-RXUQDORI Clinical & Experimental Cardiology

Knapp et al., J Clin Exp Cardiology 2016, 7:12
DOI: 10.4172/2155-9880.1000482

Research Article OMICS International

Blood Bioactive Sphingolipids and Activity of Acid Sphingomyelinase in Patients with Multivessel Coronary Artery Disease

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Abstract

Aim: Ceramide is claimed to participate in development of atherosclerosis. Another bioactive sphingolipid, namely sphingosine-1-phosphate has anti-atherogenic properties. The aim of the present study was to examine the level of ceramide, sphingosine-1-phosphate, sphinganine-1-phosphate, sphingosine and sphinganine in plasma, erythrocytes and platelets of patients with multivessel coronary artery disease.

Material and methods: The group of patients consisted of 36 people of both sexes with multivessel coronary artery disease recommended to coronary artery by-pass grafting. The control group consisted of 20 age-matched subjects without symptoms of the disease. The blood samples were collected and fractionated in plasma, erythrocytes and platelets. The levels of the above listed sphingolipids were determined by means of high performance liquid chromatography. The activity of acidic secretory sphingomyelinase in plasma was determined using 14C-sphingomyelin.

Results: It was shown in the group of patients that the plasma levels of sphingosine-1-phosphate and sphinganine-1-phosphate were reduced whereas the levels of other sphingolipids did not differ from the respective values in the control group. The plasma activity of acidic secretory sphingomyelinase was elevated in the group of patients. The levels of the examined sphingolipids in erythrocytes were similar in both groups. In platelets, the level of sphingosine and sphinganine in the group of patients was higher than in the control group. The plasma ceramide/sphingosine-1-phosphate ratio is elevated by 54% in the group of patients.

Conclusion: The results obtained indicate that metabolism of ceratain bioactive sphingolipids in plasma, and platelets in patients with multivessel coronary artery disease are changed as compared to the control group.

Keywords: Atherosclerosis; Bioactive sphingolipids; Plasma; Erythrocytes; Platelets

Introduction

There is a bulk of evidence obtained in human and animal studies indicating that certain bioactive sphingolipids, namely ceramide and sphingosine-1-phosphate, may be involved in development of atherosclerosis.

There are two main sources of ceramide in the cell: synthesis *de novo* and hydrolysis of sphingomyelin by the action of the enzyme sphingomyelinase. Sphinganine is main precursor of ceramide on the de novo synthesis pathway. Ceramide is deacylated by ceramidase to sphingosine. Both sphingosine and sphinganine can be phosphorylated to sphingosine-1-phosphate and sphinganine-1-phosphate. The content of the latter in the tissues is low and it exerts only a weak physiological activity [1]. There are three isoforms of sphingomyelinase: acidic (or secretory), neutral and alkaline [2]. It was shown that endothelial cells covering atherosclerotic plaque as well as macrophages and fibroblasts secrete acid-sphingomyelinase (S-smase). Especially, endothelial cells secrete much of the enzyme. The enzyme, locally, hydrolyzes LDL-bound sphingomyelin. Sphingomyelin-deprived LDL easily aggregate and induces formation of foam cells which promotes development of atherosclerosis. Ceramide activates apoptosis of macrophages and endothelial cells either itself or contributes to this process. As a result it accelerates development of atherosclerosis [3-6]. Pro-inflamatory cytokines seem to play crucial role in activation of S-smase in the atherosclerotic plaques [7]. Plasma ceramide level was shown to be a risk factor at the early stages of atherosclerosis [8]. In sphingomyelin synthase 2 and apolipoprotein E (apoE) double knockout mice the plasma level of sphingomyelin in atherogenic lipoproteins was reduced and it was accompanied by reduction of atherosclerotic lesions. Interestingly, the plasma ceramide level was elevated in the knockout mice [9]. Inhibition of *de novo* ceramide synthesis by myriocin results in regression of atherosclerotic plaques in hyperlipidemic ApoE-deficient mice [10]. Elevation in the plasma level of ceramide and activity of S-smase was observed in patients with acute coronary syndrome compared to in healthy subjects and patients with stable angina pectoris [11].

