

Upregulation of mRNA Myocardium Calcium Handling in Rats Submitted to Exercise and Food Restriction

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Abstract

Background: Chronic exercise and food restriction (FR) have directionally opposite changes in transcription of molecular structures of calcium handling and thyroid hormone (TH) status.

Objective: Evaluate the association of chronic exercise and FR on serum thyroid hormones and gene transcription of molecular structures of intracellular calcium transients and thyroid receptors in myocardium of rats.

Methods: Male Wistar Kyoto rats, divided into two groups: control (C, n = 7), FR (R₅₀, n = 7), chronic exercise (EX, n = 7) and chronic exercise + FR (EX₅₀, n = 7). FR was of 50% and exercise was swimming (1 hour/day, 5 days/week, during 12 weeks). Serum concentrations of T₃, T₄ and TSH were determined. The mRNA gene expression of the sarcoplasmic reticulum calcium pump (SERCA2a), phospholamban (PLB), Na⁺/Ca²⁺ exchanger (NCX), calcium channel L-type (L-channel), ryanodine (RYR), calsequestrin (CQS) and HT receptor (TR_{α1} and TR_{β1}) of the myocardium was performed by PCR real-time.

Results: FR reduced serum levels of T₄ and TSH and TR_{α1} mRNA and increased the expression of PLB, NCX and L-channel. Exercise increased the TR_{β1} receptor, L-channel and NCX. The association of exercise and FR reduced plasma T₄ and TSH, TR_{β1} mRNA increase, SERCA2a, NCX and PLB, and there was a significant correlation of TR_{β1} with CQS and NCX.

Conclusion: Chronic exercise and food restriction increased the mRNA of transient Ca²⁺ proteins; however, TH – receptor axis cannot participate in the transcription of mRNA of myocardial calcium transient proteins. (Arq Bras Cardiol. 2011; [online].ahead print, PP.0-0)

Keywords: Exercise; caloric restriction; thyroid hormones; myocardium; carrier proteins; calcium.

Introduction

Interventions such as physical activity and food restriction (FR) have often been used to maintain health or esthetics. However, depending on the intensity, duration and frequency of FR and physical activity, there may be damage to the organism¹⁻⁷. Research shows that this association may depress⁸ or improve cardiac performance^{9,10}.

Molecular structures found in the sarcolemma, slow L-type calcium channel (L-channel) and sodium/potassium exchanger (NCX), also found in the sarcoplasmic reticulum, ryanodine channel, the calsequestrin (CQS), the sarcoplasmic reticulum calcium pump (SERCA2a) and phospholamban, are involved in the intracellular calcium handling and participate in the cardiac muscle contraction and relaxation¹¹. Studies show that FR decreases the gene expression of ryanodine¹², SERCA2¹³,

L-channel¹⁴, while physical training (PT) increases the mRNA of SERCA2¹⁵⁻¹⁷, ryanodine^{18,19} and L-channel²⁰. The transcription of the molecular structures involved in the intracellular calcium handling can be modulated by thyroid hormones (TH), via nuclear receptors of HT²¹⁻²³. It is known that food restriction reduces the synthesis and release of TH hormones¹³ and exercise can increase the release and/or the sensitivity of these hormones²¹. However, the relationship between FR, PT and thyroid hormones has not been established.

There is little literature that examines the relationship between FR, physical training, thyroid hormones and gene transcription of molecular structures of myocardial calcium handling. Katzeff et al¹³ found decreased plasma levels of TH hormones and decreased gene expression of SERCA2 in restricted rats. Iemitsu et al²² showed that exercise training increased the expression of TR_{α1} and TR_{β1} receptors and the gene transcription of SERCA2 in the myocardium of aged rats.

The objective of this study was to evaluate the effects of the combination of physical training and FR on plasma of thyroid hormones and the mRNA of TH receptors, SERCA2a, L-channel, NCX, ryanodine, calsequestrin and phospholamban in the myocardium of rats.

