JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2015, 66, 3, 385-389
www.jpp.krakow.pl

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EFFECT OF ATRIAL PACING ON THE LEVEL OF BIOACTIVE SPHINGOLIPIDS IN THE HEART VENTRICLES OF THE RAT

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Bioactive sphingolipids play important role in regulation of the function of the cardiomyocytes. There are no data available on metabolism of the sphingolipids in the heart under increased work-load produced by tachycardia. The aim of the present study was to examine effect of tachycardia on the level of the principal bioactive sphingolipids in the left and right ventricles. The experiments were carried out on male Wistar rats. After anesthesia, two electrodes were administered into the right common jugular vein so that their tips were placed at the vein's aperture. The resting heart rate was 355 ± 24/min and the rate of stimulation was 600/min. EKG was continuously monitored. The stimulation time was 30 and 60 min. Thereafter, blood from the abdominal aorta and samples of the left and right ventricle were taken. The following bioactive sphingolipids were quantified by means of high performance liquid chromatography: sphinganine, ceramide, sphingosine, sphingosine-1-phosphate and sphinganine-1-phosphate. In the left ventricle, 30 and 60 min tachycardia elevated the level of sphingosine, reduced the level of sphingosine-1-phosphate and sphinganine-1-phosphate. The level of ceramide was reduced only after 60 min. In the right ventricle, 60 min pacing resulted in elevation in the level of sphingosine and sphinganine and reduction in the level of other compounds studied. It is concluded that tachycardia induces changes in metabolism of bioactive sphingolipids in each ventricle. The changes may affect cardiomyocyte functions. Also, differences in sphingolipid metabolism between both ventricles are reported.

Key words: *tachycardia, bioactive sphingolipids, left and right ventricles, high performance liquid chromatography, sphinganine, ceramide, sphingosine, sphingosine-1-phosphate, sphinganine-1-phosphate*

INTRODUCTION

Principal bioactive sphingolipids, namely sphingosine-1-phosphate (S1P), ceramide and sphingosine play a very important regulatory role in the myocardium. S1P was repeatedly shown to exert strong cardioprotective action against ischemia-reperfusion injury (1). Preincubation of isolated cardiomyocytes with S1P increases their viability during hypoxia (2) and ischemia/normoxia procedures (3, 4). In perfused rat heart, addition of S1P to the perfusion medium reduces the infarct size and increases the left ventricular developed pressure after ischemia/reperfusion (5). Ischemia/reperfusion increases the content of several ceramide species in the rat myocardium (6). Ceramide promotes apoptosis of cardiomyocytes (7, 8). Accumulation of ceramide contributes to development of cardiomyopathy and loss of myocardial function (9) whereas reduction in ceramide synthesis is accompanied by improvement of cardiac function (10). Sphingosine (the product of deacylation of ceramide), in a high dose, was shown to be cardiotoxic in isolated rat heart subjected to ischemia/reperfusion injury. The same compound at physiological dose proved to be cardioprotective in the same experimental conditions (11). The results presented above indicate that changes in metabolism of sphingolipids in the myocardium may have important impact on the myocardial functioning. It was previously shown that 30 min exercise of moderate intensity reduced the content of ceramide in the left heart ventricle. Thereafter, continuation of exercise until exhaustion resulted in elevation in its content above the resting value. In the latter case, the content of sphinganine (a precursor of ceramide) was elevated thus suggesting increased *de novo* synthesis of the compound. Concomitant reduction of the activity of acid ceramidase might indicate a reduction in its removal. The content of sphingosine-1-phosphate in the myocardium remained stable (12). Tachycardia puts much burden on the heart. However, there is no data on effect of tachycardia on the level of bioactive sphingolipids in myocardium. Therefore, the aim of the present study was to examine effect of experimental tachycardia on the level of sphingosine-1-phosphate, ceramide, sphingosine, sphinganine and sphinganine-1-phosphate in left and right ventricles of the rat.