Sphingosine-1-phosphate was shown, in experimental studies, to exert complex athero-protective action [12]. However, the data on the plasma level of S1P in atherosclerosis are not uniform. Argraves et al. [13] reported an inverse correlation between the level of sphingosine-1-phosphate (S1P), dihydro-S1P (sphinganine-1-phosphate, SA1P) and C24:1 ceramide in HDL fraction and occurrence of ischemic heart disease. However, the total serum S1P level in the patients was similar to the healthy ones. Kizitunic et al. [14] showed higher level of serum total S1P in patients with pre-infarction angina than without the symptom. According to Deutschman et al. [15] the serum plasma level of S1P was increasing with advancement of coronary artery disease.

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Received October 26, 2016; **Accepted** December 05, 2016; **Published** December 10, 2016

Citation: Knapp M, Lisowska A, Baranowski M, Lewkowicz J, Krajewska A, et al. (2016) Blood Bioactive Sphingolipids and Activity of Acid Sphingomyelinase in Patients with Multivessel Coronary Artery Disease. J Clin Exp Cardiolog 7: 482. doi:10.4172/2155-9880.1000482

Sattler et al. [16] found reduction in the total plasma HDL-bound S1P and elevation in the level of non-HDL bound S1P in patients with stable coronary heart disease and after myocardial infarction. Further work of the authors [17] revealed that the level of HDL-bound S1P remained stable with the advancement of the disease. It was previously reported that myocardial infarction in humans affects metabolism of bioactive sphingolipids not only in plasma but also in erythrocytes and platelets. In erythrocytes, it manifests with elevation in the level of S1P, SA1P, sphingosine, sphinganine and ceramide. In platelets, only the level of S1P and SA1P increased [18]. No data are available on the level of different bioactive sphingolipids in the blood cells in patients with multivessel coronary artery disease. It was, therefore, the aim of the present study to examine the level of S1P, SA1P, ceramide, sphingosine and sphinganine in plasma, erythrocytes and platelets of patients with the disease. The plasma activity of acidic secretory sphingomyelinase was also measured.

Material and Methods

Subjects

The study conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee for Human studies of the Medical University of Białystok. All subjects gave their informed consent prior to their inclusion in the study. The study includes 36 subjects of both sexes with multivessel coronary artery disease confirmed angiographically recommended to coronary artery by-pass grafting at the Department of Cardiosurgery at the Medical University of Bialystok. According to ESC guidelines significant stenosis of coronary artery vessel was regarded when the vessel lumen was reduced by more than 50%. Patients were excluded when they had prior coronary intervention. The control group consisted of 20 age-matched subjects without changes in the coronary arteries. Clinical characteristics of each group are given in Table 1. Blood was taken from the antecubital vein into heparinized syringes after an overnight fast. All clinical laboratory data were collected and processed in a manner consistent with ESC guidelines.

Blood fractionation

Blood was fractionated as described previously [18]. 4 ml of the fresh blood was centrifuged at 300 × g for 10 min at room temperature and the platelet-rich plasma was transferred to another tube. The leukocyte-rich buffy coat was thoroughly removed. Erythrocytes were washed with phosphate-buffered saline (PBS) and then flash frozen in liquid nitrogen. Platelet-rich plasma was centrifuged at 1000 xg for 10 min to isolate thrombocytes. Supernatant was then transferred to a new plastic tube and recentrifuged at 5000 xg for 10 min to obtain platelet-free plasma. Isolated platelets were washed with platelet wash buffer (5 mM KH_2PO_4, 5 mM Na_2HPO_4, 0.1 M NaCl, 1% glucose, 0.63% sodium citrate, pH 6.6), suspensed in PBS, and flash frozen in liquid nitrogen. All samples were stored at -80˚C until analysis.