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Methods

Animal models and experimental protocol

This study used 28 Wistar-Kyoto (WKY) 60 day-old rats from the Experimental Animal Laboratory, Department of Clinical Medicine, Faculty of Medicine of Botucatu. During the experiment, the animals were kept in individual cages in a temperature-controlled environment ($24 \pm 2^\circ\text{C}$), light-dark cycle (12:12 h) and fed with commercial food and water. The project followed the guidelines established in Guide for the Care and Use of Laboratory Animals and the Ethical Principles in Animal Experiments of the Brazilian College of Animal Experimentation (Cobea), and was approved by the Ethics Committee of the Faculty of Medicine of Botucatu - UNESP, under protocol 575/2006.

The rats were divided into four groups: control with *ad libitum* food (C); food restricted to 50% (R_{50}); chronic exercise with *ad libitum* food (EX); chronic exercise with food restriction of 50% (EX_{50}).

Animals in groups C and EX were fed with commercial food Labina (Purina, Paulina, SP, Brazil) consisting of 3.76% fat, 20.96% protein, 52.28% carbohydrate, 9.60% ash and 13.40% humidity. Animals in groups R_{50} and EX_{50} received the same commercial diet but with a 50% reduction of the average amount consumed by the control group. The FR period was 90 days. Food intake was monitored daily and the weight of the animals was monitored weekly.

Training was swimming with a load of 5% of body weight. For swimming sessions, we used two tanks of fiber glass with the following dimensions: 100 cm long, 80 cm wide and 80 cm high containing heated water ($32 \pm 1^\circ\text{C}$), maintained at the level of 60 cm. The weight overload was performed using fishing sinkers wrapped in bandages and attached to the thorax of the rats by means of elastic bands. Up to six animals per training session were maintained.

Animals in groups EX and EX_{50} underwent five weekly sessions of swimming for 12 consecutive weeks. In the first, second and third weeks, the animals swam for 30, 45 and 60 minutes, respectively, without any additional load. From the fourth week, the swimming sessions were 60 minutes and overloaded. The rats of these groups had been previously subjected to a period of adaptation to the water. During one week, they were kept for 15 minutes inside the tank containing heated water, whose initial level of water was 7.0 cm, and gradually increased throughout the week, reaching a height of 60 cm.

T3, T4 and TSH serum level

Prior to sacrifice, animals were fasted for 12 hours and then were killed by decapitation, anesthetized with sodium pentobarbital (0.1 ml/kg) and blood was immediately transferred into test tubes. The doses of triiodothyronine (T_3), thyroxine (T_4) and thyroid-stimulating hormone (TSH) were determined using a specific commercial kit for rats, thyroid panel (LINCOpex) at the Gênese clinical laboratory, São Paulo - Brazil.

Assessment of general characteristics of animals

After collecting blood, the heart was removed, and the atrium, right ventricle and left ventricle were dissected and

then weighed and frozen for analyses of gene expression. The morphological variables used to characterize each group of animals were: initial body weight (IW) and final body weight (FW), left ventricular weight (LV), ratio of left ventricular weight and final body weight ($LV/FW \times 10^3$), right ventricular weight (RV), ratio of right ventricular weight and final body weight ($RV/FW \times 10^3$).

Evaluation of gene expression by real-time polymerase chain reaction after reverse transcription

RNA extraction

Left ventricular fragments were quickly frozen in liquid nitrogen and stored in a freezer at -80°C . The frozen sample was homogenized in Polytron apparatus (Ika Ultra Turrax™ T25 Basic, Wilmington, USA) after adding 1 ml of TRIzol™ (Invitrogen Brasil, São Paulo) for each 100 mg of tissue. The TRIzol™, monophasic solution of phenol and guanidine isothiocyanate, is intended to maintain the integrity of RNA during the cell lysis that occurs in the process of homogenization²⁴.

The homogenized sample was transferred to a tube and incubated at room temperature for 5 minutes to allow complete dissociation of core-protein. Then, chloroform was added (Merck KGaA, Darmstadt, Germany) at a rate of 0.2 ml/1 ml TRIzol™; the sample was shaken by hand vigorously for 15 seconds and incubated for 3 minutes at room temperature. After this second incubation, the material were centrifuged (Eppendorf 5804R, Hamburg, Germany) at 14,000 rpm for 15 minutes at 4°C . In this process, the sample was separated into three phases: a) a lower, pinkish phase of phenol-chloroform, containing DNA; b) a white interphase protein; and c) an upper phase, aqueous, colorless, containing RNA.