MATERIAL AND METHODS

Animals

The experimental protocol was approved by the Ethical Committee on the Animal Research at the Medical University of

Bialystok. The experiments were carried out on male Wistar rats, 7 – 8 weeks of age and 220 – 250-grams of body weight. The rats were housed in standard conditions: temperature 21°C, 12 h light/12 h dark/light cycle, had free access to tap water and commercially available rat pellet diet.

Experimental procedures

The rats were anaesthetized with thiopental (80 mg/100 g of body weight). After anesthesia, the right jugular vein was exposed and two electrodes were inserted in it. The electrodes were located at the aperture of the vein. The location of the tips of the electrodes was checked after the experiment. The electrodes were connected to SC-04 stimulator. The heart rate was continuously monitored. To do so, electrodes were administered into skeletal muscles of the four limbs and connected to a standard electrocardiograph. The resting heart rate was 355 ± 24/min. The rats were divided into three groups (n = 10 rats in each group). In control group the rats were prepared as above and kept rested for 60 min. In the second and third group the heart was stimulated for 30 and 60 min, respectively. The parameters of the stimuli were: frequency 600/min, 4V, 100 ms of duration.

Blood from the abdominal aorta and samples of the right and left ventricle were taken. The level of sphingosine, sphinganine, sphingosine-1-phosphate, sphinganine-1-phosphate and ceramide in the muscle samples was quantified as described previously in detail (12).

Briefly, the samples were homogenized in a solution composed of 25 mM HCl and 1M NaCl and acidified with methanol. Internal standards of C_{17} - sphingosine and C_{17} - sphingosine 1-phosphate (Avanti Polar Lipids, Alabaster, AL., USA) were added and the samples were ultrasonicated. Lipids were extracted by means of chloroform, 1M NaCl and 3N NaOH. The aqueous phase containing S1P and sphinganine-1-phosphate was transferred to a fresh tube and the compounds were dephosphorylated with the use of alkaline phosphatase (bovine intestinal mucosa, Fluka). Free sphinganine and sphingosine were converted to their O-phthalaldehyde derivatives and analyzed by means of high performance liquid chromatography (HPLC) system equipped with fluorescence detector and C18 reversed-phase column (Varian Inc., OmniSpher 5, 4.6 × 150 mm).

To quantify ceramide, a small volume of the chloroform phase containing lipids was transferred to a tube containing N-palmitoyl-D-erythro-sphingosine (C17 base) as an internal standard. Ceramide was subjected to alkaline hydrolysis and the amount of sphingosine released was determined as above. The content of ceramide was corrected for the level of free sphingosine present in the same sample.

In the plasma, the level of the sphingolipids was determined as above, omitting the step of homogenization.

Statistical analysis

The data are evaluated statistically using two-way ANOVA followed by post-hoc multiple comparisons using the Newman-Keuls test. $P < 0.05$ was considered statistically different.

RESULTS

Left ventricle

In the left ventricle, pacing lasting for 30 min increased the level of sphingosine as compared to the control value shown in