Hemoglobin concentration in erythrocyte suspensions was determined colorimetrically using Drabkin's reagent kit (Sigma). Protein concentration in platelet samples was measured with the BCA protein assay kit (Sigma). Bovine serum albumin (fatty acid free, Sigma) was used as a standard.

Determination of the concentration of sphingolipids

Sphingoid bases: Concentration of free sphingoid bases (sphingosine and sphinganine) and sphingoid base-1-phosphates (S1P and SA1P) was measured simultaneously by the method of Min et al. [19]. Briefly, 750 µl of acidified methanol and internal standards (10 pmol of C17-sphingosine and 30 pmol of C17-S1P, Avanti Polar Lipids) were added to 250 µl of plasma or 150 µl of erythrocyte or platelet suspension, and the samples were ultrasonicated in ice-cold water for 1 min. Lipids were then extracted by addition of 750 µl of chloroform, 750 µl of 1 M NaCl and 75 µl of 3N NaOH. The alkaline aqueous phase containing S1P and SA1P was transferred to a fresh tube. The amount of sphingoid base-1-phosphates was determined indirectly after dephosphorylation to sphingosine and sphinganine with the use of alkaline phosphatase (bovine intestinal mucosa, Sigma). To improve the extraction yield of released free sphingoid bases some chloroform was carefully placed at the bottom of the reaction tubes. The chloroform fractions containing free sphingosine and sphinganine or dephosphorylated sphingoid bases were washed with alkalized water (pH adjusted to 10 with ammonium hydroxide) and then evaporated under a nitrogen stream. The dried lipid residues were redissolved in ethanol, converted to their o-phthalaldehyde derivatives and analyzed using HPLC system (ProStar, Varian Inc.) equipped with a fluorescence detector and C18 reversed-phase column (Varian Inc., OmniSpher 5, 4.6 × 150 mm). The isocratic eluent composition of acetonitrile (Merck): water (9:1, v/v) and a flow rate of 1 ml/min were used. Column temperature was maintained at 30°C.

Ceramide: Fifty µl of the chloroform phase containing lipids extracted as described above was transferred to a fresh tube containing 40 pmol of N-palmitoyl-D-erythro-sphingosine (C17 base) (a kind gift of Dr. Z. Szulc, Medical University of South Carolina) as an internal standard. Samples were then evaporated under a nitrogen stream, redissolved in 1.2 ml of 1 M KOH in 90% methanol and heated at 90°C for 60 minutes to convert ceramide into sphingosine. This digestion procedure does not convert complex sphingolipids, such as sphingomyelin, galactosylceramide or glucosylceramide, into free sphingoid bases [20]. Samples were then partitioned by the addition of chloroform and water and the upper phase was discarded. The lower phase was evaporated under nitrogen and then redissolved in

	Study group	Control	p
Age (years)	63 ± 11	65 ± 10	0.46
Sex (males/females)	24/12	12/8	0.39
BMI	28 ± 3	27 ± 3	0.62
Hypertension (%)	92	50	0.0059
Hyperlipidemia (%)	77	50	0.14
Smoking (%)	19	0	0.17
Diabetes (%)	37	6	0.085
Total cholesterol (mg%)	185 ± 54	185 ± 41	0.97
LDL cholesterol (mg%)	114 ± 19	117 ± 42	0.89
HDL cholesterol (mg%)	49 ± 9	51 ± 10	0.69
Triglyceride (mg%)	137 ± 39	132 ± 73	0.8
Blood glucose (mg%)	121 ± 27	96 ± 12	0.06
Hemoglobin (g/dl)	13,7 ± 1,2	12,9 ± 1,5	0.09
Red blood cell count (× 10^6/µl)	4,6 ± 0,4	4,2 ± 0,5	0.015
Platelet count (× 10^3/µl)	251 ± 70	212 ± 70	0.09
GFR (ml/min/1.73 m^2)	74 ± 14	67 ± 17	0.26
EF %	54 ± 7	58 ± 7	0.06
ACE inhibitors (%)	39	15	0.27
Aspirin (%)	100	15	0.000001
Proton-pump inhibitors (%)	100	85	0.24
Beta-blockers (%)	100	85	0.24
Statins (%)	100	25	0.032

