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# Dose-dependent effect of aspirin on the level of sphingolipids in human blood

Knapp M[1], Lisowska A[1], Knapp P[2], Baranowski M[3,*]

1 Department of Cardiology, Medical University of Bialystok, Bialystok, Poland
2 Department of Gynecology, Medical University of Bialystok, Bialystok, Poland
3 Department of Physiology, Medical University of Bialystok, Bialystok, Poland

* CORRESPONDING AUTHOR:
Department of Physiology
Medical University of Bialystok
Mickiewicza 2c,
15-222 Białystok,Poland
Tel.: +4885 748 5624
Fax.: +4885 748 5585
email: marcinb@umb.edu.pl (Marcin Baranowski)

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## ABSTRACT

**Purpose:** Aspirin is an antiplatelet drug which is commonly used in secondary prevention in ischemic heart disease and cerebrovascular events, and in newly diagnosed myocardial infarction. The aim of the present study was to examine effect of aspirin on the level of selected sphingolipid intermediates in plasma, erythrocytes and platelets.

**Material and Method:** Forty two healthy volunteers participated in the study. They were divided into two groups. In one group aspirin was given orally, daily, for one week in a dose of 75mg (n=25). The subjects from the second group received one 300mg dose of the drug (n=17). In both groups the blood was taken 4h after the last dose of aspirin. The following sphingolipid intermediates were quantified using high-pressure liquid chromatography: sphinganine, sphingosine, sphingosine-1-phosphate (S1P), sphinganine-1-phosphate (SA1P) and ceramide.

**Results:** It was found that lower dose of aspirin increased the level of S1P and ceramide in erythrocytes (by 23 and 37%, respectively) having no effect on plasma and platelet sphingolipid levels. Higher dose of the drug reduced S1P and SA1P concentration in the plasma (by 16 and 10%, respectively).

**Conclusion:** We conclude that aspirin interferes with sphingolipid metabolism in blood and that this effect depends on a dose of the drug. Since S1P is a potent cardioprotectant, the reduction in its plasma concentration after the loading dose of aspirin could be undesired side effect of the drug.

**Key words:** acetylsalicylic acid, NSAID, red blood cells, sphingoid base-1-phosphate, thrombocytes

## INTRODUCTION

Aspirin (acetylsalicylic acid, ASA) irreversibly inactivates the cyclooxygenase activity of prostaglandin H synthase 1 and 2 (COX1 and COX2, respectively). As a result, it blocks formation of thromboxane $A_2$ ($TXA_2$) in platelets, and inhibits thrombocyte activation and aggregation [1,2]. Since platelets do not contain the nucleus they cannot synthesize new COX after inhibition. Newly formed platelets contain the active enzyme and thus are able to synthetize $TXA_2$. Newly formed platelets contain both COX1 and COX2 but mature platelets contain only COX1. Therefore, inhibition of platelet COX1 is sufficient to explain the antithrombotic effects of a low dose of aspirin [1]. Daily intake of a low dose (75-

100mg) of aspirin is recommended by the European Society of Cardiology in secondary prevention in ischemic heart disease and cerebrovascular events. Higher (loading) single dose (150-300mg) is recommended by the Society for patients with newly diagnosed myocardial infarction [3].

Recent data clearly indicate that certain sphingolipid intermediates, namely sphingosine-1-phosphate (S1P), ceramide and sphingosine strongly affect the heart's response to ischemia/reperfusion injury. S1P exerts cardioprotective action, whereas ceramide augments (induces) apoptosis [4,5]. On the other hand, sphingosine in a low dose is cardioprotective but in a high dose is cardiotoxic [6]. We have previously shown that plasma sphingolipid levels are altered in patients with acute myocardial infarction or chronic systolic heart failure [7,8]. Sphingolipids are present in platelets and can be released upon their activation [9-11]. So far, there is only one report showing that celecoxib, a non-steroidal anti-inflammatory drug, reduces the concentration of S1P and sphinganine-1-phosphate (SA1P) in human plasma [12]. The aim of the present study was to examine effect of aspirin on the level of different sphingolipids in the plasma, erythrocytes and platelets. The data obtained clearly indicate that aspirin affects the level of the sphingolipid intermediates in different blood compartments.

