Exercise Training and Caloric Restriction Prevent Reduction in Cardiac Ca\textsuperscript{2+}-Handling Protein Profile in Obese Rats

Ellena Christina Paulino, Julio Cesar Batista Ferreira, Luiz Roberto Bechara, Jeane Mike Tsutsui, Wilson Mathias, Jr, Fabio Bessa Lima, Dulce Elena Casarini, Antonio Carlos Cicogna, Patricia Chakur Brum, Carlos Eduardo Negro

Abstract—Previous studies show that exercise training and caloric restriction improve cardiac function in obesity. However, the molecular mechanisms underlying this effect on cardiac function remain unknown. Thus, we studied the effect of exercise training and/or caloric restriction on cardiac function and Ca\textsuperscript{2+} handling protein expression in obese rats. To accomplish this goal, male rats fed with a high-fat and sucrose diet for 25 weeks were randomly assigned into 4 groups: high-fat and sucrose diet, high-fat and sucrose diet and exercise training, caloric restriction, and exercise training and caloric restriction. An additional lean group was studied. The study was conducted for 10 weeks. Cardiac function was evaluated by echocardiography and Ca\textsuperscript{2+} handling protein expression by Western blotting. Our results showed that visceral fat mass, circulating leptin, epinephrine, and norepinephrine levels were higher in rats on the high-fat and sucrose diet compared with the lean rats. Cardiac nitrate levels, reduced/oxidized glutathione, left ventricular fractional shortening, and protein expression of phosphorylated Ser\textsuperscript{2808}-ryanodine receptor and Thr\textsuperscript{17}-phospholamban were lower in rats on the high-fat and sucrose diet compared with lean rats. Exercise training and/or caloric restriction prevented increases in visceral fat mass, circulating leptin, epinephrine, and norepinephrine levels and prevented reduction in cardiac nitrate levels and reduced/oxidized glutathione ratio. Exercise training and/or caloric restriction prevented reduction in left ventricular fractional shortening and in phosphorylation of the Ser\textsuperscript{2808}-ryanodine receptor and Thr\textsuperscript{17}-phospholamban. These findings show that exercise training and/or caloric restriction prevent cardiac dysfunction in high-fat and sucrose diet rats, which seems to be attributed to decreased circulating neurohormone levels. In addition, this nonpharmacological paradigm prevents a reduction in the Ser\textsuperscript{2808}-ryanodine receptor and Thr\textsuperscript{17}-phospholamban phosphorylation and redox status. (Hypertension. 2010;56:00-00.)

Key Words: list obesity exercise training caloric restriction cardiac dysfunction Ca\textsuperscript{2+} handling

Recently, epidemiological studies have demonstrated that the prevalence of overweight among adults exceeds 1 billion worldwide. In addition, statistics show that 300 million of these individuals are obese (body mass index: >30 kg/m\textsuperscript{2}).

Obesity is a complex syndrome linked to an elevated risk of diabetes mellitus, dyslipidemia, and cardiovascular disease. Class III obese patients (body mass index: >40 kg/m\textsuperscript{2}) display a 2-fold increase in the risk of developing heart failure. Previous studies demonstrated that the depressed cardiac contractile function in obesity is associated with Ca\textsuperscript{2+} handling abnormalities. Decreased cytosolic Ca\textsuperscript{2+} removal and myocyte relaxation associated with reduced sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2) function have been reported in sucrose-fed rats. Furthermore, some investigators observed that the decreased cardiac contraction in high-fat diet-induced obesity was linked with reduced phosphorylated phospholamban (PLN) expression level. These obesity-deleterious effects dramatically raise the need for new strategies to improve Ca\textsuperscript{2+} handling abnormalities and cardiac contractile function in obesity.

Accumulated evidence shows that exercise training and caloric restriction are useful strategies to maintain long-term body weight reduction and to improve cardiovascular functioning associated with autonomic control in human obesity. Indeed, exercise training improves the net balance of cardiac Ca\textsuperscript{2+} handling–related proteins in heart failure. Whether exercise training and/or caloric restriction cause Ca\textsuperscript{2+} handling protein changes in obesity is an unknown issue. However, it seems reasonable to expect that these nonpharmacological interventions represent attractive strategies to improve cardiomyocyte contractility because of better Ca\textsuperscript{2+} handling–related protein profiles.
The impact of obesity on cardiovascular and metabolic profiles has been a matter of current interest. However, many of the obesity studies have been conducted in genetic animal models, which may not reflect most human obesity causes. Thus, in the present study, we tested whether 10 weeks of exercise training and/or caloric restriction would improve cardiac function in obese rats fed with a high-fat and sucrose diet that mimics food habits of humans living in Western countries.