The RNA portion was transferred to a tube containing 0.5 ml of isopropyl alcohol (Merck KGaA, Darmstadt, Germany). The sample was manually shaken 10 times by inversion, incubated for 10 minutes at room temperature and then centrifuged at 14,000 rpm for 10 minutes at 4°C . The pellet was washed with 75% ethanol (Merck KGaA, Darmstadt, Germany) at the rate of 1 ml/1 ml of TRIzol™ and centrifuged at 7,500 rpm for 5 min at 4°C . After ethanol was disposed of, the pellet was dried for 10 minutes at room temperature. The RNA pellet was dissolved in 30 μl of ultrapure water and incubated for 10 minutes at 60°C water bath (Fanem mod 100, São Paulo, Brazil); but this procedure aimed to inactivate the possible presence of RNase.

The RNA was analyzed with the aid of a spectrophotometer (GeneQuant™ RNA/DNA Calculator, Amersham Pharmacia Biotech, Cambridge, England) by absorbance at 260 nm. The purity of RNA was verified by absorbance ratio at 260/280 nm. The samples whose ratios were below 1.6 were disposed of due to their contamination by protein.

Testing the integrity of RNA by electrophoresis

Samples of 1.0 μl of total RNA diluted in 8.0 μl of ultrapure water and 1.0 μl of dye (Orange G, Acros Organics, New Jersey, USA) were applied in agarose gel 1% (0.3 g agarose, 30

ml of 1x TAE Buffer, 3.0 ml of ethidium bromide) and subjected to a voltage of 60 mV (Power Pac Basic™ Bio-Rad) for 20 minutes. The integrity of RNA was verified by visualization of rRNA bands, 28S and 18S, and no traces of RNA in the gel. The samples that remained intact were used as substrates for reverse transcription.

RNA reverse transcription (RT)

The RNA samples of the heart muscle were subjected to reverse transcription by the action of the enzyme reverse transcriptase, using the kit SuperScript First-Strand Synthesis System for RT-PCR™ (Invitrogen, Brazil, São Paulo). The samples used in the experiment were incubated in a thermocycler (Mastercycler Gradient Eppendorf™ Hamburg, Germany). Initially, a mixture containing 1,000 ng/ml total RNA, 1.0 ml I dNTP mix 10 mM, 1.0 ml of random hexamers (50 ng/ml) and 8.0 ml of H₂O DEPC (diethyl pyrocarbonate) was incubated for 5 minutes at 65° C. Then, after adding 9.0 ml of a solution containing 2.0 ml of RT 10x buffer, 4.0 ml of MgCl₂ 25 mM, 2.0 ml of DTT 0.1 M and 1.0 ml of RNase inhibitor, RNaseOUT™, the mixture was incubated for 2 minutes at 25° C. After adding 1.0 ml of the enzyme SuperScript II™, we proceeded to further incubation for 10, 50 and 15 minutes at 25° C, 42° C and 70° C, respectively. After adding 1.0 ml of RNase H, the solution was incubated for 20 minutes at 37° C.

To check the quality of reverse transcription, we employed two methods:

1) *Positive control* - The kit contains an RNA transcribed from the gene of chloramphenicol acetyltransferase and control primers A and B. These primers in polymerase chain reaction generate a product of 500 base pairs (bp);

2) *Negative control* - To prove the absence of a residual genomic DNA, a sample of RNA was subjected to RT reaction, but the enzyme SuperScript II™ was replaced with 1.0 ml of H₂O DEPC. This product was used in the PCR reactions and the absence of residual genomic DNA was confirmed by the absence of amplification products.

