Lakomkin V. L.[1], Abramov A. A.[1], Studneva I. M.[1], Ulanova A. D.[2], Vikhlyantsev I. M.[2, 3], Prosvirnin A. V.[1], Lukoshkova E. V.[1], Kapelko V. I.[1]

[1] National Cardiology Research Center, Moscow, Russia

[2] Institute for Theoretical and Experimental Biophysics of the Russian Academy of Sciences, Pushchino, Moscow Region, Russia

[3] Pushchino State Institute for Natural Science, Pushchino, Moscow Region, Russia

Early changes of energy metabolism, isoformic content and level of titin phosphorylation at diastolic dysfunction

Background	Diastolic dysfunction occurring in hypertension, obesity, diabetes, or treatment with doxorubicin tends to prevail in all patterns with chronic heart failure. Lack of effective therapy necessitates further investigation of the metabolic processes in cardiomyocytes.
Objective	Assess energy metabolism in cardiomyocytes and changes in titin, a giant myofibril protein that is responsible for their elasticity.
Materials and Methods	The study model was cardiomyopathy occurring after the 4-week administration of doxorubicin (2 mg/kg weekly). Diastolic dysfunction was identified by echocardiography and catheterization with the simultaneous measurement of pressure and volume of the left ventricle (LV).
Results	The levels of adenine nucleotides and phosphocreatine in the hearts of animals treated with doxorubicin differed little from the normal values, but lactate levels were increased manifold. A 50% increase in the level of titin phosphorylation was detected, which correlated (r=0.94) with a nearly twofold increase in the share of a more elastic N2BA isoform of this protein.
Conclusion	This form of diastolic dysfunction involves the activation of anaerobic metabolism and increased stretching of myofibrils, facilitating LV filling.

Keywords	Doxorubicin; heart failure; energy metabolism; titin isoforms and phosphorylation; diastolic dysfunction
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Corresponding author	Lakomkin Vladimir. E-mail: v.lakomkin@yandex.ru

In the 21st century, patients with chronic heart failure (CHF) with preserved ejection fraction (EF) have become the prevalent group in the total number of CHF cases due to the high prevalence of hypertension, obesity, and diabetes. These patients mainly have minor changes in minute volume, nor mal volume of the left ventricle (LV), and normal ejection fraction [2], but slow LV relaxation and increased diastolic LV pressure. These features are characteristic of diastolic myocardial dysfunction.

Myocardial metabolism has been studied mainly in association with more severe forms of CHF. The bioenergy of the heart is known to undergo significant changes in CHF: a gradual transition takes place from the burning of fatty acids to aerobic glycolysis. This transition during the development of severe CHF coincides with an increase in end-diastolic LV pressure and decompensation of CHF [1].

With the further remodeling of the LV, anaerobic glycolysis causes lactate accumulation [2]. A relationship between cellular indicators of bioenergy (levels of macroergic phosphate compounds, the ratio of phosphocreatine to adenosine triphosphate [PCr/ATP] in the heart) and the New York Heart Association's functional classes (NYHA FCs) of CHF or indices of systolic and diastolic dysfunction was demonstrated. A low PCr/ATP ratio is considered a predictor of cardiovascular mortality [3].

Cardiomyopathy caused by doxorubicin therapy is an adequate model of CHF, as it naturally occurs in cancer patients [4, 5]. It was demonstrated that four weeks of injection with doxorubicin caused diastolic and systolic dysfunction in two-thirds and one-third of rats, respectively. With more prolonged administration of doxorubicin, systolic dysfunction prevails [7]. An in vivo study of energy-exchange metabolites showed that the total content of adenine nucleotides was very close to reference levels. However, the PCr/ATP ratio was reliably reduced by 37% due to decreased levels of phosphocreatine [8]. Total creatine was reduced

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for the same reason. These changes were in line with the significantly elevated lactate levels in the heart.