To ascertain the mechanisms responsible for this improvement, we also studied the effect of these interventions on cardiac Ca\(^{2+}\)-handling–related protein levels and on the neurohumoral control mechanisms responsible for the modulation of their expression.

**Methods**

**Sampling**

Thirty-day-old male Wistar rats were assigned into 2 groups: rats fed with standard chow (Purina) and rats fed with a high-fat and sucrose diet for 25 weeks (Figure 1A). Animals fed with a high-fat and sucrose diet received 5 different diets (more details in the online Data Supplement). Every high-fat and sucrose diet lasted for 1 week. At the end of the fifth week, a new cycle of 5 diets was again initiated. Our goal with this procedure was to induce obesity in rats with a normal genetic background and mimic the Western occidental dietary habits. In addition, we thought that the change in diet every week could reinforce the urge for food intake. Both groups received water and food ad libitum. At the 25th week, rats fed with a high-fat and sucrose diet were randomly assigned into 4 groups: high-fat and sucrose diet (O; n = 13), high-fat and sucrose diet and exercise training (OT; n = 13), caloric restriction (OR; n = 14), and exercise training and caloric restriction (OTR; n = 14). The lean control group continued with standard chow feeding (L; n = 13). This new period was conducted for 10 weeks. The study was approved by the Scientific Committee from the Heart Institute and Human Subject Protection Committee of the Clinical Hospital, Medical School of University of São Paulo (No. 993/05). At the end of the protocol, all of the animals were killed by decapitation, and their tissues were harvested. Cardiac chambers were either snap frozen in liquid nitrogen (and stored at −80°C) for biochemical analysis or fixed by immersion in 4% buffered formalin and embedded in paraffin for routine histological processing.

**Figure 1.** Exercise training and/or caloric restriction prevent reduction in ventricular function in obese rats. A, Experimental design showing rats submitted to high-fat and sucrose diets or standard control diet for 25 weeks and an additional 10-week period in which the rats fed with high-fat and sucrose diets were randomly divided into O, OT, OR, and OTR groups. The control group continued with standard chow feeding. Animals fed with high-fat and sucrose diets received 5 different diets (more details in the online Data Supplement). Every high-fat and sucrose diet lasted for 1 week. B, Body weight; (C) fractional shortening, (D) total distance run, and (E) peak oxygen uptake before (25th week) and after 10 weeks of exercise training and/or caloric restriction in obese rats (35th week). Note that O rats display decreased left ventricular fractional shortening and total distance run. Exercise training and/or caloric restriction prevent reduction in left ventricular fractional shortening. No significant difference in left ventricular fractional shortening is observed among OT, OR, ORT, and L rats. Exercise training improves maximal oxygen uptake (peak VO\(_2\)) and total distance run in obese rats. Data are presented as mean ± SE. *P < 0.05 vs L rats; ‡ P < 0.05 vs O rats. Data were analyzed by unpaired Student t test (25th week) or 1-way ANOVA with post hoc testing by Bonferroni (35th week).

The impact of obesity on cardiovascular and metabolic profiles has been a matter of current interest. However, many of the obesity studies have been conducted in genetic animal models, which may not reflect most human obesity causes. Thus, in the present study, we tested whether 10 weeks of exercise training and/or caloric restriction would improve cardiac function in obese rats fed with a high-fat and sucrose diet that mimics food habits of humans living in Western countries. To ascertain the mechanisms responsible for this improvement, we also studied the effect of these interventions on cardiac Ca\(^{2+}\)-handling–related protein levels and on the neurohumoral control mechanisms responsible for the modulation of their expression.
Untrained rats were submitted to treadmill exercise for 5 minutes every day and at the end of the exercise training protocol, an exercise test was performed at the beginning, at the fourth week to adjust the caloric restricted diet. A progressive exercise test that consisted of 5-m/min speed increments was continuously measured by means of expired gas analysis during rapid-flow, open-circuit indirect calorimetry during a progressive oxygen uptake. Oxygen consumption was measured by means of expired gas during a progressive exercise test that consisted of 5-m/min speed increments at the fourth week, as described previously. Exercise training was performed on a motor treadmill over 10 weeks, progressively increased to elicit 60% maximal oxygen uptake, for 60 minutes at the fourth week, as described previously. The maximal exercise test was performed at the beginning, at the fourth week to adjust the training intensity, and at the end of the exercise training protocol. Untrained rats were submitted to treadmill exercise for 5 minutes every day to become accustomed to exercise protocol and handling.