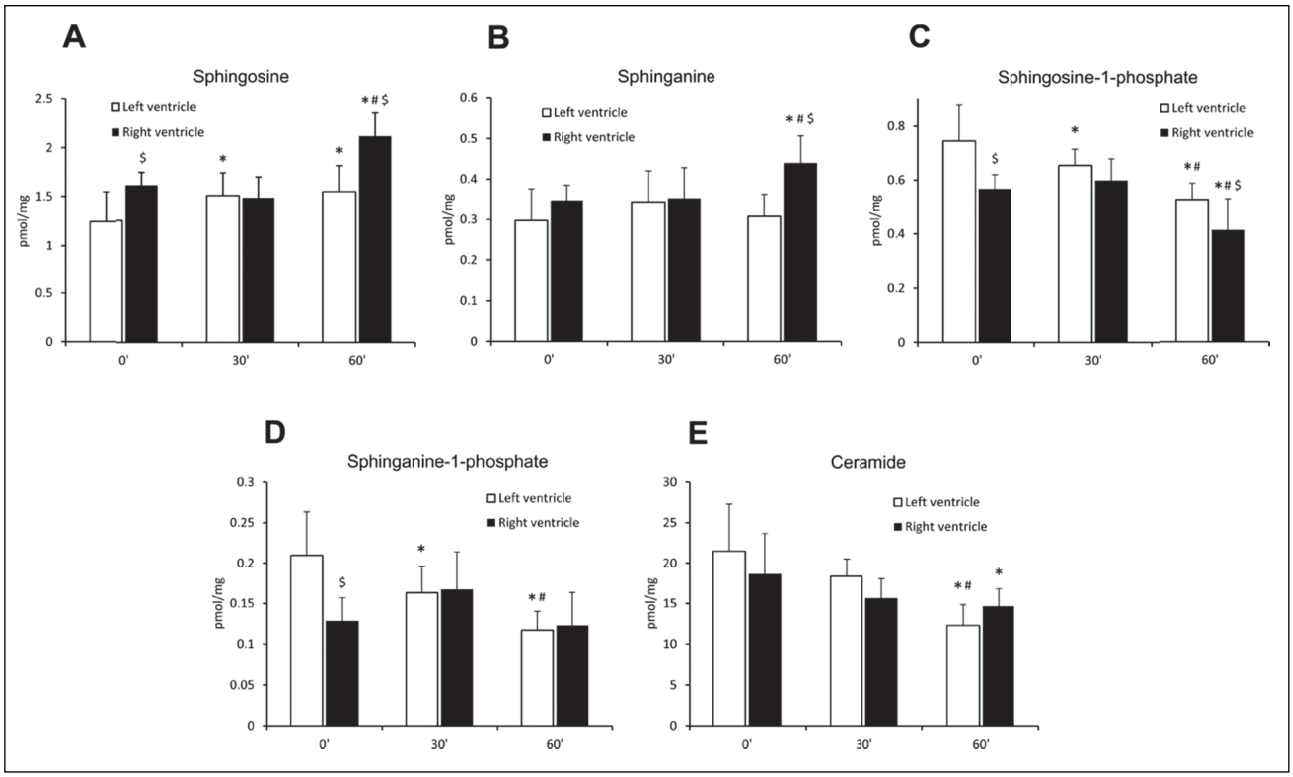

Fig. 1. Effect of 30 and 60 min pacing on the level of sphingosine (A), sphinganine (B), sphingosine-1-phosphate (C), sphinganine-1-phosphate (D) and ceramide (E) in the rat ventricles. Values are means ± S.D. (n = 10). *P < 0.05 vs. the respective control value, #P < 0.05 vs. the respective value after 30-min stimulation. $P < 0.05 vs. the respective value in the left ventricle.

Table 1. Effect of tachycardia on the level of sphingosine-1-phosphate/the level of ceramide ratio in the rat ventricles.

Time of pacing	0’	30’	60’
Left ventricle	0.034 ± 0.007	0.034 ± 0.008	0.045 ± 0.009*#
Right ventricle	0.037 ± 0.011	0.037 ± 0.006	0.029 ± 0.007*$

Values are means ± S.D. (n = 10). *P < 0.05 vs. the respective control value; #P < 0.05 vs. the respective value after 30-min pacing; $P < 0.05 vs. the respective value in the left ventricle.