The state of titin, the largest and most elastic protein in the cross-striated muscles of vertebrates, is responsible for diastolic myocardial elasticity within the physiological range of the length of sarcomeres [9, 10]. It binds with myosin (thick) filaments in the A-band of sarcomeres. Some regions of the titin molecule can interact with actin (thin) filaments in the I-band of the sarcomere. However, a larger portion of the molecule passes freely in this zone and connects the ends of myosin filaments with the Z-line. Titin was shown to be a framework for the assembly of myosin filaments and sarcomere, to participate in maintaining the extremely ordered sarcomere structure and, thus, the contractile function of a muscle, and to participate in controlling the actin-myosin interaction [9]. In heart muscle, titin exists in two isoforms: an elastic N2BA form (with a longer, extensible region in the I-band of the sarcomere) and a stiff N2B form (with a shorter, stiffer, less extensible region in the I-band of the sarcomere). The phosphorylation of titin and other post-translational modifications cause changes in its elasticity [11]. It is anticipated that titin in combination with signaling proteins acts as a sensor of stretching and tension, and participates in the processes of intracellular signalings, such as the regulation of the expression of muscle protein genes and protein metabolism in the sarcomere [9, 11]. In this regard, the role of titin in the formation of a hypertrophic stimulus has been discussed. The N2BA/N2B ratio varies in the hearts of mammals from the smallest in small animals to the largest in the myocardium of large animals and humans [12]. It is approximately 20%/80% in the rat's heart [12].

This study aimed to measure the levels of macroergic phosphate compounds in the rat myocardium and simultaneously evaluate the contractile function of the heart and determine the isoformic content and levels of titin phosphorylation after the 4-week administration of doxorubicin with predominant diastolic dysfunction.

Materials and Methods

Male Wistar rats weighing 250–300 g were used. All manipulations with laboratory animals were carried out following the International Guiding Principles for Biomedical Research Involving Animals, the requirements of the ethical committee of the Russian National Cardiology Research Center, and the principles of the GOSTP 53434-2009 national standard. Doxorubicin (2 mg/kg) was injected subcutaneously in 20 rats once a week for 4 weeks; 10 rats were injected with saline. Transthoracic echocardiography was carried out in all rats using Zoletil (5 mg/kg) before and after the 4-week administration of doxorubicin (VUJIFILM VisualSonic Vevo 1100). A linear sensor 24-13 MHz with a maximum location depth of 30 mm was used. The diastolic and systolic dimensions of LV were measured, based on which the diastolic and systolic volumes and ejection fraction were calculated.

The LV was catheterized through the right carotid for 4 weeks using a standard PV-catheter FTH-1912B-8018, ADV500 amplifier (Transonic, Canada), and Zoletil (5 mg/kg) anesthesia. The relevant software was used, which allowed calculating more than 20 parameters of contractile function during a cardiac cycle. In addition to these data, the index of myocardial contractility was calculated by dividing the maximum rate of pressure development by the value of pressure at the moment of the maximum rate; an index of relaxation was calculated by dividing the maximum rate of pressure reduction by the value of developing pressure.

To study the metabolic state of the hearts of anesthetized animals that were examined only by echocardiography, thoracotomy under mechanical ventilation was performed, and the hearts were rapidly frozen in situ using Vollenberger's forceps cooled in liquid nitrogen. The frozen tissue was homogenized in cold 6% HClO4 solution (10 mL/g of tissue) in Ultra-Turrax T-25 (IKA-Labortechnik, Germany). Proteins were precipitated by centrifuging (Sorvall RT1, Thermo Fisher Scientific, USA) at 2,800 g for 10 minutes at 4°C. The supernatants were neutralized to pH 7.4 using K2CO3 5M. KClO4 was precipitated by centrifuging in the same conditions. Protein-free extracts were stored at -20°C until the identification of metabolites. The dry weight of the homogenized tissue was determined after the samples had been dried for 24 hours at 110°C. The metabolite levels were determined by the methods of enzymatic analysis [13] and presented as μmol/L of the dry weight.

The isoforms of titin were identified in the frozen samples taken after the acute experiment. Large-pore polyacrylamide gel 2.1–2.3% with agasrose 0.5–0.6% prepared as described in Hamdani et al. [14] was used for the electrophoretic separation of the high-molecular-weight isoforms of titin in the presence of sodium dodecyl sulfate (SDS). Gels dyed with Coomassie Brilliant Blue (G-250 and R-250 mixed at a ratio of 1:1) were digitized, and densitometric data were processed using Total Lab v1.11.

Titin content was evaluated with respect to the content of myosin heavy chains. The native levels of protein phosphorylation in the gel were evaluated using Pro-Diamond (Invitrogen) phosphoprotein dye. Phosphate-containing protein bands were visualized using the Bio-Rad ChemiDoc Touch Imaging System. The gels then were stained with Coomassie Brilliant Blue G-250 and R-250 mixed at a ratio of 1:1 for the estimation of protein content.

