JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2017, 68, 5, 731-736
www.jpp.krakow.pl

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EFFECT OF TACHYCARDIA ON mRNA AND PROTEIN EXPRESSION OF THE PRINCIPAL COMPONENTS OF THE LIPOLYTIC SYSTEM IN THE RAT'S HEART VENTRICLES

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There is a convincing piece of evidence showing that most of free fatty acids (FFA) entering cardiomyocytes are first esterified into triacylglycerols (TG) before being oxidized or used for synthesis of complex lipids. The enzyme adipose triglyceride lipase (ATGL) initiates lipolysis of TG by hydrolyzing the first ester bond of the compound. As a result, free fatty acid and diacylglycerol (DG) are released in that process. Finally, DG may be further hydrolyzed by the enzyme hormone sensitive lipase (HSL). The aim of the present study was to examine effect of tachycardia on mRNA and protein expression of ATGL, CGI-58 (an activator of ATGL), G0S2 (an inhibitor of ATGL) and HSL in the left and right ventricle of the rat. The experiments were carried out on male Wistar rats, 240 – 260 grams of body weight. After anesthesia, two electrodes were inserted in the right jugular vein and connected to SC-04 stimulator. The rats were randomly allocated into one of the three groups, namely: control, 30 min and 60 min of the heart stimulation at the rate of 600 times/min. The expressions of ATGL, CGI-58, G0S2 and HSL were evaluated at both gene and protein levels using real-time PCR and Western Blot analysis, respectively. Both 30 and 60 min stimulation reduced ATGL, HSL, CGI-58 and G0S2 mRNA content in the left ventricle. The stimulation had only insignificant impact on ATGL, HSL and CGI-58 transcript levels in the right ventricle. Interestingly, Western Blot analysis showed that the stimulation, regardless of the time, reduced the ATGL and G0S2 protein expression, but did not change the CGI-58 and HSL expression in the left ventricle. Furthermore, in the right ventricle, the protein expressions of ATGL, HSL and G0S2 decreased after stimulation. In conclusion, the obtained results clearly show that tachycardia affects both mRNA and protein expression of particular compounds of the TG lipolytic system in the heart ventricles. Additionally, there are marked differences in the expressions of the examined compounds between the ventricles.

Key words: *adipose triglyceride lipase, cardiomyocytes, hormone sensitive lipase, triacylglycerols, comparative gene identification 58, G0/G1 switch protein 2, heart ventricles, tachycardia*

INTRODUCTION

It is well known that cardiomyocytes contain triacylglycerols (TG), which are stored in lipid droplets. The droplet is around 0.5 µm in size and it is coated by a monolayer of phospholipids with a number of proteins inserted in the layer. Perilipins are the most abundant group of proteins present in the layer (1, 2). Interestingly, for a long time, the heart TG were considered to be rather an inert sink protecting against harmful elevation in content of intracellular pool of free fatty acids (FFA). The plasma borne FFA entering cardiomyocytes were thought to be directly channeled for oxidation and only a small portion was used for synthesis of complex lipids. However, this view has been challenged presently. Recent year's investigations have proven that considerable portion of FFA entering cardiomyocytes is incorporated into triacylglycerols before being oxidized. Moreover, studies in mice showed that TG-derived fatty acids are preferentially utilized in the heart. Notably, a magnitude of FFA utilization seems to depend on experimental conditions (3-6). It would suggest that turnover of the heart TG must be high. TG breakdown is initiated by the enzyme adipose triglyceride lipase (ATGL). The enzyme hydrolyzes the first ester bond of the TG moiety. As a result FFA and diacylglycerol (DG) moiety are released. DG can be further hydrolyzed to monoacylglycerol and FFA by the enzyme hormone-sensitive lipoprotein lipase (HSL) (3, 7, 8). Importantly, the ATGL activity is regulated by two factors: comparative gene identification 58 (CGI-58) and G0/G1 switch protein 2 (G0S2). The first one activates the enzyme (9) and the second one inhibits it (10, 11). Haemmerle *et al.* (12) showed that inactivation of ATGL gene in mice results in severe accumulation of TG in the myocardium, elevation of the heart weight, increase in number and size of lipid droplets and eventually causes heavy cardiac insufficiency. Additionally, the results obtained in humans with mutations of the ATGL gene provided further important data regarding the role of ATGL in regulation of the heart TG metabolism. For instance, Fisher *et al.* (13) reported that mutations of the gene led to development of neutral lipid storage disease (NLSD) with myopathy and cardiomyopathy. Further data showed that symptoms of ATGL

