM-1, ICAM-1, E- and P-selectins, tPA and PAI-1 concentration at baseline were significantly higher than in the control group. Concentration of ICAM-1 and PAI-1 significantly increased at month 3. At the end of the wash-out period, ICAM-1 was significantly lower compared to baseline and no significant change was observed in other parameters of endothelium and fibrinolysis. Because of technical reasons, no results of these parameters are available at month 12. Statistically significant increase was observed at month 36 in VCAM-1 and PAI-1 concentration compared to baseline values while E-selectin concentration was significantly decreased (Table 2).

Despite the profound lipid lowering, no relationship between lipid levels and MVR parameters was found either at baseline or during simvastatin therapy. MVR was not related to cell adhesion molecules and fibrinolysis. Similarly, no relationship was detected between the parameters of lipid metabolism and endothelial function or fibrinolysis.

Table 2 – Lipid profile, skin microvascular reactivity during post-occlusive reactive hyperemia (PORH), thermal hyperemia (TH), and endothelial function in Type 2 diabetic patients at baseline, after 3 months of treatment, after wash-out period and then after 12 and 36 months of treatment with simvastatin and in the control group

	Type 2 diabetic patients (n=20)					Controls (n=20)
	baseline	simvastatin 3 months	after wash-out	simvastatin 12 months	simvastatin 36 months	
TC (mmol/l)	6.85 ± 0.71 ^a	4.99 ± 0.71 ^z	6.36 ± 0.82	5.29 ± 0.94 ^z	5.12 ± 1.09 ^z	4.99 ± 0.60
HDL-C (mmol/l)	1.33 ± 0.24 ^b	1.26 ± 0.15	1.40 ± 0.28	1.32 ± 0.28	1.28 ± 0.35	1.62 ± 0.34
LDL-C (mmol/l)	4.42 ± 1.02 ^c	2.77 ± 0.57 ^z	3.85 ± 0.57	2.86 ± 0.76 ^z	2.98 ± 0.93 ^z	2.87 ± 0.56
TG (mmol/l)	3.81 ± 2.68 ^c	2.69 ± 1.61	2.86 ± 2.00	3.12 ± 2.50	2.93 ± 2.52	1.11 ± 0.6
PORHmax (PU)	56 ± 30	26 ± 12 ^x	40 ± 18	35 ± 17 ^x	26 ± 11 ^x	51 ± 20
PORHmax/t (PU/s)	7.7 ± 7.4	2.0 ± 1.4 ^x	4.7 ± 3.7	2.8 ± 1.7 ^x	1.9 ± 1.3 ^y	4.0 ± 2.3
PORH% (%)	476 ± 179	220 ± 127 ^y	538 ± 293	383 ± 264	214 ± 119 ^z	564 ± 306
THmax (PU)	134 ± 70	67 ± 19 ^y	101 ± 38	81 ± 37 ^y	58 ± 24 ^z	115 ± 41
THmax/t (PU/s)	1.5 ± 0.7	1.1 ± 0.6	1.4 ± 0.5	1.0 ± 0.4 ^x	0.7 ± 0.4 ^z	1.5 ± 0.7
TH% (%)	1312 ± 484	794 ± 396 ^y	1484 ± 541	1060 ± 748	622 ± 303 ^z	1401 ± 694
VCAM-1 (μg/l)	533 ± 170 ^b	589 ± 195	570 ± 112	–	658 ± 177 ^y	365 ± 56
ICAM-1 (μg/l)	257 ± 62 ^a	306 ± 90 ^x	199 ± 46 ^x	–	301 ± 106	214 ± 43
E-selectin (μg/l)	66 ± 27 ^b	70 ± 27	51 ± 18	–	33 ± 10 ^z	40 ± 12
P-selectin (μg/l)	203 ± 64 ^b	205 ± 64	174 ± 35	–	178 ± 51	140 ± 48
tPA (μg/l)	6.3 ± 1.9 ^a	6.8 ± 2.6	5.6 ± 2.1	–	5.6 ± 2.3	4.9 ± 2.0
PAI-1 (μg/l)	92 ± 23 ^c	109 ± 37 ^x	87 ± 28	–	132 ± 76 ^x	45 ± 18