## METHODS

The investigation conforms with the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee for Human Studies of the Medical University of Bialystok. All patients gave their informed consent prior to their inclusion in the study. Forty two healthy volunteers of both sexes, aged 23-24 years were recruited for the study. The subjects were divided into two groups: one group (n=25, 11 males and 14 females) received aspirin (Polfa) orally, at a dose of 75mg daily for one week. This period of low dose of aspirin intake was selected after Patrignani et al. [13]. They showed a cumulative and complete inhibition of platelet $TXB_2$ (it is stable breakdown product of $TXA_2$) after 7-day intake of a low dose of aspirin. The second group (n=17, 7 males and 10 females) received a single, loading dose (300mg) of chewed, plain aspirin (3). In both groups the blood was taken from the antecubital vein before the treatment and 4h after the last dose of the drug.

### Blood fractionation

Blood samples (4ml) were collected into EDTA coated vacutainers, centrifuged at 300×g for 10 minutes, and the platelet-rich plasma was transferred to a fresh tube. The buffy coat was then thoroughly removed. Separated erythrocytes were suspended in 3ml of phosphate-buffered saline (PBS), centrifuged at 800×g for 10 minutes and the upper layer as well as the remaining buffy coat were discarded. Red blood cells were then re-suspended in 2 ml of PBS and flash frozen in liquid nitrogen. Platelet-rich plasma was centrifuged at 1000× g for 10 minutes to separate platelets. Supernatant was then transferred to a fresh tube and re-centrifuged at 5000× g for 10 minutes to obtain platelet-free plasma. Isolated thrombocytes were washed with 1ml of platelet wash buffer (5mM $KH_2PO_4$, 5mM $Na_2HPO_4$, 0.1M NaCl, 1% glucose, 0.63% sodium citrate, pH 6.6). The cells were then suspended in 0.3ml of PBS, and flash frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

Hemoglobin concentration in erythrocyte suspensions was determined using Drabkin's reagent kit (Sigma). Protein concentration in platelet suspensions was measured with the BCA protein assay kit (Sigma).

### Concentration of blood sphingolipids

The level of ceramide, free sphingoid bases and sphingoid base-1-phosphates was determined as described previously in detail [14]. Briefly, 750µl of acidified methanol and internal standards (10 pmol of $C_{17}$-sphingosine and 30 pmol of $C_{17}$-S1P, Avanti Polar Lipids) were added to 250µl of plasma or 150µl of erythrocyte or platelet suspension, and the samples were ultrasonicated in ice-cold water for 1 min. Lipids were then extracted by addition of chloroform, 1M NaCl and 3N NaOH and the aqueous phase containing sphingoid base-1-phosphates 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, Fluka). Free sphingoid bases as well as sphingosine and sphinganine formed during dephosphorylation of sphingoid base-1-phosphates were converted to their o-phthalaldehyde derivatives and analyzed using HPLC system equipped with a fluorescence detector and C18 reversed-phase column (Varian Inc. OmniSpher 5, 4.6×150mm). The isocratic eluent composition of acetonitrile (Merck): water (9:1, v/v) and a flow rate of 1 ml/min were used.

For ceramide assay 50µl of the chloroform phase containing extracted lipids was transferred to a fresh tube with pre-added 40pmol of N-palmitoyl-D-erythro-sphingosine (C17 base) (a kind gift of Dr Z. Szulc, Medical University of South Carolina) as an internal standard. The samples were then subjected to alkaline hydrolysis to deacylate ceramide. The content of free sphingosine liberated from ceramide was then analyzed by means of HPLC as described above.

### Statistical analysis

All data are presented as means ± SD. Statistical comparisons were made by using the paired samples t test. $P<0.05$ was considered statistically significant.

***Table 1.*** **Hematological parameters of the two groups of subjects studied.**

| | Low dose of aspirin | | High dose of aspirin |
|---|---|---|---|
| | Basal | After treatment | |
| Red blood cell count ($\times 10^6/\mu l$) | 4.77±0.43 | 4.68±0.45 | 4.60±0.48 |
| Hemoglobin (g/dl) | 14.0±1.5 | 13.8±1.6 | 14.2±1.4 |
| Platelet count ($\times 10^3/\mu l$) | 251±52 | 250±55 | 244±47 |

*The results are means ± SD. Healthy volunteers were treated with either the low (75mg daily for one week, n=25) or high (single loading dose of 300mg, n=17) dose of aspirin.*

## RESULTS

There were no significant differences in erythrocyte count, platelet count or hemoglobin concentration between the experimental groups. In addition, no change in any of the above parameters was observed after one week of treatment with the low dose of aspirin (*Tab. 1*).