gene mutation depend on its type. It turned out that complete deficiency of ATGL, due to mutations of its gene, produces severe cardiomyopathy (14). Interestingly, Tavian *et al.* (15) showed that small amounts of properly functioning the enzyme in patients with ATGL mutations protects against cardiac myopathy, nevertheless it does not protect against development of NLSD with myopathy. A very recent study (16) in a patient diagnosed for NLSD with myopathy has shown that ATGL gene mutation blocking the enzyme function only mild cardiac complication existed. Another mutation of the ATGL gene resulted in a NLSD form called triglyceride deposit cardiomyovasculopathy. Finally, samples of the myocardium obtained from a patient with the ATGL mutation showed massive accumulation of TG in the heart muscle (17).

As mentioned above, most of free fatty acid entering cardiomyocytes are esterified in TG before being utilized. It is well recognized that increased heart work output enhances utilization of energy substrates (18, 19). We previously showed that tachycardia produced by atrial pacing did not affect TG content in the left ventricle but reduced it in the right ventricle (20). Interestingly, incorporation of the plasma borne 14C-FFA in TG moiety increased in both ventricles during tachycardia. Therefore, we may speculate that during pacing the synthesis of TG matched their hydrolysis in the left ventricle but not in the right ventricle. However, no data are available on behavior the heart TG lipolytic complex during tachycardia. Therefore the aim of our present work was to study that question. We measured the mRNA and protein expression of ATGL, CGI-58, G0S2 and HSL in both ventricles of the rat after 30 and 60 min tachycardia.

MATERIALS AND METHODS

The experiments were carried out on male Wistar rats 240 – 260 g of body weight. All experimental procedures were approved by the Ethical Committee on the Animal Research at the Medical University of Bialystok. The animals were housed in standard conditions, namely: stable temperature 21°C, humidity, reverse 12 h light/12 h dark cycle, free access to commercially available rat pellet diet and tap water. The experimental procedure was described before (21). After anesthesia (thiopental 80 mg/100 g of body weight) two electrodes were administered in the right jugular vein and connected to SC-04 stimulator. The tips of the electrodes were located at the aperture of the vein. Additionally, electrodes were inserted in skeletal muscles of the four limbs, connected to a standard electrocardiograph and the heart rate was continuously monitored. The resting heart rate was 349 ± 25/min. Next, the rats were randomly allocated into one of the three groups (n = 8 rats in each group), namely: control, stimulated for 30 min and stimulated for 60 min at the rate of 600 times/min. The parameters of the stimuli were: 4V and 100ms duration. The control rats were prepared as above and maintained without pacing for 60 min. The animals were kept on heating pads. The samples of the left and right ventricle were taken, blotted dry, immediately frozen in liquid nitrogen and stored at – 80°C until further analysis.

Protein extraction and Western Blot

Western blotting procedures were used to detect protein content (i.e. ATGL, G0S2, CGI-58, and HSL) as described in details elsewhere (22, 23). Briefly, the right and left ventricle were homogenized in ice-cold RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris-HCl, 150 M NaCl, 1 mM EDTA, 1% NP-40, 0,25% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride) for 1 min at 4°C. Protein concentration was then determined by means of bicinchonic acid (BCA) method with bovine serum albumin serving as a standard. Next, the samples were boiled at 95°C for 10 minutes in sample buffer containing 2-mercaptoethanol. Then the proteins (40 µg of the total protein) were subjected to SDS-PAGE and transferred to PVDF membranes, followed by blocking membranes in TTBS buffer containing 5% nonfat dry milk. Thereafter membranes were incubated overnight with the corresponding antibodies at a dilution of 1:1000. The primary antibodies were purchased from Abcam (ATGL, HSL, G0S2, Cyclophilin A) and Novus Biologicals (CGI-58). Subsequently, the membranes were incubated with anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:3000, Santa Cruz Biotechnology, USA). Immunoreactive protein bands were visualized by using an enhanced chemiluminescence substrate (ThermoScientific, Waltham, MA) and quantified densitometrically (Bio-Rad Laboratories, Hercules, CA). Equal protein concentrations were loaded in each lane as confirmed by Ponceau S staining technique on the blot membrane. Protein expression was then normalized to Cyclophilin A expression. Eventually, the control group was set as 100% and the experimental groups were expressed relatively to the control.