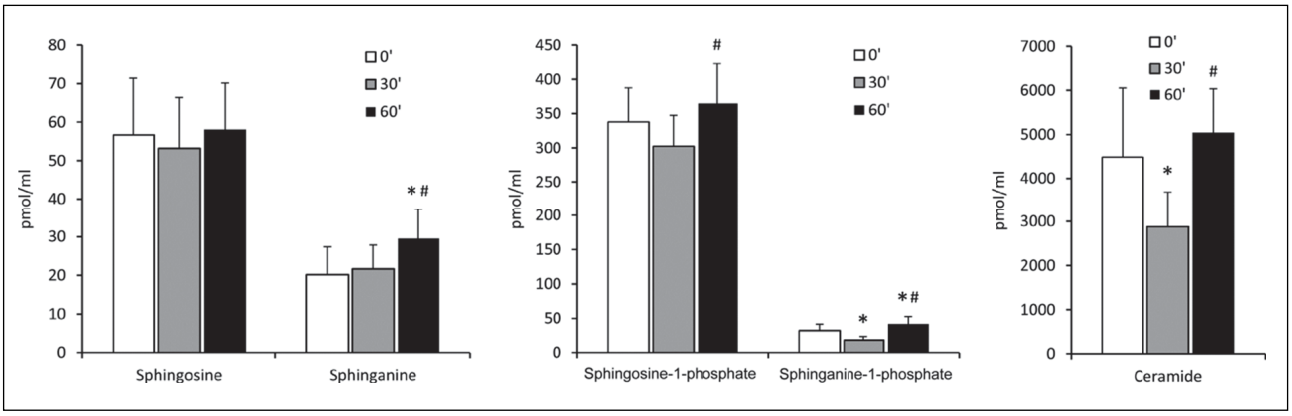

Fig. 2. Effect of 30 and 60 min pacing on the concentration of sphingosine, sphinganine,sphingosine-1-phosphate, sphinganine-1-phosphate and ceramide in plasma. Values are means ± S.D. (n = 10). *P < 0.05 vs. the respective control value, #P < 0.05 vs. the respective value after 30-min stimulation.

Table 2. Effect of pacing on the ratio between individual sphingolipid content in the plasma and in the heart ventricles.

Time of pacing	0’	30’	60’
Plasma/left ventricle			
Sphingosine	0.047 ± 0.017	0.035 ± 0.007	0.039 ± 0.010
Sphinganine	0.070 ± 0.028	0.065 ± 0.019	0.098 ± 0.030*#
S1P	0.466 ± 0.141	0.465 ± 0.067	0.702 ± 0.155*#
SA1P	0.153 ± 0.050	0.112 ± 0.021	0.374 ± 0.130*#
Ceramide	0.208 ± 0.060	0.150 ± 0.046	0.420 ± 0.088*#
Plasma/right ventricle			
Sphingosine	0.037 ± 0.007	0.036 ± 0.009	0.028 ± 0.008
Sphinganine	0.063 ± 0.017	0.064 ± 0.021	0.069 ± 0.024
S1P	0.595 ± 0.080	0.518 ± 0.119	0.957 ± 0.404*#
SA1P	0.291 ± 0.054	0.117 ± 0.042*	0.357 ± 0.143#
Ceramide	0.398 ± 0.133	0.184 ± 0.073*	0.355 ± 0.107#

Values are means ± S.D. (n = 10). *P < 0.05 vs. the respective control value; #P < 0.05 vs. the respective value after 30-min pacing. S1P-sphingosine-1-phosphate, SA1P-sphinganine-1-phosphate. Values in plasma expressed in pmol/ml and values in the ventricles expressed in pmol/g were used for calculation of the ratio.

Fig. 1A. It remained further stable after 60 min pacing. The pacing did not affect the level of sphinganine (*Fig. 1B*). The level of sphingosine-1-phosphate (*Fig. 1C*) and sphinganine-1-phosphate (*Fig. 1D*) was reduced after 30 min pacing. sixty min pacing resulted in further reduction in the level of each compound, as compared to the respective level after 30 min. The level of ceramide (*Fig. 1E*) decreased after 60 min of pacing.

Right ventricle

In the right ventricle, 30 min pacing had no effect on the level of either compound studied. 60 min pacing increased the level of sphingosine (*Fig. 1A*) and sphinganine (*Fig. 1B*) and reduced the level of sphingosine-1-phosphate (*Fig. 1C*) and ceramide (*Fig. 1E*) comparing to the respective control value.