Table 1: Clinical characteristics of study and control group.

ethanol. The content of free sphingosine liberated from ceramide was then analyzed by means of HPLC as described above. The calibration curve was prepared using N-palmitoylsphingosine (Avanti Polar Lipids) as a standard. The chloroform extract used for the analysis of ceramide contains small amounts of free sphingoid bases. Therefore, the concentration of ceramide was corrected for the level of free sphingosine determined in the same sample.

The average within-run variations (%CV) for plasma sphingolipids quantified using the above method ranged from 4.1 to 9.8% (for ceramide and sphinganine, respectively). The between-run variations ranged from 0.8 to 10.7% (for ceramide and sphinganine, respectively).

Determination of the plasma activity of secretory acidic sphingomyelinase

The plasma activity of secretory acidic sphingomyelinase (S-smase) was measured as described previously [21,22]. Briefly, 50 µl of plasma was incubated for 1 h in 100 mM Sodium acetate, pH=5.0, with 1% Triton X-100, 40 µM EDTA and 1 mM [Choline-Methyl-14C]-Sphingomyelin (specific activity 1200 DPM/nmol). For zinc-dependent S-smase, zinc chloride was added to a final concentration of 100 µM. For zinc-independent S-smase, zinc chloride was omitted from incubation buffer. Reaction was stopped by addition of 1400 µl of $CHCl_3$/Methanol (2:1, v/v) and reaction product (methyl-14C-phosphorylcholine) was extracted by vortexing and centrifugation. Radioactivity of released product was estimated in 500 µl of aqueous phase with Packard Tri-Carb 1900 Liquid Scintillation Counter. Activity was expressed in nmol of liberated methyl-14C-phosphorylcholine x hour-1 x ml-1 of plasma.

Statistical analysis

Descriptive statistics (percentages for discrete variables and mean ± SD for continuous variables) was done for baseline characteristics. Parameters distribution was assessed using Shapiro-Wilk test. Continuous variables between groups were compared using ANOVA test and the χ^2 test was used for categorical variables. The ROC curves and area under curve (AUC) were performed to determine optimal value of S1P and SA1P for recognition significant IHD. A p value ≤ 0.05 was considered statistically significant. Statistic software Statistical 10PL was applied.

Results

In the group of patients the plasma level of S1P and SA1P was reduced as compared to the respective values in the control group ($p<0.001$). The level of sphingosine, sphinganine and ceramide was similar in both groups (Figure 1). The level of S1P and SA1P below which indicates the presence of significant coronary lesions was determined. It is properly 180.73 pmol/ml for S1P (sensitivity 84%; specificity 62%) (Figure 2) and 23.02 pmol/ml for SA1P (sensitivity 80%; specificity 81%) (Figure 3). The plasma ceramide/sphingosine-1-phosphate ratio is elevated by 54% in the group of patients.

In erythrocytes, the level of each sphingolipid examined in the group of patients was similar to the comparable value in the control group (Figure 4).

In platelets, the level of sphingosine and sphinganine in the group of patients was elevated as compared to the respective control value ($p<0.001$) whereas the levels of other sphingolipids were similar in both groups (Figure 5).

The activity of plasma acidic sphingomyelinase in the group of patients was higher than in the control group ($p<0.03$; Figure 6).