Real-time polymerase chain reaction

Aliquots of RT reaction containing 2.0 µg of cDNA were added to a mixture containing TaqMan™ Universal PCR Master Mix (Applied Biosystems) and custom assay containing sense and anti-sense primers and Taqman™ probe (Applied Biosystems, Foster City, CA, USA) specific for each gene, and the volume completed to 25ml with water treated with DEPC. The reactions were performed in triplicates for each target gene in the Real Time PCR System 7500 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. For each specimen, an amplification graph showing increased fluorescent reporter dye (ΔRn) was plotted in each cycle of PCR. From this graph, we determined the cycle in which the reaction crosses the threshold of detection (cycle threshold - C_t) based on the variability of baseline data obtained from the initial cycles of PCR. The primers for the genes analyzed (Box 1) were obtained using the software Primer Express™ (Applied Biosystems, Foster City, CA, USA) from published

sequences in the GenBank (www.pubmed.com). The relative quantification of each gene normalized by endogenous reference (cyclophilin) was performed according to the User Bulletin # 2 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The experimental results were expressed as mean ± SD. Comparisons between groups were performed by two-way analysis of variance (ANOVA), exercise and diet, followed by the Tukey's test. Relations between the mRNA of SERCA2a, PLB, CQS, NCX, L-channel and RYR2 and thyroid hormone receptors, TR_{β1} and TR_{α1}, were measured by Pearson's correlation coefficient. The significance level was 5%.

Results

The general characteristics of the animals are shown in Table 1. The initial body weights and the LV/FW were not different between the groups. The animals restricted, R₅₀ and EX_{50'}, had final body weights, LV, RV, atrium and feed consumption significantly lower than their respective controls (C vs R₅₀ and EX vs EX_{50'}). The trained animals, EX and EX_{50'}, showed a reduction in final body weights compared to C and R₅₀ rats, respectively. The EX rats increased their atrium weight in relation to animals C. The LV/FW ratio significantly increased in EX₅₀ animals compared to EX animals.

Table 2 presents the results of the evaluation of mRNA by the real-time PCR technique. It was found that R₅₀ animals increased the expression of NCX, L-calcium channel and PLB, and reduced the expression of TR_{α1} compared to control rats. Animals in the EX group increased the expression of NCX, L-channel and TR_{β1} receptor compared to control animals. The association of physical training and FR, in the EX₅₀ group, increased expression of SERCA2a, NCX and PLB over animals

Box 1 - Primers employed

Gene	Code
SERCA2a	Rn00568762_m1
PLB	Rn01434045m_1
RyR2	Rn01470303_m1
CSQ2	Rn00567508_m1
NCX	Rn00570527_m1
L-type calcium channel	Rn00709287_m1
TR _{α1}	Rn00579692m1
TR _{β1}	Rn00562044_m1
Cyclophilin	Rn00690933_m1

SERCA2a - Calcium ATPase of sarcoplasmic reticulum (SR), cardiac isoform responsible for the uptake of Ca²⁺ to the SR; PLB - phospholamban, a protein that inhibits the uptake of Ca²⁺ of SERCA2a; RyR2 - ryanodine receptor, SR Ca²⁺ outlet to the cytosol; CSQ, calsequestrin, an abundant protein in the SR that acts as a reservoir of Ca²⁺; NCX - Na⁺/Ca²⁺ exchanger present in the sarcolemma, responsible for expelling the Ca²⁺ to the extracellular medium; L-type calcium channel, slow-channel of Ca²⁺ of the sarcolemma; it is opened by the potential action arrival; TR_{α1} - thyroid hormone receptor; TR_{β1} - thyroid hormone receptor; Cyclophilin - housekeeping gene used in the standardization of the target gene transcript product quantity.