RNA extraction and real-time quantitative PCR analysis

Expression of the key genes involved in fatty acid metabolism was measured with the use of real-time quantitative PCR, as it was described previously (24). Total RNA was isolated from the right and left ventricle using the NucleoSpin RNA Plus Kit (Macherey Nagel GmbH & Co.KG, Duren, Germany) according to the manufacturer's instructions. After RNA purification, DNase treatment (Ambion, Thermo Fisher Scientific,Waltham, MA) was done to ensure that there was no contamination of genomic DNA. The total RNA amount was determined by spectrophotometry and RNA quality was verified by measuring the samples absorbencies at 260 and 280 nm and assessed by running the agarose electrophoresis with ethidium bromide. First strand cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), while specific primers were designed using Beacon Designer Software (Premier Biosoft, Palo Alto, CA, *Table 1* shows primers sequences). Real-time PCR was performed using SYBR GreenJumpStart Taq ReadyMix (Sigma-Aldrich) by Bio-Rad Chromo4system. The PCR reaction was carried out under following conditions: 15 s denaturation at 94°C, 30 s annealing at 60°C for Cyclophilin A, 61°C for ATGL, HSL, G0S2 and 62°C for CGI-58 and 30 s extension at 72°C for 40 cycles. PCR effciency was examined by serially diluting template cDNA, and a melt curve was performed at the end of each reaction to verify PCR product specificity. A sample containing no cDNA was used as a negative control to verify absence of contamination due to exogenous DNA and cDNA. The relative expression of genes was determined by normalizing to Cyclophilin A, the housekeeping gene, and calculated according to Pfaffl (25).

Statistical analysis

The results were statistically analyzed using Statistica 13.1 (StatSoft, Cracow, Poland). The data were analyzed using one-way ANOVA with a following post hoc Tukey's test for groups with a normal distribution and homogeneity of variances. If these assumptions did not hold the non-parametric Kruskal-Wallis rank test with the subsequent pairwise Wilcoxon test were conducted. The normality of the distribution was checked with the Shapiro-Wilk test, and the homogeneity of variances was checked with Levene's test. The results are presented as mean ± S.E.M. Statistical significance level was set at 0.05.

RESULTS

mRNA expression

1. The left ventricle

The mRNA expression of ATGL was reduced by 65.5% and by 47.2% after 30 and 60 min of pacing, respectively ($P < 0.05$) (*Fig. 1A*). Also, the mRNA expression of HSL was significantly reduced ($P < 0.05$), both after 30 min (by 52.3%) and after 60 min (by 69.5%) (*Fig. 1D*). Similarly, CGI-58 mRNA expression was diminished by 42% and 39.3% after two terms of pacing, respectively, ($P < 0.05$) (*Fig. 1B*). Additionally, the expression of G0S2 also dropped down by 46% after 30 min and by 69.4% after 60 min pacing ($P < 0.05$ at each time point) (*Fig. 1C*).

2. The right ventricle

There were only insignificant changes in ATGL and HSL mRNA expressions in the right ventricle both after 30 and 60 min pacing (*Fig. 1A* and *1D*). Again, 30 and 60 min pacing did not significantly affect the CGI-58 mRNA expression as compared to the control value. However, the expression after 60 min was higher than after 30 min (60 min versus 30 min, by 66.4%, $P < 0.05$) (*Fig. 1B*). The mRNA expression of G0S2 decreased after pacing but only the reduction in 60 min was significant (by 40.3%, $P < 0.05$) (*Fig. 1C*).