Low dose of aspirin did not induce statistically significant changes in the concentration of either examined compound in the plasma. On the other hand, high dose of aspirin significantly decreased plasma concentration of S1P and SA1P (by 16 and 10%, respectively). This effect was observed in 88 and 81% of the subjects, respectively. On the other hand, the concentration of sphingosine and sphinganine remained stable. There was also a trend towards an increase in plasma ceramide level (by 16%), however, it did not reach statistical significance (*Fig. 1*).

Low dose of aspirin increased the level of ceramide, S1P and SA1P in erythrocytes (by 37, 23 and 17%, respectively), however, in the latter case the difference was of borderline significance. This trend was observed in 67, 63 and 67% of the subjects, respectively. The erythrocyte level of all examined compounds after the high dose of the drug remained stable (*Fig. 2*). Neither low nor high dose of aspirin induced statistically significant changes in the content of the examined sphingolipids in platelets. There was, however, a trend towards an increase in thrombocyte sphingosine and ceramide level (by 25 and 18%, respectively) after treatment with the low dose of the drug (*Fig. 3*).

## DISCUSSION

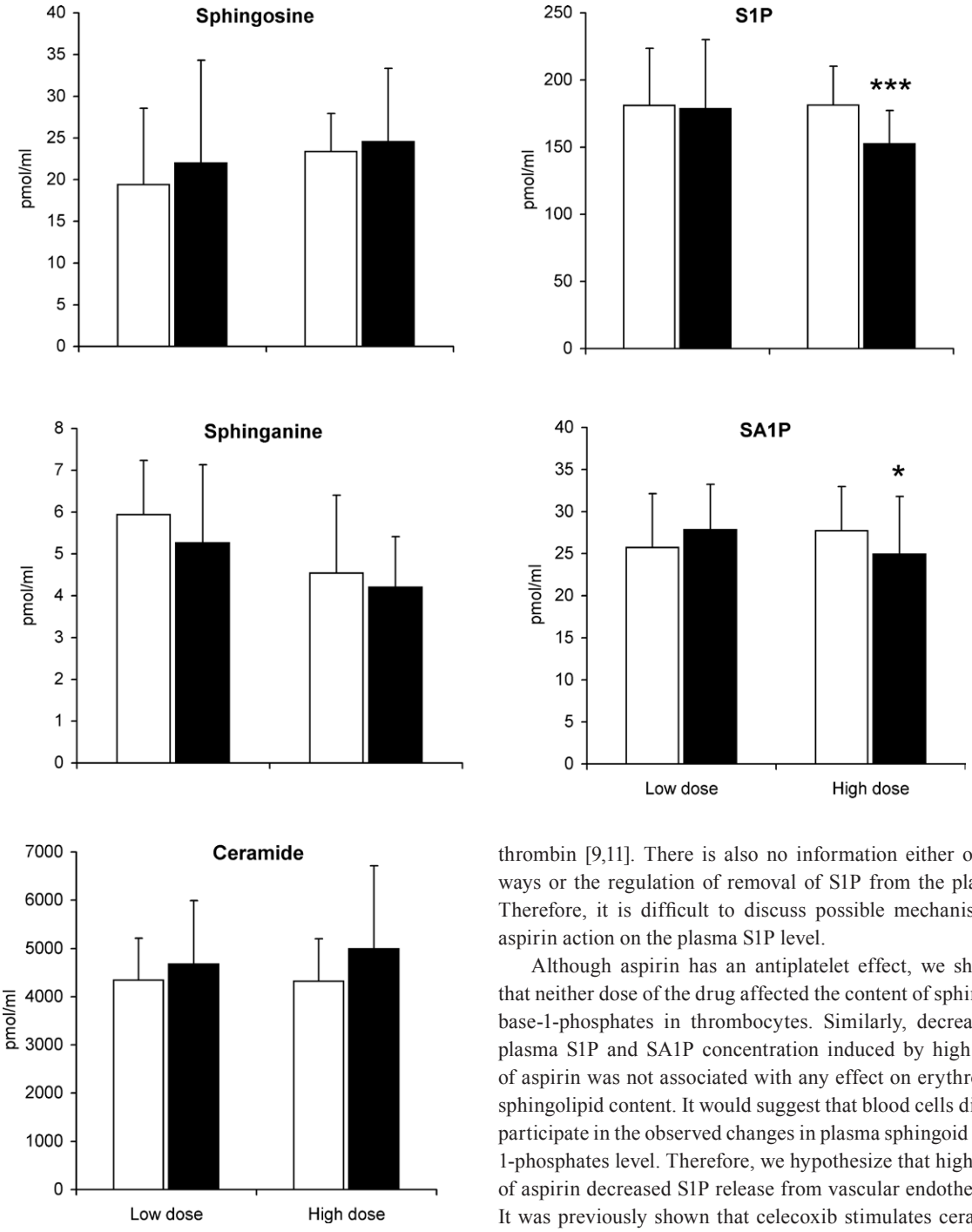
This study is the first one to report data on effect of aspirin on the level of different sphingolipid intermediates in the plasma, erythrocytes and platelets. As already mentioned in the introduction, Schmidt et al. [12] found reduced level of plasma S1P and SA1P in healthy subjects after a single dose of 200mg of celecoxib, a non-steroidal anti-inflammatory drug. We confirmed it presently for aspirin in a single dose of 300mg. Interestingly, a lower dose (75mg) which is routinely used in secondary prevention had no effect.