Table 1 - General characteristics of animals

	C n = 7	R ₅₀ n = 7	EX n = 7	EX ₅₀ n = 7
Food intake (g)	20.2 ± 2.3	10.1 ± 1.1 *	20.7 ± 2.5	10.4 ± 1.2 *
IW (g)	292 ± 12	291 ± 21	294 ± 10	293 ± 17
FW (g)	354 ± 19	219 ± 8*	333 ± 16 *	207 ± 8 *†‡
LV (g)	0.694 ± 0.064	0.462 ± 0.016 *	0.672 ± 0.053	0.474 ± 0.024 *
RV (g)	0.213 ± 0.030	0.126 ± 0.019 *	0.217 ± 0.050	0.149 ± 0.022 *
Atrium (g)	0.059 ± 0.006	0.042 ± 0.005 *	0.077 ± 0.013 *	0.049 ± 0.006 *‡
LV/FW (mg/g)	1.962 ± 0.139	2.135 ± 0.142	2.017 ± 0.122	2.284 ± 0.100 *‡
RV/FW (mg/g)	0.601 ± 0.069	0.571 ± 0.069	0.654 ± 0.177	0.721 ± 0.121

Values expressed as mean ± standard deviation; C - control rats; R₅₀ - rats with restricted food (FR) of 50%; EX - trained rats; EX₅₀ - rats trained with 50% FR; IW - initial body weight (g); FW - final body weight (g); LV - left ventricle weight (g); RV - right ventricle weight (g) * versus C; † versus R₅₀; ‡ versus EX - ANOVA. Tukey. *p* < 0.05.

EX and R₅₀. Moreover, the EX₅₀ animals showed increased expression of TR_{β1} compared to R₅₀ rats.

Serum levels of T₃, T₄ and TSH are presented in Table 3. There was no change in serum concentration of T₃ by the action of FR or physical training. The trained rats, EX, showed no change in serum concentrations of T₄ and TSH. However, there was significant reduction of T₄ and TSH in the groups R₅₀ and EX₅₀ compared to respective controls (C vs R₅₀ and EX vs EX₅₀).

The correlation coefficients and significances between mRNA of calcium handling structures and thyroid hormone receptors are shown in table 4. Only the positive correlations (*r* > 0.70) in group EX₅₀ are presented, since the objective of this study was to evaluate the association and physical training and food restriction on the gene transcription of molecular structures involved in the calcium handling and the influence of the TH receptor in the myocardium. Thus, the correlation coefficients for the groups C, R₅₀ and EX were presented to define the participations of the factors exercise, food restriction and association between two factors.

Table 2 - Evaluation of mRNA by real time PCR

	C n = 7	R ₅₀ n = 7	EX n = 7	EX ₅₀ n = 7
L-channel	1.00 ± 0.34	2.34 ± 0.48 *	3.60 ± 0.76 *	2.74 ± 0.76*
NCX	1.00 ± 0.32	1.89 ± 0.56 *	2.48 ± 0.33*	3.13 ± 0.52 *†‡
RyR2	1.00 ± 0.18	1.08 ± 0.17	1.11 ± 0.23	1.26 ± 0.23
Serca2a	1.00 ± 0.36	0.92 ± 0.27	1.04 ± 0.24	1.42 ± 0.10 *†‡
PLB	1.00 ± 0.09	1.25 ± 0.19 *	1.14 ± 0.22	1.51 ± 0.26 *†‡
CSQ2	1.00 ± 0.29	1.03 ± 0.21	1.07 ± 0.22	1.27 ± 0.25
TR _{α1}	1.00 ± 0.29	0.67 ± 0.16 *	0.78 ± 0.23	0.86 ± 0.26
TR _{β1}	1.00 ± 0.29	0.96 ± 0.25	1.47 ± 0.40*	1.66 ± 0.17*†

Values expressed as mean ± standard deviation; C - control rats; R₅₀ - rats with restricted food (FR) of 50%; EX - trained rats; EX₅₀ - rats trained with 50% FR; RyR2 - ryanodine channel; Serca2a - sarcoplasmic reticulum calcium pump; PLB - phospholamban; CSQ2 - calsequestrin; NCX - Na⁺/Ca²⁺; TR - thyroid hormone receptor; * versus C; † versus R₅₀; ‡ versus EX. ANOVA. Tukey. *p* < 0.05.