Data are mean ± SD. Statistical significance between baseline values of control group and diabetic subjects: ^ap<0.05, ^bp<0.01, ^cp<0.001, and baseline vs. post treatment values in diabetic patients: ^xp<0.05, ^yp<0.01, ^zp<0.001

Discussion

In current study, maximal perfusion (expressed as absolute number) during heating and after arterial occlusion in patients was non-significantly higher compared to control group at baseline. Maximal perfusion expressed in percents of basal perfusion (expressing vasodilatory reserve) was decreased nonsignificantly. The microvascular reactivity in patients was comparable to control group at baseline. A tendency to increased perfusion in maximal hyperemia in diabetic patients probably reflects an increased blood flow through non-nutritive vessels due functional and structural changes in microvascular bed. The maximal vasodilatory capacity is slightly diminished as can be seen from parameters that are calculated using basal, pre-provocation flow [36]. Significant decrease of MVR parameters following simvastatin treatment can be hypothetically explained by decreased blood flow through functional microvascular network. Partial reversibility of these changes following the three months of simvastatin removal may show relatively early abolishment of simvastatin effect after its discontinuation. After the next three years of simvastatin therapy significantly lower values of several MVR parameters still persisted, although a trend toward the increase could be observed. The effect of simvastatin on microvascular reactivity persisted for at least three years of treatment. However, we are not aware of mechanism(s) causing such a large decrease in capillary blood flow. Statins increase the production and bioavailability of NO in vessels [19, 20]. This should be reflected in increased maximal perfusion during provocative tests. However, this was not observed in our study. Similarly, increased production of vasoactive prostaglandins has been reported following treatment with different statins [33, 37]. This should also probably lead to increased maximal perfusion which was not present in our experiment.

PAI-1 is involved in the regulation of fibrinolysis by inhibiting the tissue plasminogen activator (tPA). This prevents systemic plasmin generation and inhibits fibrinolysis. In an experimental model, an increased fibrinolysis was observed after simvastatin in human peritoneal mesothelial cells [38]. Similar effect of simvastatin was observed in a clinical study in Type 2 diabetic patients [39]. However, in current study, opposite effect of statin treatment on PAI-1 was observed in diabetic patients while the tPA concentration did not changed significantly. Possible mechanism contributing to these changes may be an interaction of fibrinolysis and oxidative stress as was suggested in our previously published results [40].

Changes in concentration of ICAM-1, VCAM-1 and selectins indicate a different degree of endothelial activation in different stages of treatment with simvastatin. After three years of follow-up, significant increase in VCAM-1 and non significant increase in ICAM-1 were present, contradictory to significant lowering of E-selectin. Regarding to the effect of simvastatin on cell adhesion molecules (CAM), literary data are inconclusive. Several studies have shown a decrease of CAM after simvastatin in different settings [41, 42], however, no effect of simvastatin on CAM was observed in two randomized studies [43, 44] with 6 and 3 months of

simvastatin treatment, respectively. No conclusion regarding possible influence of simvastatin on CAM can be drawn based on data observed in current study.

The limitation of the present study is the fact that it was not randomized and placebo-controlled. However, the study was designed as descriptive and exploratory, and all MVR records and all samples were blinded prior to the assessment. The observed differences were large, and the reproducibility of the laser Doppler method is acceptable. It should be mentioned that laser Doppler flowmetry provides different information than methods used in examinations of large vessels and macrovascular function like for example flow mediated dilation. In microvasculature, this method can represent an easy and non-invasive test available for large scale of experiments.

Conclusion

In conclusion, a significant decrease of microvascular reactivity was observed in Type 2 diabetic patients with poor metabolic control and hyperlipidemia in both post-occlusive and thermal hyperemia after simvastatin therapy. This effect was reversible after discontinuation of simvastatin and persisted for three years of simvastatin treatment. Changes in concentrations of some cell adhesion molecules and PAI-1 were observed during the study, however, changes in MVR were not associated with those in lipid concentrations and parameters of endothelial activity or fibrinolysis. Attribution of these changes to pleiotropic effects of simvastatin is questionable. No conclusion can be drawn on a possible relationship between observed findings and their relevance to vascular impairment and prognosis of patients and the results should be interpreted with caution. Further research in that field is needed.

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