Protein expression

1. The left ventricle

The ATGL protein expression was significantly reduced by 53.2% after 60 min pacing ($P < 0.05$), whereas the HSL protein expression was not affected by the pacing (*Fig. 2A* and *2D*). The expression of CGI-58 remained stable during tachycardia (*Fig. 2B*). Furthermore, both 30 and 60 min of tachycardia reduced the G0S2 protein expression (by 28.4% and 27.8%, respectively, $P < 0.05$) (*Fig. 2C*).

Table 1. Primers sequences used for particular expression of genes.

Gene	Primer sequence	
	forward	reverse
Cyclophilin A	5'-TGTCTCTTTTCGCCGCTTGCTG-3'	5'- CACCACCCTGGCACATGAATCC-3'
ATGL	5'- CCCTGACTCGAGTTTCGGAT-3'	5'- CACATAGCGCACCCCTTGAA-3'
G0S2	5'- TGACCTCCTTCAGCGAGTG-3'	5'- TCGGGACTTCTGCGTCATC-3'
CGI-58	5'- AACCCCAAGTGGTGAGACAG-3'	5'- GCGCCGAAGATGACTGAAAC-3'
HSL	5'- AATGACACAGTCGCTGGTGGCG-3'	5'- TGCCACACCCAAGAGCTGACCT-3'