**Table. Physiological Parameters in Lean and Obese Rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>25 wk</th>
<th>25 + 10 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>O</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>VFM, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>420.2 ± 11.3</td>
<td>429.6 ± 4.9</td>
</tr>
<tr>
<td>BP, mm Hg</td>
<td>120.9 ± 3.1</td>
<td>123.3 ± 1.4</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>6.0 ± 0.1</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.3 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>RWT, mm</td>
<td>0.40 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>HW/TL, g/mm</td>
<td>2.6 ± 0.1</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>CW, µm</td>
<td>18.5 ± 0.2</td>
<td>19.0 ± 0.3</td>
</tr>
<tr>
<td>CC, %</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

Groups are divided into L, O, OT, OR, and OTR. VFM indicates visceral fat mass; HR, heart rate; BP, blood pressure; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; RWT, relative wall thickness; E/A ratio, rate of the peak early to late diastolic filling velocity; HW/TL, heart weight/tibia length ratio; CW, cardiomyocyte width; CC, collagen content. *P<0.05 vs lean. †P<0.05 vs obese. †‡P<0.05 vs trained obese.

**Oxygen Uptake**

Oxygen consumption was measured by means of expired gas during a rapid-flow, open-circuit indirect calorimetry during a progressive running exercise test on a motor treadmill. Oxygen consumption was continuously measured by means of expired gas analysis during a progressive exercise test that consisted of 5-m/min speed increment every 3 minutes and no grade until exhaustion.

**Exercise Training Protocol**

Exercise training was performed on a motor treadmill over 10 weeks, 5 days per week. The running speed and exercise duration were progressively increased to elicit 60% maximal oxygen uptake, for 60 minutes at the fourth week, as described previously. The maximal exercise test was performed at the beginning, at the fourth week to adjust the training intensity, and at the end of the exercise training protocol. Untrained rats were submitted to treadmill exercise for 5 minutes every day to become accustomed to exercise protocol and handling.

**Caloric Restriction Protocol**

The caloric restricted diet consisted of standard chow in a quantity of 20 kcal/day lower than that consumed by control L rats, as described elsewhere.

**Cardiovascular Measurements**

Systolic blood pressure was evaluated noninvasively using a computerized tail-cuff system (Kent Scientific). Noninvasive cardiac function was assessed by 2D guided M-mode echocardiography in xylazine/ketamine-anesthetized rats. Transthoracic echocardiography was performed using a commercially available ultrasound scanner (SONOS 5500, Philips Medical Systems) equipped with a 5- to 12-MHz linear transducer that allows images to be 2- to 3-cm deep. Left ventricular systolic function was estimated by fractional shortening (FS) as follows: FS (%) = [(LVEDD - LVESD)/LVEDD] × 100, where LVEDD means left ventricular end-diastolic diameter and LVESD means left ventricular end-systolic diameter. Left ventricular diastolic filling pattern was evaluated by E/A ratio, where E wave is characterized by an early peak and A wave by a late peak, which is caused by atrial contraction. Cardiac hypertrophy was assessed by relative wall thickness (RWT); RWT = (anterior wall thickness + posterior wall thickness)/LVEDD. Echocardiography was performed in all of the rats by the same investigator (J.M.T.) in a blind manner.

**Histological Analysis**

Paraffin embedded midventricular sections were deparaffinized, rehydrated, and further stained with hematoxylin-eosin or Sirius red stain. Cardiomyocyte width and cardiac collagen deposition, indexes of cardiomyocyte hypertrophy and myocardial fibrosis, were observed and quantified by a computer-assisted image analysis system (Quantimet 520, Cambridge Instruments), as described previously. See more details in the online Data Supplement (please see http://hyper.ahajournals.org).