It was observed that the TR_{β1} gene has more correlations with the molecular structures of the calcium handling than the TR_{α1} gene (Table 4). Although the L-channel mRNA and PLB have shown significant increase in EX₅₀ rats, these genes did not show any correlation with the genes TR_{α1} or TR_{β1}. The mRNA of ryanodine was significantly correlated with mRNA of TR_{β1} in all groups and was higher in the combination of FR with physical training. The SERCA2a mRNA also showed

Table 3 - Plasma concentration of thyroid hormones

	C n = 7	R ₅₀ n = 7	EX n = 7	EX ₅₀ n = 7
T ₃ (mg/dl)	4.35 ± 1.32	3.11 ± 1.34	5.52 ± 2.72	3.96 ± 2.36
T ₄ (mg/dl)	242 ± 29	113 ± 17 *	191 ± 61	126 ± 6 *‡
TSH (mg/dl)	6.35 ± 1.77	1.94 ± 0.75 *	5.07 ± 3.13	1.54 ± 0.66 *†‡

Values expressed as mean ± standard deviation; C - control rats; R₅₀ - rats with restricted food (FR) of 50%; EX - trained rats; EX₅₀ - rats trained with 50% FR; RyR2 - ryanodine channel; Serca2a - sarcoplasmic reticulum calcium pump; PLB - phospholamban; CSQ2 - calsequestrin; NCX - Na⁺/Ca²⁺ exchanger; TR - thyroid hormone receptor; * versus C; † versus R₅₀; ‡ versus EX. ANOVA. Tukey. *p* < 0.05.

Table 4 - Pearson correlation coefficient between mRNA of calcium handling proteins and thyroid hormone receptors

	C n = 7	R ₅₀ n = 7	EX n = 7	EX ₅₀ n = 7
NCX x TR _{α1}	<i>r</i> = 0.09 <i>p</i> = 0.85	<i>r</i> = 0.18 <i>p</i> = 0.71	<i>r</i> = 0.16 <i>p</i> = 0.73	<i>r</i> = 0.73 <i>p</i> = 0.06
RyR2 x TR _{β1}	<i>r</i> = 0.78 <i>p</i> = 0.037	<i>r</i> = 0.80 <i>p</i> = 0.03	<i>r</i> = 0.69 <i>p</i> = 0.09	<i>r</i> = 0.96 <i>p</i> = 0.0007
Serca2a x TR _{β1}	<i>r</i> = 0.11 <i>p</i> = 0.82	<i>r</i> = 0.67 <i>p</i> = 0.09	<i>r</i> = 0.69 <i>p</i> = 0.09	<i>r</i> = 0.72 <i>p</i> = 0.07
CSQ2 x TR _{β1}	<i>r</i> = 0.32 <i>p</i> = 0.49	<i>r</i> = -0.46 <i>p</i> = 0.30	<i>r</i> = 0.04 <i>p</i> = 0.93	<i>r</i> = 0.91 <i>p</i> = 0.004

C - control rats; R₅₀ - rats with restricted food (FR) of 50%; EX - trained rats; EX₅₀ - rats trained with 50% FR; RyR2 - ryanodine; Serca2a - sarcoplasmic reticulum calcium pump; PLB - phospholamban; CSQ2 - calsequestrin; NCX - Na⁺/Ca²⁺ exchanger; TR - thyroid hormone receptor; *r* - correlation coefficient; *p* - significance level.

a good correlation with the mRNA of TR_{β1} in the groups R₅₀, EX and EX₅₀ although it is not statistically significant, which may be due to the low number of samples. Calsequestrin and NCX genes correlated well with the mRNA TR_{β1} and TR_{α1'} respectively, only in the group EX₅₀ indicating the dependence of the association of both factors. It is interesting to note that the mRNA of CQS and RYR2 were strongly correlated with TR_{β1} in EX₅₀ animals. However, there was no significant increase in gene expression of CQS and RYR2 in this group. Conversely, the NCX gene increased but correlated only with the gene TR_{α1'}. The SERCA2a gene was the only one to correlate with the mRNA of TR_{β1} and increase in gene expression in animals EX₅₀.