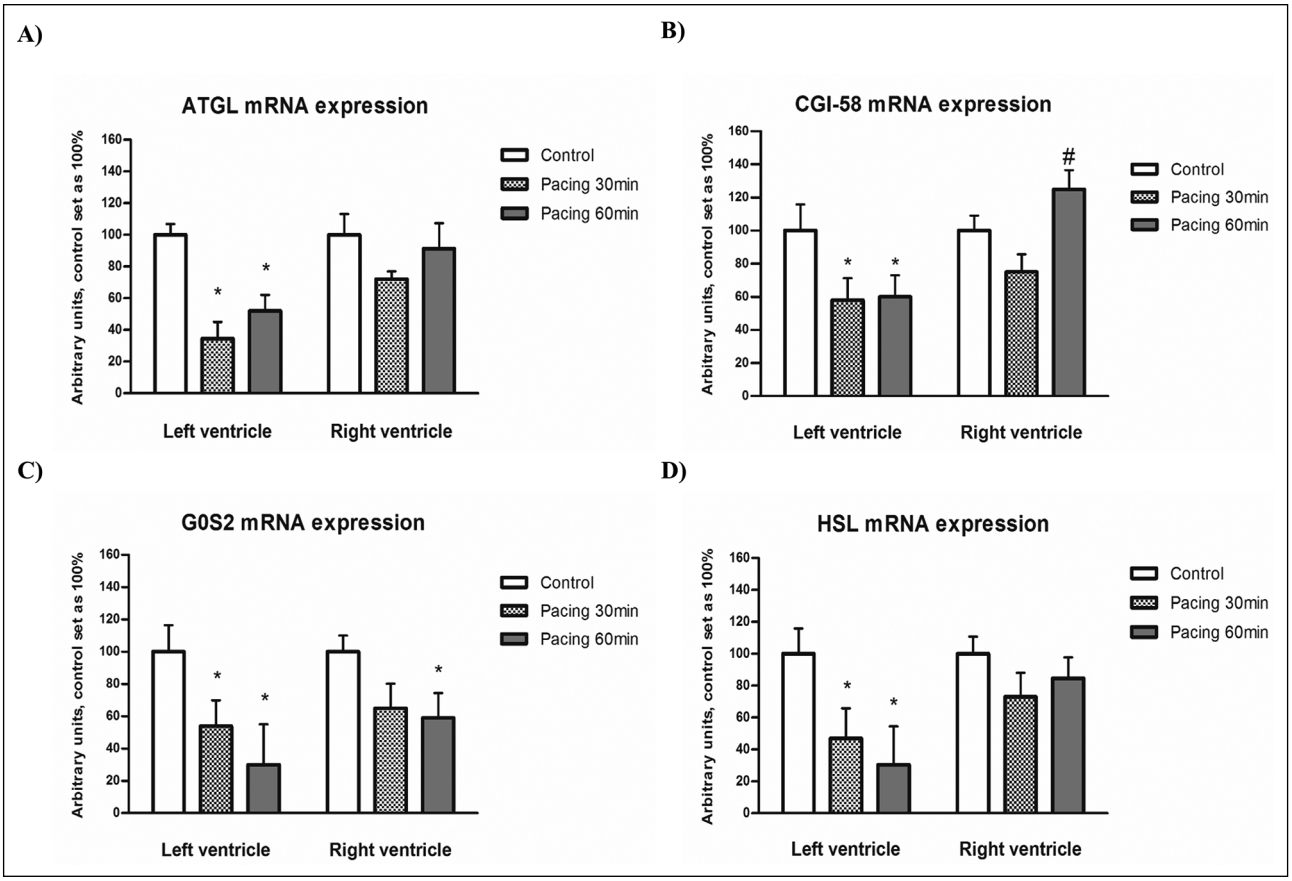

Fig. 1. The effect of atrial pacing on the mRNA expression of ATGL (*A*), CGI-58 (*B*), G0S2 (*C*) and HSL (*D*) in the left and right ventricle. Values are mean ± S.E.M., n = 8 per group. Control group was set as 100%. *$P < 0.05$ significant difference: control versus pacing (within ventricle type), #$P < 0.05$ significant difference: pacing 60 min versus pacing 30 min of stimulation (within ventricle type).