**NO Bioavailability**

Heart homogenate was prepared under liquid nitrogen and centrifuged at 3000g for 5 minutes, and 10 µL aliquots of the supernatant were injected into a Sievers chemiluminescence analyzer (model 280, Sievers Instruments Inc) with VCl3 and HCl (at 95°C) as reductants, described previously. NO results were normalized by total protein concentration.

**Statistical Analysis**

Data are shown as mean±SE. Statistical significance was determined by unpaired Student t test and 1-way and 2-way ANOVA, as appropriate. When significant differences were found, the post hoc Bonferroni multiple comparisons tests were carried out. P<0.05 was considered statistically significant.

**Results**

**Effects of Exercise Training and/or Caloric Restriction on Hormones and Left Ventricular Function**

Physiological parameters of obese and lean rats at 25th and 35th weeks are shown in the Table. At the 25th and 35th weeks, animals submitted to a high-fat and sucrose diet presented a significant increase in body weight when compared with animals fed with standard chow (Figure 1B). No differences in left ventricular function were observed between O and L rats at the 25th week (Figure 1C and Table). However, at the 35th week, left ventricular fractional shortening was significantly lower in O rats when compared with L rats (Figure 1C). Exercise tolerance, assessed by running...
distance, was significantly lower in O rats compared with L control rats at the 25th and 35th weeks (Figure 1D), whereas peak oxygen uptake, blood pressure, and heart rate were similar in both groups (Figure 1E and Table).

The effect of exercise training and/or caloric restriction (10 weeks) on physiological parameters of obese rats (35th week) is depicted in the Table. Exercise training and/or caloric restriction had lower body weight (OT: −9%, OR: −16%, and OTR: −20%; Figure 1B). Exercise training and/or caloric restriction prevented increase in visceral fat mass (Table). No changes in skeletal muscle mass were observed in all of the groups studied (data not shown). Exercise training and/or caloric restriction did not significantly change left ventricular diameters, relative wall thickness, cardiomyocyte width, or cardiac collagen content (Table). Exercise training and/or caloric restriction prevented a reduction in left ventricular fractional shortening. No significant difference in left ventricular fractional shortening was observed among OT, OR, and OTR and L rats (Figure 1C). Exercise training alone or associated with caloric restriction increased running distance and peak oxygen uptake in obese rats (Figure 1D and 1E). Blood pressure was similar in all of the groups studied. Heart rate was lower in OTR rats than in OT and O rats (Table). Exercise training and/or caloric restriction prevented an increase in circulating norepinephrine, epinephrine, and leptin levels (Figure 2).

**Effects of Exercise Training and/or Caloric Restriction on Cardiac PLN_{thr17} and RyR_{ser2808} Phosphorylation**

Because Ca^{2+} handling–related protein profile contributes to cardiac function, we investigated whether the expression of these proteins was altered in obese rats. In addition, we investigated whether exercise training and caloric restriction would improve cardiomyocyte Ca^{2+} handling protein expression in obese rats.

α-Tubulin protein expression was not different among all of the groups studied and was used to normalize Ca^{2+} handling–related protein expression. SERCA2, Na^{+}-Ca^{2+} exchanger, and phospho-Ser^{16}-PLN/PLN expression were not different between O and L rats (Figure 3A through 3D). Exercise training and/or caloric restriction had no effect onSERCA2, Na^{+}-Ca^{2+} exchanger, and phospho-Ser^{16}-PLN/PLN protein expression (Figure 3A through 3D). SERCA2, PLN, and RyRmRNA levels were also similar in all of the groups studied (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). O rats displayed no changes in protein kinase A protein expression and activity when compared with L rats (Figure S2A through S2D). Exercise training and/or caloric restriction did not change protein kinase A expression and activity in obese rats. Both phospho–Thr^{17}-PLN/PLN and phospho–Ser^{2808}-RyR/RyR expressions were significantly lower in O compared with L rats. Exercise training and/or caloric restriction prevented reduction in phospho–Thr^{17}-PLN/PLN:PLN ratio (Figure 3A and 3E) and phospho–Ser^{2808}-RyR:RyR ratio (Figure 3A and 3F). No significant difference in phospho–Thr^{17}-PLN:PLN ratio (Figure 3A and 3E) and phospho–Ser^{2808}-RyR:RyR ratio was observed among OT, OR, and OTR and L rats. Phospho–Thr^{17}-CaMKII/CaMKII was unchanged in all of the groups studied (Figure S2A and S2E).