There are some significant differences in the levels of examined sphingolipids between the right and left ventricle. The resting level of sphingosine was higher (*Fig. 1A*) and that of sphingosine-1-phosphate (*Fig. 1C*) and sphinganine-1-phosphate (*Fig. 1D*) was lower than in the left ventricle. There were no difference in the level of either sphingolipid between the ventricles after 30 min pacing. However, after 60 min pacing the level of sphingosine (*Fig. 1A*), and sphinganine (*Fig. 1B*) was higher and the level of sphingosine-1-phosphate was lower from the respective values in the left ventricle.

Ratio of the level of sphingosine-1-phosphate/the level of ceramide in the ventricles is shown in *Table 1*. Thirty min pacing did not affect the sphingosine-1-phosphate/ceramide ratio in either ventricle. After 60 min pacing, the ratio increased in the left ventricle and decreased in the right one as compared to the

respective control values. At this time point the ratio in the left ventricle was higher than in the left one by 35.6%.

Plasma

Thirty min pacing reduced the level of sphinganine-1-phosphate and ceramide shown in *Fig. 2* as compared to the respective control values. The level of sphinganine and sphinganine-1-phosphate after 60 min pacing was higher than appropriate control value. The level of each compound, with the exception of sphingosine, was higher after 60 min pacing than the respective value after 30 min pacing (*Fig. 2*).

Ratio of the level of plasma sphingolipids/the level of sphingolipids in the ventricles is shown in *Table 2*.

Thirty min pacing did not affect the ratios in the left ventricle. In the right ventricle, it was reduced in case of sphinganine-1-phosphate and ceramide and remained stable for other sphingolipids. After 60 min pacing, the ratio in the left ventricle was elevated for each sphingolipid, with the exception of sphingosine, comparing to the respective control values. In the right ventricle, only the ratio of sphingosine-1-phosphate was elevated above the control. It did not differ from the respective controls for other sphingolipids.

DISCUSSION

The results obtained clearly show that tachycardia produced by atrial pacing influenced metabolism of the examined sphingolipids in both ventricles. Moreover, we revealed existence of some differences both at rest and in response to pacing between the right and left ventricle. Ceramide is the key compound on the pathways of sphingolipid metabolism. The main source of ceramide is its *de novo* synthesis and sphinganine is a precursor of ceramide in this pathway. Ceramide is deacylated to sphingosine and the latter may be phosphorylated to sphingosine-1-phosphate (13, 14). In the left ventricle, the level of ceramide was reduced, the level of sphinganine was stable and the level of sphingosine was elevated after 60 min pacing (*Fig. 1*). It would suggest that the rate of *de novo* ceramide synthesis was unchanged during tachycardia and that catabolism of the compound was activated what contributed to the reduction in its level. In the right ventricle, the level of ceramide was also reduced after 60 min pacing. However, the level of both sphinganine and sphingosine was elevated (*Fig. 1*). It would indicate that the pacing increased not only synthesis but also breakdown of ceramide and that the balance was shifted in the direction of its catabolism. The present data differ from the results obtained after treadmill exercise of moderate intensity. As mentioned in the introduction, 30 min exercise reduced the level of ceramide in the left ventricle. It exceeded the resting value after exercise until exhaustion (12). Thirty min pacing (the present work) did not affect the level of ceramide in either ventricle. It was reduced in both ventricles after 60 min pacing. The differences could be caused by different experimental conditions (treadmill running vs. atrial pacing).

As already mentioned, sphingosine in higher dose was shown to be cardiotoxic (11). Therefore, the elevation in the level of the compound during pacing could be detrimental to the myocardium. Sphingosine-1-phosphate is the product of phosphorylation of sphingosine by the enzyme sphingosine kinase. The compound is irreversibly cleaved by sphingosine-1-phosphate lyase and reversibly dephosphorylated by both specific phosphatases (SPP1 and SPP2) and nonspecific phosphatases (the LPPs) (13, 14). The reduction in the level of sphingosine-1-phosphate along with elevation in the level of sphingosine would indicate an inhibition of phosphorylation of the latter during tachycardia. However, it should be mentioned that ischemia increases activity of sphingosine-1-phosphate lyase in perfused mouse heart (15). Therefore, one cannot exclude that tachycardia would increase activity of the enzyme and thus contribute to the reduction in the content of the compound on this way.