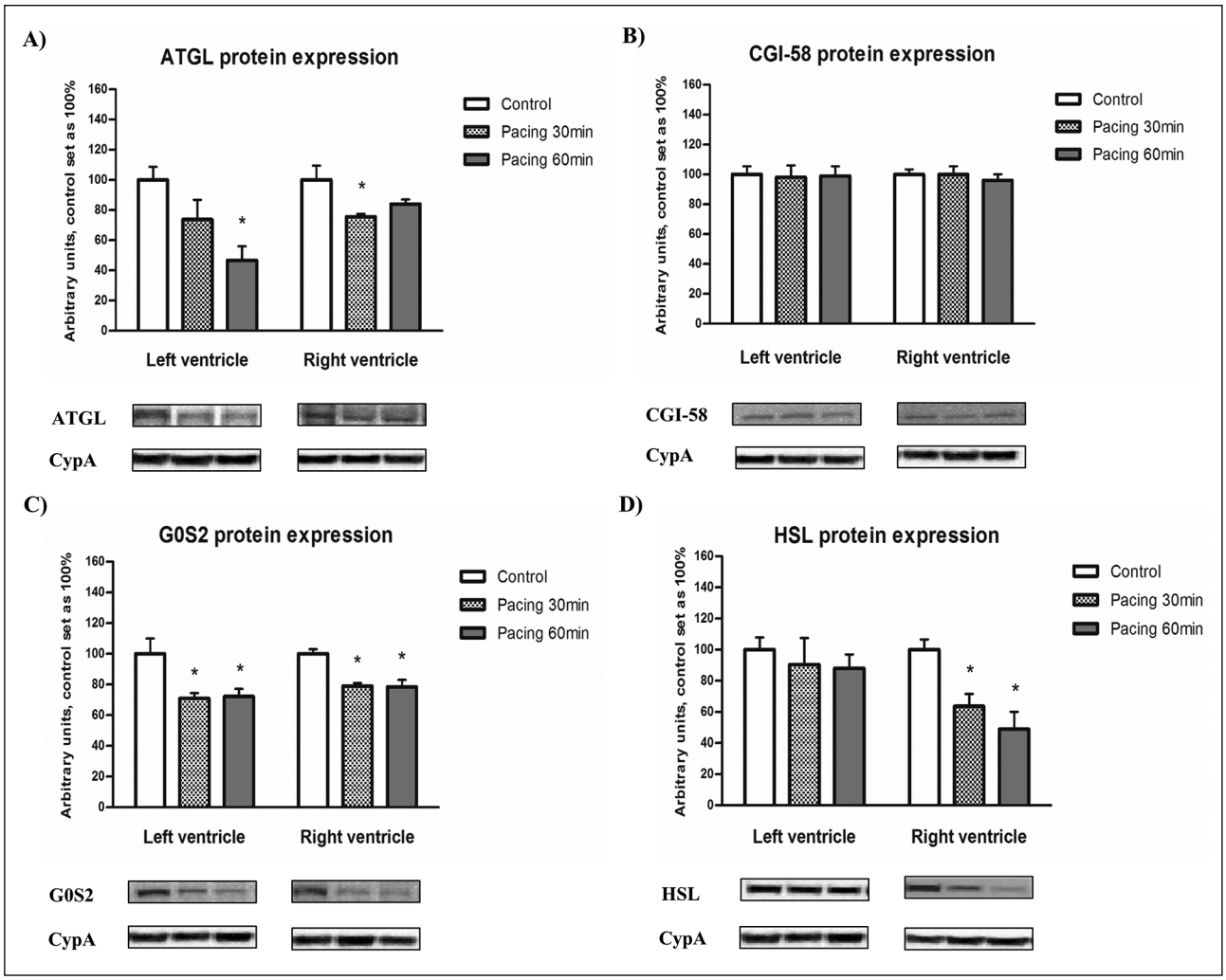

Fig. 2. The effect of atrial pacing on the protein expression of ATGL (*A*), CGI-58 (*B*), G0S2 (*C*) and HSL (*D*) in the left and right ventricle. Values are mean ± S.E.M., n = 8 per group. Control group was set as 100%. Representative Western blots are shown.*P < 0.05 significant difference: control versus pacing (within ventricle type), #P < 0.05 significant difference: pacing 60 min versus pacing 30 min of stimulation (within ventricle type).

2. The right ventricle

Tachycardia reduced the expression of protein ATGL in the right ventricle but only the difference after 30 min was significant (by 24.5%, P < 0.05) (*Fig. 2A*). The protein expression of HSL was reduced both after 30 and 60 min pacing (by 36.6% and 50.7%, respectively, P < 0.05) (*Fig. 2D*). However, the protein expression of CGI-58 was not affected by pacing (*Fig. 2B*). Finally, tachycardia caused the 20.7% and 21.5% reduction of G0S2 protein expression after 30 and 60 min, respectively (P < 0.05) (*Fig. 2C*).

DISCUSSION

The data obtained so far indicate that such factors like diabetes (26), fasting (9), high fat diet (27) and endurance training (27) increase the ATGL mRNA expression in the mice heart. However, these factors are long-term acting ones. The present study is the first one reporting the expression of the principal components of the heart lipolytic system during acute tachycardia. The mRNA and protein expression of ATGL, CGI-58, G0S2 and HSL in both heart ventricles in the rat was determined. The results obtained are very much surprising. They clearly show that tachycardia induces changes in both mRNA and protein content of the particular compounds. However, the changes are not uniform and they differ depending on the compound examined. Moreover, there are marked differences in the response of mRNA's of particular components to tachycardia between the two ventricles. Tachycardia produced deep reduction in the mRNA expression of each compound in the left ventricle whereas in the right one only a decrease in the G0S2 mRNAs was observed. For comparison, 30 min stimulation of incubated rat soleus (slow-twitch oxidative muscle) increased CGI-58 content (28). In mice, *in vivo* 30 min exercise did not significantly affect either total TG hydrolytic activity or ATGL content in the skeletal muscle (29). On the other hand, our previous data showed that already 30 min tachycardia induced upregulation of FAT/CD36 and GLUT4 protein transporters in the plasma membrane. The latter was mostly due to a shift of the transporters from the intracellular stores to the plasma membrane (20). A reason for the dramatic reduction in the mRNA's of the examined compounds in the left ventricle and lack of changes, with the exception of G0S2, in the right ventricle remains totally obscure. It should be stressed that cardiomyocytes belong to a group of so called mechanosensitive