Discussion

As indicated in the introduction the results obtained so far regarding the level of plasma S1P and ceramide in patients with coronary heart disease are not uniform. Argraves et al. [13] and Sattler et al. [16] reported reduced level of plasma HDL-bound S1P in patients with ischemic heart disease. The total level of plasma S1P was stable in the group of patients. However, the level of non-HDL bound S1P increased in patients with ischemic heart disease. According to Deutschman et al. [15] the serum level of total S1P increases with advancement of the disease. The latter was not confirmed by Sattler et al. [17]. The plasma level of ceramide in patients with coronary heart disease was reduced in one work [13] and elevated in another study [15]. Here, we showed that total plasma level of S1P and SA1P was reduced and the plasma level of ceramide remained unchanged in patients recommended to coronary artery by-pass grafting. It is difficult to explain a reason of the discrepancies. The present as well as Argraves et al. [13] data seem to be logical in the light of the anti-atherogenic properties of S1P showed in *in vitro* studies [12]. Those data suggest that reduction in S1P plasma level could accelerate development of atherosclerosis. It should be added that the total plasma concentration of S1P and SA1P was reduced after the myocardial infarction in humans. The level of sphingosine, sphinganine and ceramide remained stable [18,23]. On

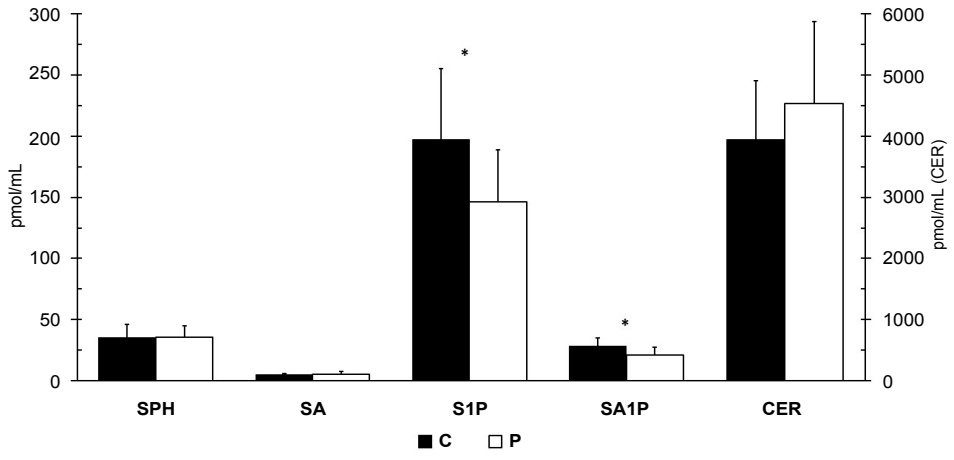

Figure 1: The plasma level of bioactive shingolipids in patients with multivessel coronary artery disease. C: Controls; P: Patients with the disease; SPH: Sphingosine: SPA: Sphinganine; S1P; Sphingosine-1-phosphate; SA1P: Sphinganine-1-phosphate; CER: Ceramide.

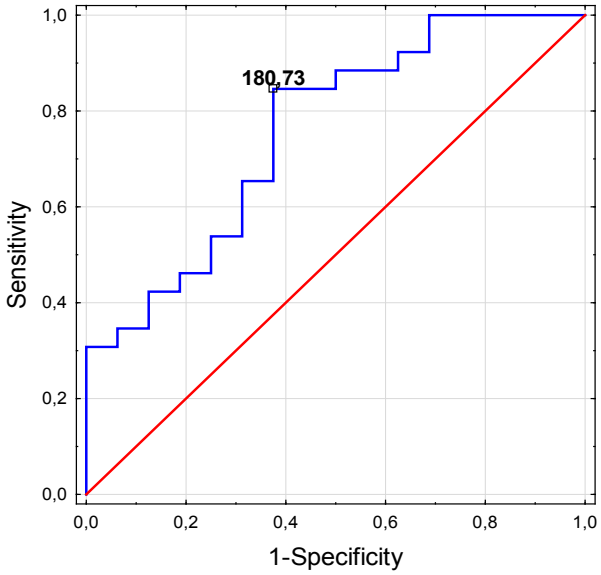

Figure 2: ROC curve for S1P level in patients with multivessel coronary artery disease vs. control group. Cutt off value 180.73 pmol/ml (sensitivity 84%; specificity 62%; area under the ROC 0.757; 95%CI 0.60-0.90).