The effects of exercise training and/or caloric restriction on cardiac reduced glutathione (GSH):oxidized glutathione (GSSG) ratio and nitrate levels are shown in Figure 4. Cardiac GSH:GSSG ratio was significantly reduced in O rats (Figure 4A). Similarly, cardiac nitrate levels were significantly lower in O rats compared with L rats (Figure 4B). Exercise training and/or caloric restriction prevented a reduction in the GSH:GSSG ratio and nitrate levels (Figure 4A and 4B). No significant difference in the GSH:GSSG ratio and nitrate levels was observed among OT, OR, ORT, and L rats.

**Discussion**

In the present study, we investigated whether exercise training and caloric restriction would improve left ventricular function in an animal model of high-fat and sucrose diet–induced obesity. We found that both nonpharmacological therapies prevented reduction in left ventricular systolic function in obese rats chronically fed with a high-fat and sucrose diet. In addition, these interventions prevented a reduction in cardiac phospho–Thr^{17}-PLN and phospho–Ser^{2808}-RyR protein expression levels.
Previous studies show that obesity causes cardiac dysfunction and decrease in SERCA2 activity and phosphorylation of PLN. More recently, Fang et al. elegantly demonstrated that cardiac dysfunction and impairment in intracellular Ca\textsuperscript{2+} handling in mice are attributed to high-fat diet–induced obesity rather than high-fat diet intake without weight gain. The present study extends these findings showing that chronic high-fat and sucrose diet–induced obesity provokes a reduction in left ventricular systolic function and phospho–Thr\textsuperscript{17}-PLN and phospho–Ser\textsuperscript{2808}-RyR protein expression levels. In addition, our study provides evidence that nonpharmacological treatment based on exercise training, caloric restriction, or both prevents a reduction in left ventricular systolic function and in phosphorylation of sarcoplasmic reticulum proteins, essential to the maintenance of cardiac Ca\textsuperscript{2+} handling.

It has been shown that PLN phosphorylation in either Ser\textsuperscript{16} or Thr\textsuperscript{17} residues increases SERCA2 activity and Ca\textsuperscript{2+} reuptake by the sarcoplasmic reticulum. In addition, some investigators report that RyR phosphorylation increases Ca\textsuperscript{2+} release from the sarcoplasmic reticulum to cytoplasm. Thus, it is reasonable to expect that the improvement in phospho–Thr\textsuperscript{17}-PLN and phospho–Ser\textsuperscript{2808}-RyR protein expression plays a role in the prevention of left ventricular systolic reduction in the present study.

The mechanisms underlying the increase in the phospho–Thr\textsuperscript{17}-PLN and phospho–Ser\textsuperscript{2808}-RyR expressions are out of the scope of our study. However, it brings about some candidates for the explanation of changes in protein expression after exercise training and caloric restriction. The increase in phospho–Thr\textsuperscript{17}-PLN and phospho–Ser\textsuperscript{2808}-RyR expressions may be attributed to the reduction in adrenergic hyperactivity (epinephrine and norepinephrine levels), because chronic cardiac exposure to elevated catecholamine desensitization. In addition, it has been reported that these alterations can reduce protein kinase A activation and cardiac force. Surprisingly, we did not find alterations in protein kinase

---

**Figure 3.** Exercise training and/or caloric restriction prevent reduction in cardiac PLN\textsubscript{Thr17} and RyR\textsubscript{Ser2808} phosphorylation in obese rats. A, Representative blots, (B) SERCA2, (C) Na+/Ca\textsuperscript{2+} exchanger, (D) phospho–Ser\textsuperscript{16}-PLN:PLN ratio, (E) phospho–Thr\textsuperscript{17}-PLN:PLN ratio, and (F) phospho–Ser\textsuperscript{2808}-RyR:RyR ratio after 10 weeks of exercise training and/or caloric restriction in obese rats (35th week). Groups are divided into L, O, OT, OR, and OTR. All of the data were normalized against α-tubulin. Note that O rats have decreased phospho–Thr\textsuperscript{17}-PLN and phospho–Ser\textsuperscript{2808}-RyR expression levels vs L rats. Exercise training and/or caloric restriction prevent reduction in phospho–Thr\textsuperscript{17}-PLN:PLN ratio and phospho–Ser\textsuperscript{2808}-RyR:RyR ratio vs L rats. Data are presented as mean±SE. *P<0.05 vs L rats; ‡P<0.05 vs O rats. Data were analyzed by 1-way ANOVA with post hoc testing by Bonferroni.