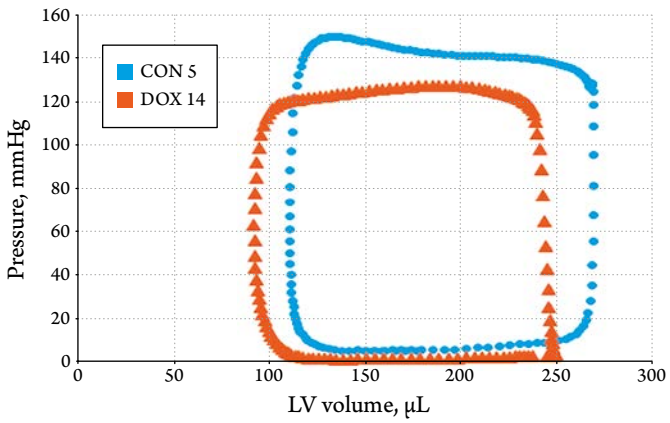
The results are presented as mean ± standard error of the mean (M±SEM). The statistical processing of the measurement results used computational algorithms available in GraphPad Prism (version 8.0.1). This statistical package

Table 1. Echocardiographic findings in the hearts of rats after the 4-week administration of doxorubicin

Parameter	Control	DOX	DOX-SD
Number of rats	8	9	7
Weight, g	343±7	330±13	309±19
EDD, mm	6.2±0.3	6.5±0.2	6.9±0.2*
ESD, mm	3.1±0.2	3.4±0.2	3.8±0.2*
EDV, μL	251±16	274±16	292±11
ESV, μL	86±16	274±16	292±9** ##
EF, %	67±3	64±2	49±1*** ###

* p <0.05, ** p <0.01, *** p <0.001 significance of differences vs. control; ## p <0.01, ### p <0.001 significance of differences vs DOX. EDD, end-diastolic dimension of LV; ESD, end-systolic dimension of LV; EDV, end-diastolic volume of LV; ESV, end-systolic volume of LV; EF, ejection fraction; DOX, without systolic dysfunction; DOX-SD, with systolic dysfunction.

Figure 1. Volume-pressure ratio during a heart cycle on control rats versus animals treated with doxorubicin for 4 weeks



LV, left ventricle of the heart; CON 5, control animal #5, DOX 14, rat #14 treated with doxorubicin.

allows effectively analyzing the results of multiple comparisons using analysis of variance (ANOVA) with F-test and Brown-Forsythe test and evaluati on of the significance of differences in mean values of the parameters measured, including taking into account the multiplicity of comparisons. The parameters of two groups of experiments were compared using the Student's t-test; for three (or more) groups, Tamhane T2 test was used. The normality of distribution of the parameters measured was confirmed by using the Kolmogorov-Smirnov and D'Agostino-Pearson tests.

Results

Echocardiographic examination of the hearts of 16 rats treated with doxorubicin showed a reliable 8% increase in the end-diastolic dimension and a significant 40% increase in end-systolic volume. Thus, LVEF was reduced by 15%. This group was heterogeneous and was divided into two subgroups according to LVEF: with normal EF (n=9) and reduced EF (n=7). In the normal EF subgroup, all parameters were close to the control values (Table 1), and in the reduced EF subgroup, a significant increase occurred in end-diastolic and end-systolic dimensions. The reduced EF subgroup was characterized by an increase in end-systolic volume by 71%, which affected EF—it was reduced by 27%. Thus, this subgroup can be regarded as animals with systolic LV dysfunction.

Catheterization of the LV showed that parameters of the pumping function (minute volume, stroke work) and parameters of contractility in the doxorubicin group are very close to control values. The only significant difference was a 19% decrease in the relaxation index (Table 2). An evident but insignificant increase in diastolic pressure should also be noted. These features describe the diastolic dysfunction. The "pressure-volume" loop curves of the typical experiments are presented in Figure 1. The heart loop for the doxorubicin group reflects a lower pressure developed and a left shift of the loop.

Measurement of the metabolite content showed that the energetic condition of the hearts of rats treated with doxorubicin was very similar to that of the control animals (Table 3). The levels of adenine nucleotides and free creatine tended to increase. The only significant difference was a greater than fivefold increase in lactate levels in the hearts of animals treated with doxorubicin. Echocardiography examination performed in these rats before the heart was frozen showed that LVEF virtually did not differ from the control values of 57±4% and 69±4%, respectively.