We previously showed that plasma level of S1P in patients with the myocardial infarction was reduced [7]. We hypothesized that it could be caused by the treatment with aspirin. This hypothesis seems to be confirmed by the present study. S1P is a potent cardioprotective compound against ischemia/reperfusion injury [4-6]. In addition, plasma S1P is an important factor regulating lymphocyte trafficking and maintaining endothelial barrier function [15,16]. Therefore, the reported reduction in plasma S1P level after the high dose of aspirin could be, yet unknown, undesirable side effect of the drug. SA1P is the product of phosphorylation of sphinganine, a precursor of ceramide in the de novo sphingolipid synthesis pathway [17]. The level of SA1P in the plasma is much lower than that of S1P [18,19]. It binds to S1P receptors and likely exerts the same effect (though much weaker, due to lower concentration) as S1P [20]. The loading dose of aspirin reduced the level of plasma SA1P. To date the role of SA1P has not been recognized. Therefore, consequences of the reduction in its plasma level are probably negligible.

We observed some statistically significant differences in basal blood cell sphingolipid levels between the two experimental groups (i.e. S1P in erythrocytes and sphingosine and sphinganine in platelets). We believe that these differences result from the fact that experiments with low and high dose of aspirin were conducted in different time periods. Samples from the former group were taken in the winter, whereas in the case of the latter group they were taken in the summer. Abnet et al. [21] showed that plasma sphingosine level is correlated with fruit and vegetable consumption. On the other hand, we have previously found that physical activity alters S1P, sphingosine and sphinganine content in erythrocytes [14]. Therefore, it is likely that seasonal variations in diet and physical activity can affect blood sphingolipid levels. In addition, it is possible that seasonal differences in the ambient temperature during the process of blood cell isolation contributed to the observed variations between the two groups of subjects. However, in our opinion the above facts do not diminish the significance of our results since effect of aspirin was determined on the basis of comparison of samples taken from the same subjects before and after treatment.

Erythrocytes are claimed to be the major source of plasma S1P [18,22-24]. Platelets and vascular endothelium also contribute to this pool of S1P, although the proportions

***Figure 1.*** **Concentrations of different sphingolipids in plasma of healthy volunteers before (white bars) and after (black bars) treatment with the low (75mg daily for one week, n=25) or high (single loading dose of 300mg, n=17) dose of aspirin.**