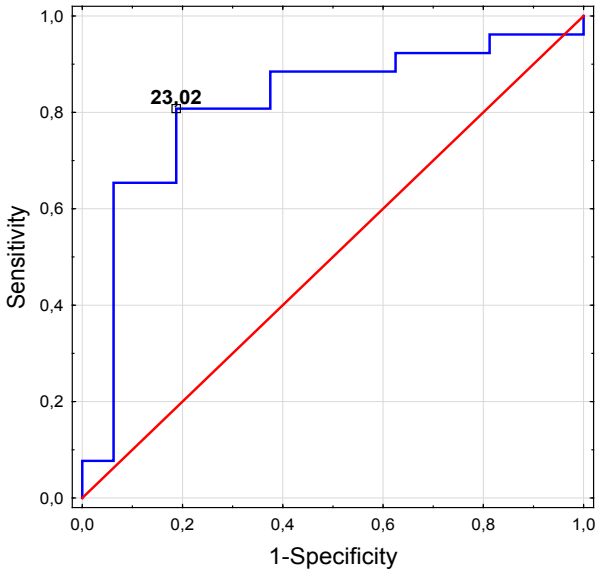

Figure 3: ROC curve for SA1P level in patients with multivessel coronary artery disease *vs.* control group. Cutt off value 23.02 pmol/ml (sensitivity 80%; specificity 81%; area under the ROC 0.813; 95%CI 0.67-0.95).

the other hand Egom et al. [24] reported severalfold elevation in the plasma level of S1P and sphinganine and two-fold elevation in the level of sphingosine already in 1 min after elective percutaneous coronary intervention (PCI). The discrepancy could be caused by different time points of the blood sampling: after infarction and before PCI in the studies of Knapp et al. [18,23] and after elective PCI in patients without the infarction in the study of Egom et al. [24]. Certainly more work is needed to delineate a role of S1P in development of atherosclerosis.

As already mentioned, ceramide activates and S1P inhibits apoptosis. It has been suggested that the final biological effect of S1P and ceramide depends rather on the relative proportion between the two compounds than on the level of either sphingolipid itself [25]. In the present study, the ratio: the plasma level of ceramide/the plasma level of S1P were calculated from the mean values. It was higher by 54% in the group patients than in the control group. It further indicates on the reduction of the anti-atherogenic potential of the bioactive sphingolipids in the plasma of patients with advanced atherosclerosis.

Erythrocytes are the main source of plasma S1P [26-30]. Platelets and endothelial cells also contribute to the plasma pool of S1P [30-33]. However, the blood cells do not synthetize sphingolipids *de novo*. They take up plasma sphingosine and phosphorylate it to sphingosine-1-

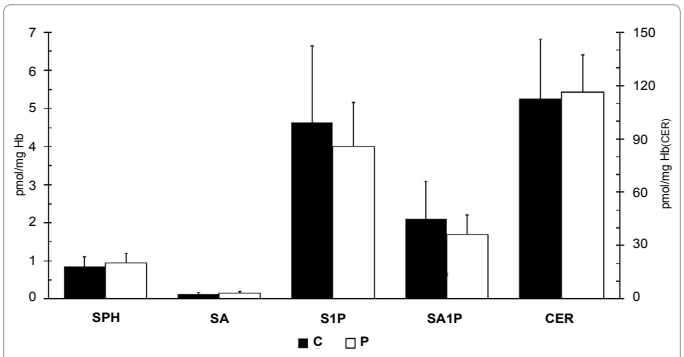

Figure 4: The level of bioactive sphingolipids in erythrocytes in patients with multivessel coronary artery disease. The denominations as in the legend to the Figure 1.