cells (along with fibroblasts, skeletal myocytes, osteoblasts and endothelial cells). In those cells a mechanism known as 'mechanotransduction' operates (30, 31). Increase in the mechanical tension induces release of active compounds. They act either on the mother cells or/and neighboring cells and induce certain functions like, e.g. increased production of collagen in case of fibroblasts (32) or increased production of mechano-growth factor (MGF) in case of skeletal myocytes (33). It should be added that in the rabbit heart mechanical overload or short-term ischemia was shown to increase expression of MGF in the myocardium (34). It might be hypothesized that the mechanism of mechanotransduction could be involved in producing changes in the expression of mRNA's of the compounds studied during tachycardia. The systolic tension developed by the left ventricle is much higher than the systolic tension developed by the right ventricle. It is likely that the tension in the left ventricle wall is high enough to induce changes in the mRNA's expression of the studied compounds. On the contrary, the systolic tension developed in the right ventricle wall would be too small to fully activate the system of mechanotransduction. In the left ventricle, the protein expression of only ATGL and G0S2 partially followed changes in the mRNA expression of the compounds. The expression of protein CGI-58 and HSL remained stable. It seems, therefore, that the time-lag needed to elapse between changes in mRNA expression and protein expression is different for each compound studied. Interestingly, in the right ventricle, pacing reduced the protein expression of G0S2 and HSL and transitorily the expression of ATGL. However, it is hard to find a reason for the differences between the ventricles.

It remains an open question of how the observed changes of the components of the lipolytic system reflect on TG metabolism in cardiomyocytes. It was shown in humans that tachycardia increases utilization both glucose and plasma free fatty acids by the heart (35-37). According to (36) carbohydrate oxidation covered 62% of myocardial energy output during pacing. As mentioned in the introduction, much of the plasma free fatty acids entering the heart is esterified in triacylglycerols before oxidation. Reduction in the functioning of the lipolytic system would decrease liberation of fatty acids from endogenous TG and thus protect against overload of cardiomyocytes with the acids. As indicated in the introduction, incorporation of the plasma free fatty acids into TG moiety increased during 60 min pacing but it did influence the TG content in the left ventricle (20). It would suggest increased turnover of the TG. On the other hand, the present study shows over 50% reduction in the ATGL protein content in the ventricle. However, simultaneously the content of CGI-58 (the stimulator of ATGL) remained stable and the content of G0S2 (the inhibitor of the enzyme) dropped down thus facilitating activation of the remaining pool of the enzyme. In the right ventricle, TG content was shown to decrease during tachycardia in spite of increased incorporation of plasma free fatty acids (20). Now, we showed that ATGL protein content in the ventricle decreased only transitorily during pacing. However, similarly to the case of the left ventricle, the CGI-58 content remained stable and G0S2 content was reduced creating favorable conditions for activation of the enzyme.

So far, the research on heart metabolism concerned the left ventricle or both ventricles when studies utilized AV difference. However, available data evidently indicate on existing certain differences between the ventricles. For instance, lower level of ceramide in the right compared to the left ventricle was reported (38). Similarly, we also showed differences between the ventricles regarding different glycerophospholipid (20) and sphingolipid (21) metabolism during tachycardia. Now, we reported clear differences in the expression of principal compounds of the lipolytic system in the response to tachycardia between the ventricles. It would suggest that further studies are warranted to accumulate more data on differences in metabolism between the two ventricles as well their physiological meaning.

It should be stressed that the results obtained in the present study cannot be directly related to the data acquired in humans with mutation of the ATGL gene. First of all, the tachycardia certainly did not permanently effect on the ATGL gene. Moreover, tachycardia lasted for relatively short period of time whereas mutations of the gene reported in humans were probably life-lasting. Nevertheless, the available results indicate that ATGL mRNA expression is very much sensitive to relative short change in the cardiac function. It remains an open question whether tachycardia in humans also reflects on the cardiac ATGL mRNA response.

It is concluded that tachycardia produces several changes in both mRNA and protein expression of the principal compounds of the lipolytic system in the rat myocardium.

Acknowledgements: This work was supported by the Medical University of Bialystok, grant no. N/ST/ZB/17/002/1118 and no N/ST/ZB/17/008/1118.

Conflict of interests: None declared.

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Received: August 8, 2017
Accepted: September 25, 2017

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