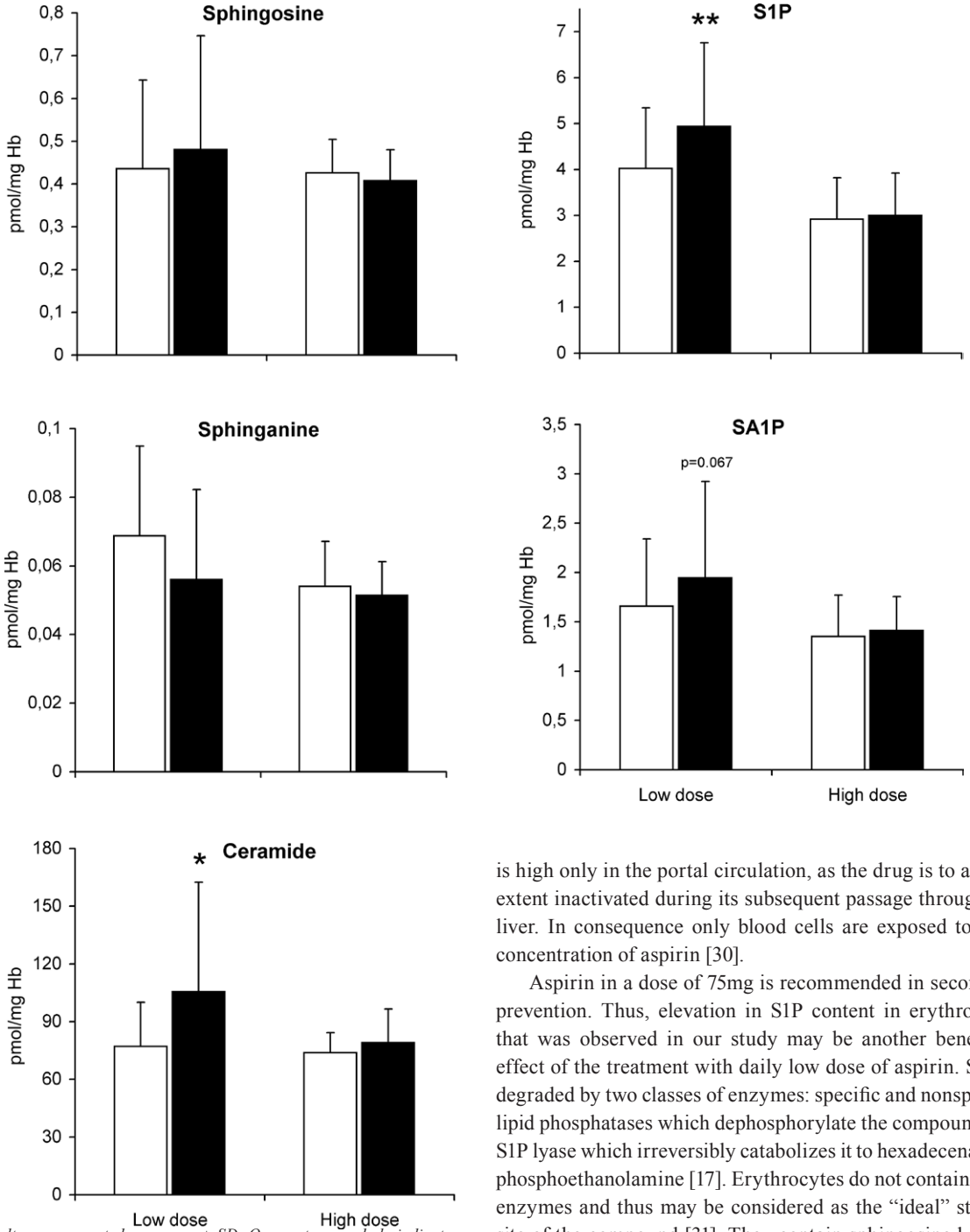

*The results are presented as means ± SD. One or three symbols indicate a significant difference vs. the basal concentration at the p<0.05 or p<0.001 levels, respectively. S1P – sphingosine-1-phosphate, SA1P – sphinganine-1-phosphate.*

of contribution remain unknown [9,25,26]. It should be emphasized that factors stimulating liberation of S1P from blood cells are not recognized so far. The only known exception is stimulation of S1P release from platelets by thrombin [9,11]. There is also no information either on the ways or the regulation of removal of S1P from the plasma. Therefore, it is difficult to discuss possible mechanism of aspirin action on the plasma S1P level.

Although aspirin has an antiplatelet effect, we showed that neither dose of the drug affected the content of sphingoid base-1-phosphates in thrombocytes. Similarly, decrease in plasma S1P and SA1P concentration induced by high dose of aspirin was not associated with any effect on erythrocyte sphingolipid content. It would suggest that blood cells did not participate in the observed changes in plasma sphingoid base-1-phosphates level. Therefore, we hypothesize that high dose of aspirin decreased S1P release from vascular endothelium. It was previously shown that celecoxib stimulates ceramide synthesis from sphingosine in mice tissues [27], which potentially could result in reduced S1P production. It was also postulated that the anticancer properties of nonsteroidal antiinflammatory drugs result, at least in part, from inhibition of S1P synthesis [28].