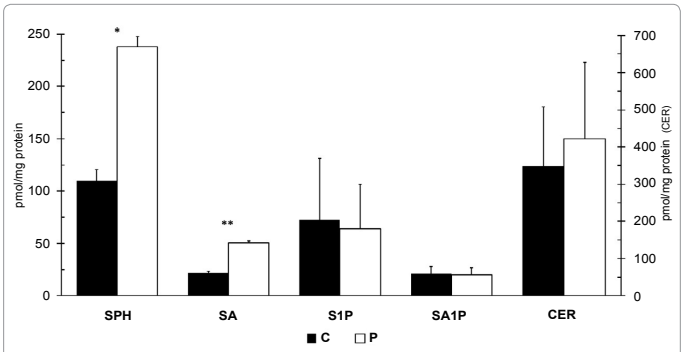

Figure 5: The level of bioactive sphingolipids in platelets in patients with multivessel coronary artery disease. The denominations as in the legend to the Figure 1.

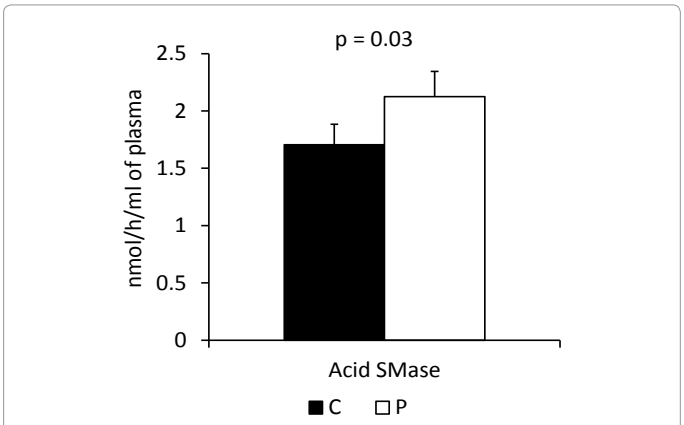

Figure 6: The activity of secretory sphingomyelinase in patients with multivessel coronary artery disease. C-control group, P- group of patients.

posphate. The compound is stored in the cells and released into plasma. Here, we showed that the levels of examined sphingolipids in erythrocytes remain unchanged in the group of patients. Also in platelets, the level of S1P was stable. It would suggest that the contribution of the blood cells to the plasma S1P remained on the same level in both groups. It is likely that release of S1P from the endothelial cells was reduced in atherosclerosis with subsequent reduction in the plasma level of the compound. The plasma S1P is dephosphorylated by different unspecific phosphatases. Their activity could increase in atherosclerosis and thus also contribute to the reduction in the plasma level of the compound.

The level of sphingosine and sphinganine in platelets was elevated in the group of patients. It was a consequence of either increased uptake of the compounds from plasma or inhibition of their phosphorylation in the cells. Anyhow, it indicates that atherosclerosis induces some changes in metabolism of sphingolipids in platelets. Its meaning for the function of the cells remains unknown.

The activity of plasma S-smase was elevated in the group of patients. Thus, we confirmed the data of Pan et al. [11]. Elevation in the plasma activity of S-smase is, certainly, caused by increased release of the enzyme from endothelial cells.

In summary, we have shown that in patients with multivessel coronary artery

a) The plasma ceramide/S1P ratio and the activity of secretory acid sphingomyelinase are elevated.

b) The disease affects metabolism of sphinganine (a precursor of ceramide) and sphingosine (product of ceramide catabolism) in the platelets.

c) The disease does not affect the content of examined sphingolipids in erythrocytes.

Conclusion

The results obtained indicate that metabolism of ceratain bioactive sphingolipids in plasma, and platelets in patients with multivessel coronary artery disease are changed as compared to the control group.

Acknowledgments

This work was supported by Medical University of Białystok, grant 133-53905L.

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Citation: Knapp M, Lisowska A, Baranowski M, Lewkowicz J, Krajewska A, et al. (2016) Blood Bioactive Sphingolipids and Activity of Acid Sphingomyelinase in Patients with Multivessel Coronary Artery Disease. J Clin Exp Cardiolog 7: 482. doi:10.4172/2155-9880.1000482