The content of the elastic N2BA isoform of titin with respect to the stiff N2B isoform in the control experiments was 14%/86%. In the experiments with doxorubicin rats, this was shifted toward the predominance of the elastic N2BA isoform; its content was 26±2% (Figure 2).

The titin content was slightly reduced, but the levels of its proteolytic fragments (T2) increased by 36%, indicating a more intensive metabolism of this protein. The total level of titin phosphorylation was higher than the control by 50±16%. The degree of titin phosphorylation directly correlated (r=0.94) with the levels of the elastic N2BA isoform of titin and inversely correlated with the levels of the stiff N2B isoform.

Discussion

After 4 weeks of doxorubicin administration, most rats, as shown by echocardiography and catheterization, had LV diastolic dysfunction. This agrees with our previous results: the predominance of systolic dysfunction occurs later [6, 7]. The characteristic signs of diastolic dysfunction were slow relaxation and increased pressure in LV at the beginning and end of diastole. Systolic dysfunction was characterized by increased dimensions of the LV but with relatively less modified diastolic volume. This suggests remodeling of the LV chamber.

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The state of energy metabolism in LV diastolic dysfunction was generally similar to the control values. Previously, the study of systolic myocardial dysfunction identified evident changes in the phosphocreatine/creatine system. In this work, these indicators changed little in diastolic dysfunction, but, as in systolic dysfunction, lactate levels were significantly increased, even much more than in systolic dysfunction. This suggests the impaired oxidative phosphorylation and activation of anaerobic glycolysis.

The N2BA/N2B titin isoform ratio in our experiments with the control rats was 14%/86% and was similar to that in other experiments with rats (20%/80%) [12]. The contents of the elastic N2BA titin isoform in the myocardium of rats with doxorubicin cardiomyopathy was almost twofold, which suggests a decrease in diastolic myocardial elasticity. At the same time, a significant increase (50%) in titin phosphorylation was observed. It is known that the elasticity of titin molecules is regulated not only by changing the length in the I-band of the sarcomere but also with post-translational modifications [15]. The titin molecules can become stiff due to oxidative stress caused by myocardial infarction, obesity, or diabetes, which impairs LV diastolic function. This is associated with the formation of disulfide bonds in the N2B sequence of titin, which causes a higher stiffness of the molecule.

Increased stiffness of titin can be compensated by reversible S-glutathionylation of cysteines in the deployed Ig-domains (by increasing load on the sarcomere). It was shown that N2B-region phosphorylation by cyclic guanosine monophosphate (cGMP)-dependent or cyclic adenosine monophosphate (cAMP)-dependent protein kinases reduces the stiffness of the titin molecule. However, phosphorylation of the PEVK-sequence (enriched with residues of prolin, glutamic acid, valine, and lysine) by cAMP-dependent protein kinase increases its stiffness [15].

In our study, a 50% increase in the total level of titin phosphorylation was detected, which does not allow identifying which parts of the titin molecule were phosphorylated. However, a strong correlation between the level of titin phosphorylation and the decrease in the portion of its stiff N2B isoform suggests that phosphorylation is aimed at reducing the stiffness of the titin molecules.

This was the first time when such results were obtained. The results show that, at diastolic dysfunction, myocardial relaxation slows down, diastolic pressure increases, anaerobic glycolysis mobilizes, and titin stretching increases.

Analysis of the available data on the N2BA/N2B ratio in different types of cardiomyopathy shows wide variability in different disease models. Increased N2BA/2B ratio is typical of dilated cardiomyopathy, chronic ischemic cardiomyopathy, and CHF with reduced EF [16], and decreased ratio is typical of concentric cardiomyopathy or hypertension [17]. These data show that when stretching requires mobilization, the ratio shifts toward N2BA, and when contractions must be increased, increased levels of the

Table 2. Cardiac hemodynamics in rats with diastolic dysfunction after 4-week administration of doxorubicin

Parameter	Control	DOX-DD
Number of rats	6	7
Heart rate, bpm	384±20	375±17
Stroke work, mmHg × mL/min	22.5±2.0	19.8±2.5
Ejection rate, mL/sec	5.0±0.7	4.5±0.4
End-diastolic volume of LV, mL	0.31±0.03	0.31±0.03
End-systolic volume of LV, mL	0.11±0.01	0.12±0.03
Ejection fraction, %	61±2	58±4
End-diastolic LV pressure, mmHg	2.8±0.6	5.6±1.8
Minimum LV pressure, mmHg	1.0±1.2	2.2±1.8
Maximum LV pressure, mmHg	133±5	128±5
dP/dt max, mmHg	13130±2546	12090±1203
dP/dt min, mmHg	10,940±2,476	8,480±956
Ea, arterial elastance, mmHg/mL	0.76±0.13	0.77±0.05
Contractility index, c^{-1}	137±9	130±9
Relaxation index, c^{-1}	83±2	67±6*
Relaxation time constant, msec	9.9±0.8	12.4±1.5