We can only speculate as to the reason why low dose of aspirin had no effect on plasma sphingoid base-1-phosphate concentration. Interestingly, it was found that although a

***Figure 2.*** **Content of different sphingolipids in erythrocytes isolated from healthy volunteers before (white bars) and after (black bars) treatment with the low (75mg daily for one week, n=25) or high (single loading dose of 300mg, n=17) dose of aspirin.**

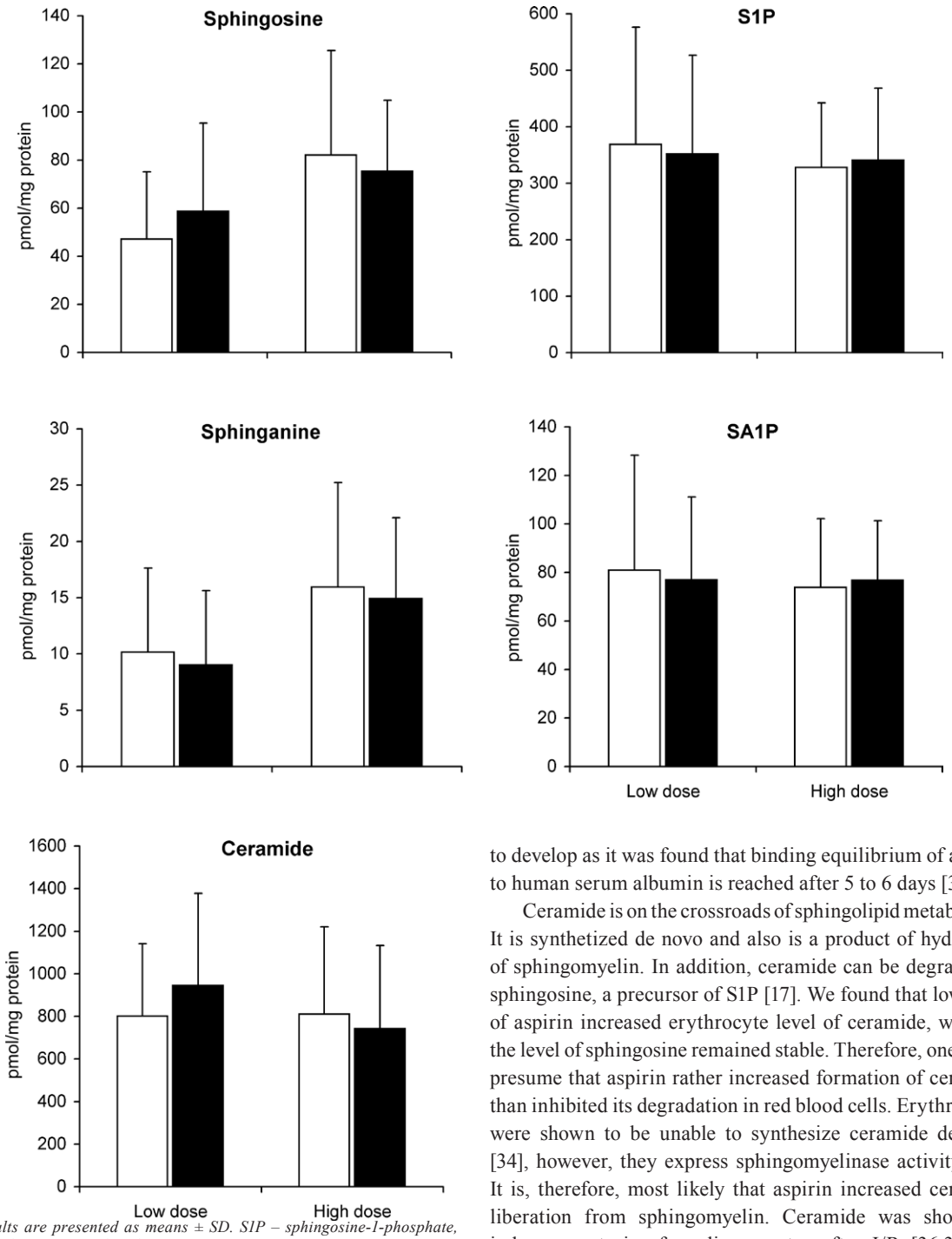



*The results are presented as means ± SD. One or two symbols indicate a significant difference vs. the basal concentration at the p<0.05 or p<0.01 levels, respectively. S1P – sphingosine-1-phosphate, SA1P – sphinganine-1-phosphate.*