Discussion

The objective of this study was to evaluate the participation of thyroid hormones in the association between severe food restriction and physical training and the correlation between TH receptors and gene expression of the molecular structures involved in myocardial intracellular calcium handling. The results of this study showed that physical training associated with severe FR promoted a significant increase of mRNA of SERCA2a, L-channel, phospholamban and Na⁺/Ca²⁺ exchanger. These changes were accompanied by an increase in the expression of thyroid hormone receptors (TR_{β1}) despite normal serum levels of T₃ and reduced serum levels of T₄ and TSH.

FR and physical training protocols promoted significant morphological changes (Table 1). The reductions in body weight, RV weight and LV weight induced by FR are associated with the degree of caloric restriction. Food restriction, excessive intake or fasting state, change energy expenditure and body composition²⁵. The reduction in energy expenditure during food restriction has been documented and represents a mechanism of energy conservation, which prevents excessive loss of body weight²⁵. The heart chambers were reduced in proportion to the reduction of body weight, as observed by the relations LV/BW and RV/BW. The decrease in body weight by exercise training is associated with increased energy expenditure resulting from physical training sessions; the LV/BW and RV/BW ratios were maintained compared to the control group. The association of PT and FR enhanced reduction in body weight, but kept the LV and RV mass and maintained the LV/BW and RV/BW ratios compared with R₅₀ rats. Thus, the results related to the cardiac chambers indicate that the FR promoted a significant reduction of cardiac mass and physical training, alone or combined with FR, did not cause cardiac hypertrophy. These results were similar to those observed in previous studies¹⁰.

FR, alone or in trained rats, reduced levels of T₄ and TSH, while physical training did not change serum levels of thyroid hormones. Although FR reduces the serum level of T₃ by 29% in both groups (R₅₀ and EX₅₀), this reduction was not of sufficient magnitude to show a statistical difference between groups. The lack of change in T₃ levels may be due to total T₃ rather than free T₃, which is the active form of the hormone. Literature data indicate that in states of fasting and severe calorie restriction, there is marked increase of reverse T₃¹³. So it is possible that the free T₃/reverse T₃ ratio was reduced in animals subjected to FR. It is believed that energy deprivation

may modulate serum concentration of T₃ and reduce the activity or concentration of iodothyronine deiodinase that converts T₄ into T₃²⁶. The significant reduction of serum levels of T₄ and TSH induced by FR indicates state of hypothyroidism, which was also observed in experimental^{8,13,27,28} and clinical studies²⁹. The mechanism responsible for decreased plasma levels of thyroid hormones induced by FR may be related to the actual reduction in energy consumption. Reduced levels of thyroid hormones relate to the mechanism of energy conservation, since these hormones are mainly responsible for the energy expenditure²⁵. Considering that energy expenditure is reduced in the food restriction, reduced levels of TH would be expected. Physical training did not promote changes in plasma levels of thyroid hormones. This result differs from that reported by Iemitsu et al²² and Katzeff et al¹³, who found an increase in serum levels of T₄. This discrepancy in results may have been due to the type, intensity and volume of physical training. In conclusion, while the FR promoted the state of hypothyroidism, physical training seems to have no direct effect on the action of thyroid hormones.

In the heart of rats, over 70% of all TH receptors are TR_{α1'} with TR_{β1} accounting for only 30%³⁰. The thyroid hormone binds to thyroid response elements (TH receptors) in regions that promote many genes, regulating transcription of target genes in the heart including Na⁺/Ca²⁺ exchanger, ryanodine, L-type calcium channel and mainly SERCA2a and fosfolamban³⁰. This regulation by TH is an important mechanism in the process of myocardial contraction and relaxation³¹.

The results of this study indicate that the state of hypothyroidism induced by FR decreased the expression of the gene TR_{α1'}, a receptor prevalent in the myocardium. This result was expected since the reduction of TH causes a negative feedback in the TH receptor. Exercise training, alone or combined with FR, increased expression of TR_{β1} without changing the levels of TR_{α1'}. There is limited literature on the role of TR_{α1'} and TR_{β1} receptors on the gene transcription of proteins of the myocardial calcium handling. Kinugawa et al²¹ showed that the TR_{α1'} relates to the transcription of α-MHC and myocardial protein synthesis, while TR_{β1} relates to SERCA2a, TR_{β1} itself and inhibition of β-MHC. Thus, our data suggest that the reduction of the TR_{α1'} gene by FR could be a mechanism associated with loss of heart mass; absence of hypertrophy in trained animals could be due to maintenance of TR_{α1'} levels.