**Figure 4.** Exercise training and/or caloric restriction prevent reduction in cardiac NO bioavailability and redox status in obese rats. A, Reduced GSH/GSSG ratio and (B) cardiac nitrate levels after 10 weeks of exercise training and/or caloric restriction in obese rats (35th week). Groups are divided into L, O, OT, OR, and OTR. Note that O rats display decreased nitrate levels and GSH/GSSG ratio vs L rats. Exercise training and/or caloric restriction prevent reduction in GSH/GSSG ratio and in nitrate levels. Data are presented as mean±SE. *P<0.05 vs L rats; ‡P<0.05 vs O rats. Data were analyzed by 1-way ANOVA with post hoc testing by Bonferroni.
A and CaMKII expression and activity, both components of the adrenergic signaling pathway.

An alternative explanation for the prevention of phospho-Thr\textsuperscript{17}-PLN and phospho-Se\textsubscript{S2808}RyR expression reduction is the reduction in leptin levels, because leptin resistance decreases redox balance and impairs peak Ca\textsuperscript{2+} transient.\textsuperscript{24,25} In fact, elevated circulating leptin levels have been observed in myocardial infarction and heart failure. In addition, leptin causes a dose-dependent inhibition in cardiomyocyte shortening and intracellular Ca\textsuperscript{2+} handling.\textsuperscript{25,26} These findings suggest that elevated leptin plasma levels may act as pathophysiological triggers for cardiovascular diseases attributed to tissue leptin resistance. We cannot rule out the possibility that the changes in protein expression after exercise training and caloric restriction are because of an increase in NO bioavailability. Previous studies show that reduction in NO bioavailability decreases its inhibitory effect on xanthine oxidase, which, in consequence, increases superoxide production.\textsuperscript{27,28} Some investigators have suggested that oxidized RyR by superoxide and peroxynitrite causes Ca\textsuperscript{2+} leaking from the sarcoplasmic reticulum, whereas oxidized SERCA by superoxide decreases sarcoplasmic reticulum Ca\textsuperscript{2+} uptake.\textsuperscript{27,29} Our study shows that exercise training and caloric restriction prevent reduction in cardiac nitrate levels and the GSH:GSSG ratio, which are consistent with higher NO bioavailability. Altogether, these findings favor the idea that the prevention of Ca\textsuperscript{2+} handling and cardiac function reduction after exercise training and/or caloric restriction in obese rats are associated with the amelioration in leptin resistance and redox balance.

An unexpected finding was the lack of additive effects on the association of exercise training and caloric restriction on cardiac function and Ca\textsuperscript{2+} handling protein expression. One clear explanation for such a result is not available. However, it is possible that exercise or caloric restriction is sufficient to reduce body weight in obese rats.

Surprisingly, 25 weeks of a high-fat and sucrose diet did not cause alteration in left ventricular function in obese rats. Alteration in left ventricular function was only observed at the 35th week, which suggests that the impact of obesity on cardiac function occurs in the chronic stage of obesity. In fact, in a previous study, some investigators found myocardial contractile dysfunction in rats fed with high-fat diet-induced obesity for 6 months.\textsuperscript{20}

The increase in total distance running and peak oxygen consumption shows the effectiveness of our exercise training paradigm. Interestingly, the increase in total distance running was proportionally greater than the increase in peak oxygen consumption in exercise-trained rats. This finding suggests that exercise training, other than increasing peak oxygen uptake, substantially improves metabolic efficiency in obese rats. Another interesting finding in our study is the decreased total distance running in untrained O rats compared with L rats, despite the similarity in peak oxygen consumption between them. This information suggests that obesity deteriorates metabolic efficiency.