single dose of 80mg of aspirin is sufficient for almost complete inhibition of thromboxane generation in platelets, a dose of 325mg is required for efficient inhibition of prostacyclin production in vascular endothelium [29]. It is generally thought that after low oral dose of aspirin its concentration is high only in the portal circulation, as the drug is to a large extent inactivated during its subsequent passage through the liver. In consequence only blood cells are exposed to high concentration of aspirin [30].

Aspirin in a dose of 75mg is recommended in secondary prevention. Thus, elevation in S1P content in erythrocytes that was observed in our study may be another beneficial effect of the treatment with daily low dose of aspirin. S1P is degraded by two classes of enzymes: specific and nonspecific lipid phosphatases which dephosphorylate the compound and S1P lyase which irreversibly catabolizes it to hexadecenal and phosphoethanolamine [17]. Erythrocytes do not contain these enzymes and thus may be considered as the "ideal" storage site of the compound [31]. They contain sphingosine kinase, the enzyme responsible for phosphorylation of sphingosine to S1P. It is also likely that they can take up S1P from plasma [18]. In our study, the content of S1P in erythrocytes did not mirror the changes in the level of the compound in the plasma. Therefore, one may presume that the elevation in S1P was not caused by its increased uptake from the plasma. Similarly to S1P, both aspirin and its primary metabolite – salicylic acid bind to plasma albumin [32,33]. It is, therefore, possible

***Figure 3.*** **Content of different sphingolipids in platelets isolated from healthy volunteers before (white bars) and after (black bars) treatment with the low (75mg daily for one week, n=25) or high (single loading dose of 300mg, n=17) dose of aspirin.**




*The results are presented as means ± SD. S1P – sphingosine-1-phosphate, SA1P – sphinganine-1-phosphate.*

that aspirin-induced elevation in erythrocyte S1P level was a result of slightly impaired albumin-driven export of S1P from these cells. It is an open question why the single loading dose of the drug was ineffective in this regard. However, we speculate that longer treatment may be required for this effect to develop as it was found that binding equilibrium of aspirin to human serum albumin is reached after 5 to 6 days [33].

Ceramide is on the crossroads of sphingolipid metabolism. It is synthetized de novo and also is a product of hydrolysis of sphingomyelin. In addition, ceramide can be degraded to sphingosine, a precursor of S1P [17]. We found that low dose of aspirin increased erythrocyte level of ceramide, whereas the level of sphingosine remained stable. Therefore, one could presume that aspirin rather increased formation of ceramide than inhibited its degradation in red blood cells. Erythrocytes were shown to be unable to synthesize ceramide de novo [34], however, they express sphingomyelinase activity [35]. It is, therefore, most likely that aspirin increased ceramide liberation from sphingomyelin. Ceramide was shown to induce apoptosis of cardiomyocytes after I/R [36,37]. Its role in erythrocyte physiology remains unknown. So, it is impossible to evaluate a significance of elevation in its content in red blood cells.

Although the aspirin-induced changes in blood sphingolipid levels were relatively small the available data suggests that they can be biologically relevant. For instance, it was shown that minor changes in the level of ceramide in cells

are sufficient to induce its biological actions [38]. Moreover, Vessey et al. [39] reported that 28% increase in myocardial S1P level was associated with significant protective effect against ischemia/reperfusion injury.

## CONCLUSIONS

In summary, we showed that loading dose of aspirin reduced the plasma level of S1P and SA1P. Aspirin in a dose recommended in secondary prevention of atherosclerosis increases the level of S1P and ceramide in erythrocytes having no effect on the level of either examined compound in other blood compartments. Therefore, we showed, for the first time, that aspirin affects metabolism of sphingolipids in the blood. S1P is a potent cardioprotective factor. Therefore, reduction in the blood level of the compound by loading dose of aspirin can possibly diminish its cardioprotective action.

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