The effects of interventions, FR and PT, resulted in changes in the mRNA of molecular structures that regulate the calcium handling (Table 2). The increase in SERCA2a and PLB in EX₅₀ rats observed in this study suggests that the process of calcium reuptake by the sarcoplasmic reticulum (SR) can be facilitated. The explanation for this hypothesis is based on data on the effects of FR which, alone, caused only an increase in PLB indicating a worsening in the process of reuptake of calcium by the SR. Because this process is dependent on ATP and, under severe food restriction, it requires energy savings, increased phospholamban may lead to reduced activity of SERCA2a. When FR was associated with physical training there was a proportional increase of SERCA2a and PLB, indicating that exercise training protected the myocardium from damage caused by the FR. The improvement in the process of reuptake of calcium by the SR induced by physical training is consistent

with studies by Kemi et al³² who reported an increase in the reuptake of calcium in the myocardium derived from the increase of SERCA2a and phosphorylated phospholamban (PLB-thr17) in trained rats. The increased L-channel observed in EX₅₀ animals suggests an increased influx of calcium to improve the contractile response. The increase in the NCX gene would aim to offset the increased influx of calcium through sarcolemmal slow calcium channels.

In this study, we could not relate TH receptors with the molecular structures of the myocardial calcium handling in rats trained and subjected to severe FR, considering that the correlation test showed no significant difference in the mRNA of SERCA2a, PLB, NCX, L-channel with TR_{α1} or TR_{β1} receptors. Literature data show an association of the molecular structures involved in calcium handling with TH receptors induced by training or state of hypothyroidism; lemitsu et al²² found a significantly increased expression of TR_{α1} and TR_{β1} receptors accompanied by increased expression of SERCA2a by physical training. In conditions of hypothyroidism, thyroid failure is associated with decreased SERCA2a and increased phospholamban²³, increased expression of NCX³³ and unchanged³⁴ or increased³⁵ levels of L-channel.

The molecular mechanisms for increasing gene transcription of SERCA2, PLB and NCX in the exercise alone or combined with FR remain unclear; as previously mentioned, our findings suggest that TH and/or TH receptors do not participate in the gene transcription of molecular structures of calcium handling in this group of animals. The process of cardiac remodeling derives from extracellular stimuli that activate complex cytosolic signals with a number of integration points, and nuclear transcriptional processes. Among the extracellular

stimuli, agonists of G protein-coupled receptors (angiotensin, endothelin, catecholamines), cytokines and growth factors (IGF, TGF, FGF) are the main candidates that could therefore stimulate the gene transcription of calcium handling structures^{36,37}. However, there is no literature data that allow speculating the mechanisms responsible for increased gene transcription of SERCA2, PLB and NCX in the interaction between exercise and food restriction.

Study limitations

Our findings of gene expression of mRNA obtained by quantitative PCR of SERCA2a, phospholamban, Na⁺/Ca²⁺ exchanger, L-type calcium channel, and calsequestrin and TH receptors may not be reflected in protein formation. Recent data obtained in our laboratory found a significant reduction of protein expression of L-type channel in rats with severe FR, which is contrary to our findings of increased mRNA in the same protein¹⁴. Therefore, the continuity of these findings will be the determination of the protein expression by Western blot. Nor was it possible to establish a cause-effect relationship between thyroid hormone and/or TH receptors with the mRNA of the calcium handling; studies with TH treatment or others using specific monoclonal animals for TH would be required.

In conclusion, the findings of the study showed increased mRNA expression of the molecular structures involved in the intracellular calcium handling in animals subjected to severe restriction and physical training. Although the association between PT and FR induces a state of hypothyroidism, the TH-receptor axis does not seem to participate in the transcription of mRNA of myocardial calcium handling proteins.

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