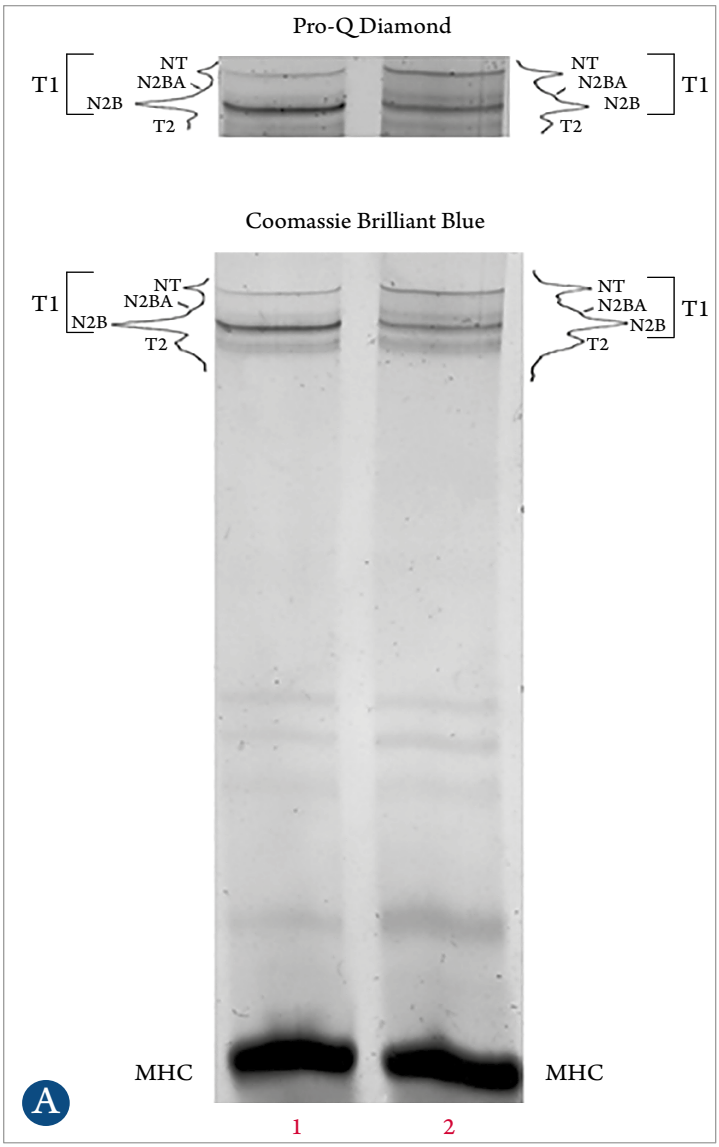
* p <0.05 significance of differences with the control.
DOX-DD, diastolic dysfunction.

Table 3. Energy metabolites in rats treated with doxorubicin for 4 weeks (in situ)

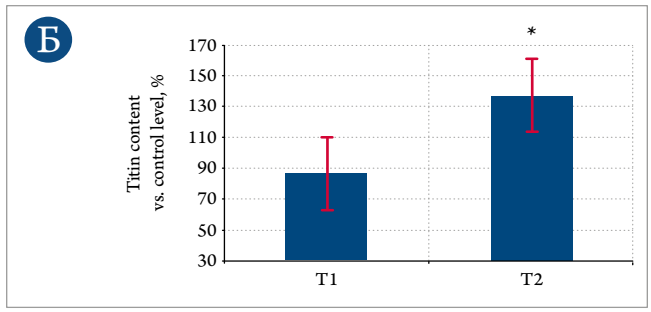
Parameter	Control	DOX	% of control
Number of rats	7	6	–
ATP	14.6±0.8	16.8±2.7	115
ADP	5.7±0.2	7.1±0.8	125
AMP	1.3±0.2	1.8±0.3	144
ΣAN	21.6±0.9	25.8±3.4	120
EC	0.81±0.01	0.78±0.02	97
PCr	27.2±1.6	25.8±4.4	95
Cr	39.2±1.6	47.5±5.7	121
ΣCr	66.4±3.2	73.3±8.6	110
PCr / ATP	1.87±0.08	1.58±0.17	85
Lactate	3.6±0.8	20.0±4.6*	552