A mechanism triggering the changes in the sphingolipid metabolism in the heart remains obscure. Muscular exercise on treadmill was shown to affect metabolism of sphingolipids in skeletal muscles of the rat. It manifested with elevation in the level of sphingosine and sphinganine and biphasic behavior of ceramide (16-18). Stimulation of the sciatic nerve resulted in marked elevation in the level of sphingosine and sphinganine in contracting muscles (17). The *in situ* data indicate that the changes in sphingolipid metabolism in working skeletal muscles are induced by contractile activity *per se*. Therefore, it is very likely that the changes in the sphingolipid content in both ventricles during tachycardia may also be produced by increased contractile activity of the myocardium.

As has been already mentioned, sphingosine-1-phosphate and ceramide exert opposite effects in myocardium: sphingosine-1-phosphate protects against ischemia/reperfusion injury whereas ceramide induces apoptosis of cardiomiocytes. The ratio: the level of S1P/the level of ceramide was suggested to play important role in creating susceptibility of cardiomyocytes to injury during ischemia or ischemia/reperfusion (19). Previous data showed that the ratio in uninfarcted section of the left ventricle was markedly reduced (20). In the present study, the ratio in both ventricles remained stable after 30 min tachycardia. It increased in the left ventricle but decreased in the right one after 60 min tachycardia (*Table 1*). It would indicate that prolonged pacing increases the pro-survival potential of cardiomyocytes in the left ventricle but reduces it in the right one.

Sphinganine-1-phosphate is claimed to mimic the action of sphingosine-1-phosphate. However its level is much lower than the level of sphingosine-1-phosphate. In consequence, biological consequences of the changes in its level are relatively low (21).

The present data revealed an existence of differences in the level of sphingolipids between the two heart ventricles. They are present both at rest and after 60 min of tachycardia. It should be noted that differences in the level of ceramide between the two ventricles, both in controls and chronically hypoxic rats were described. The differences depended on the age of the animals. In the control rats at age of 8 weeks the level in the right ventricle was significantly lower than in the left one (by 14%). The levels of other sphingolipids were not determined (22). In our study the control level of ceramide in the right ventricle was lower than in the left one by 13.2% but did not reach statistical difference. The biological meaning of the differences reported in our work remain, however, obscure.

Tachycardia produced changes in the sphingolipid content not only in the ventricles but also in the plasma. As it can be seen in the *Table 2*, the ratio: plasma level/level in each ventricle for individual sphingolipid is below one. It indicates that the plasma level of each sphingopids is lower than the respective level in the ventricles. The source of particular plasma sphingolipids is diversified. Ceramide is secreted by the liver along with lipoproteins. Sphingosine-1-phosphate and sphinganine-1-phosphate are excreted by erythrocytes, platelets and endothelial cells. The plasma membrane of other cells is impermeable for the phosphorylated sphingolipids. Sphingosine and sphinganine cross the plasma membranes but factors regulating their transport hasn’t been recognized, so far (23-26). Sixty min pacing elevated the ratio above the appropriate control value in case of the plasma membrane impermeable sphingolipids. It would suggest that systemic sources contributed to this

phenomenon. The ratio for plasma membrane permeable sphingosine and sphinganine was stable during pacing with the exception of its elevation in case of sphinganine in the left ventricle after 60 min pacing. The stability of the ratio would indicate that the balance between the plasma and myocardial level of the compounds was maintained. Its elevation in the case of sphinganine might suggest increased leak of the compound from the left ventricle muscle to the plasma.

Taken together, the results obtained clearly indicate that tachycardia produces numerous changes in metabolism of sphingolipids in each ventricle of the rat heart. Also, the existence of differences in metabolism of the compounds between the two ventricles has been revealed.

Acknowledgments: This work was supported by The Medical University of Bialystok, projects: 143-18567L and 143-18566L.

Conflict of interests: None declared.

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Received: December 3, 2104
Accepted: February 25, 2015

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