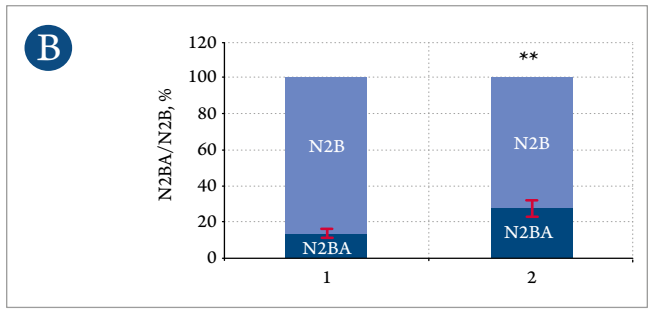
* p <0.05 significance of differences with the 4-week control.
The metabolite content is presented as μmol/L of the dry tissue weight. ΣAN, a sum of all adenine nucleotides; EC, energy charge (ATP +0,5ADP/ΣAN); PCr, phosphocreatine; Cr, creatine free; ΣCr, total creatine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

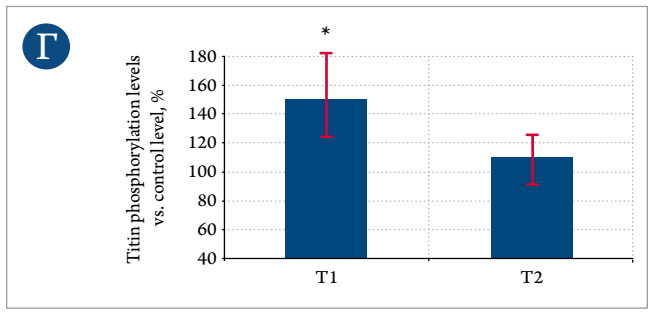
Figure 2. Content of titin and phosphorylation levels



A. SDS-gel-electrophoresis of titin (Coomassie Brilliant Blue) and phosphophoregram of titin (Pro-Diamond).
1 – control (n=7); 2 – after 4 weeks of doxorubicin administration (n=9).
Electrophoresis was performed in 2.2% polyacrylamide gel enriched with agarose.
MHC, myosin heavy chains. T2, proteolytic fragments of intact isoforms of titin-1 (T1).
N2B, N2BA, NT, T1 isoforms.







B. The T1 and T2 levels in the group of rats treated with doxorubicin vs. the control level of 100%. A 36% increase in the levels of T2 fragments in the myocardium of rats was detected after 4 weeks of doxorubicin administration; * p≤0.05 vs. the control level.

C. The N2BA/N2B ratio in the myocardium of rats. 1 – control (n=7); 2 – after 4 weeks of doxorubicin administration (n=9). A significant increase in the portion of N2BA isoform of titin in the hearts of rats after 4 weeks of doxorubicin administration of doxorubicin; ** p≤0.01 vs. the control level.

D. Changes in the levels of T1 and T2 phosphorylation in the group of rats treated with doxorubicin vs. the control level of 100%. A 50% increase i n the levels of T1 phosphorylation in the hearts of rats was detected after 4 weeks of doxorubicin administration; * p≤0.05 vs. the control level.

N2B isoform prevail. This ratio may change oppositely over time in CHF, caused by permanent high-frequency pacing: it increased in 2 weeks [18], which improved the filling of the LV, but decreased in 4 weeks [19], which contributes to the increase in force.

Conclusion

Thus, titin is a promising molecular target for therapeutic interventions that may improve the stretching of sarcomeres and thus improve the filling of the LV.

Opinion

1) The administration of doxorubicin in rats for 4 weeks caused diastolic dysfunction diagnosed via the catheterization of the LV.
2) In this group, the myocardial levels of adenine nucleotides and phosphocreatine differed little from the normal values, but lactate levels were increased manyfold.
3) The ratio of elastic N2BA isoform of titin to the stiffer N2B isoform (14%/86% in the control experiments) changed to 26%/74% in diastolic dysfunction.
4) The 50% increase in titin phosphorylation directly correlated (r=0.94) with the increase in N2BA-isoform levels and the decrease in N2B-isoform levels.

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