**Limitation**

Despite the fact that exercise training and caloric restriction prevent a reduction in phospho-Thr\textsuperscript{17}-PLN and phospho-Se\textsubscript{S2808}RyR protein expression in O rats, the present study does not provide direct evidence that these responses in protein expression reflect cardiomyocyte Ca\textsuperscript{2+} transient. Although Ca\textsuperscript{2+} transients tend to parallel changes in the expression of cardiac Ca\textsuperscript{2+} handling proteins,\textsuperscript{30–32} cardiac Ca\textsuperscript{2+} transients were not directly assessed in our study. The present findings show that exercise training and/or caloric restriction prevent reduction in left ventricular systolic function in O rats but cause no effects on left ventricular diastolic function. This last finding may be because of methodological limitations, because complete diastolic function was not evaluated in the present study. Someone could also argue that left ventricular fractional shortening is a poor index of left ventricular systolic function. Thus, a more sensitive method based on measurements of left ventricular dP/dt or end-systolic elastance could provide more information regarding the effects of exercise training and/or caloric restriction on left ventricular function. The lack of changes in left ventricular end-systolic and -diastolic diameters could lead someone to question the functional importance of exercise training and caloric restriction in left ventricular fractional shortening in the present study. Plasma norepinephrine does not closely track cardiac sympathetic stimulation. Thus, there is no guarantee that exercise training and/or caloric restriction cause a reduction in cardiac sympathetic stimulation in our obese rats. It is reasonable to raise the question that the reduction in body fatness in obese rats beyond that found in lean rats caused negative energy balance. This is unlikely, because soleus, plantar and gastrocnemius mass were similar in rats submitted to exercise training associated with caloric restriction and rats submitted to caloric restriction or exercise training alone (data not shown). In addition, adiposity index and leptin levels were not different between rats with caloric restriction and rats with caloric restriction associated with exercise training. Accumulated evidence shows that resting bradycardia is an important marker of exercise training efficacy.\textsuperscript{9,10} So, why did exercise training not cause heart rate reduction in the present study? One possibility is that moderate exercise training intensity over a 10-week period was not sufficient to provoke resting bradycardia in obesity. In fact, similar findings were observed in obese women.\textsuperscript{9} In that study, no significant reduction in resting heart rate was found in obese women submitted to moderate exercise training for 4 months. Finally, studies with a longer duration before the onset of treatment to generate worse obesity and left ventricular dysfunction (50 weeks) should be performed to determine whether exercise training and/or caloric restriction can improve left ventricular systolic function in chronic obesity.

**Perspectives**

Exercise training and caloric restriction are important strategies to prevent a reduction in left ventricular systolic function associated with Ca\textsuperscript{2+} handling protein expression abnormalities provoked by a prolonged high-fat and sucrose diet. Moreover, the changes in phospho-Thr\textsuperscript{17}-PLN and phospho-Se\textsubscript{S2808}RyR expressions provide evidence for the molecular mechanisms of the effects of exercise training and caloric restriction on cardiac function in obesity. Of course, our study gives no information regarding whether these findings are
applicable to humans, but they stress some important benefits caused by nonpharmaceutical therapy based on regular exercise and hypocaloric diet in the treatment of human obesity.

In conclusion, exercise training and/or caloric restriction prevent obesity-induced neurohormonal activation and the decline in left ventricular fractional shortening and phosphorylation of sarcoplasmic reticulum proteins.

Acknowledgments

We gratefully acknowledge André Ferreira do Nascimento, Ana Paula de Almeida Lima-Leopoldo, André Soares Leopoldo, and Silvio Assis de Oliveira, Jr, from the Department of Medicine Clinical, Botucatu School of Medicine, São Paulo State University, São Paulo, Brazil, for preparing high fat pellets. We are grateful to Katt C. Mattos for technical assistance.

Sources of Funding

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (478463/2006-5). E.C.P. and Silvio Assis de Oliveira, Jr, from the Department of Medicine Clinical, Botucatu School of Medicine, São Paulo State University, São Paulo, Brazil, for preparing high fat pellets. We are grateful to Katt C. Mattos for technical assistance.

References


Disclosures

None.
Exercise Training and Caloric Restriction Prevent Reduction in Cardiac Ca\(^{2+}\)-Handling Protein Profile in Obese Rats

Ellena Christina Paulino, Julio Cesar Batista Ferreira, Luiz Roberto Bechara, Jeane Mike Tsutsui, Wilson Mathias, Jr, Fabio Bessa Lima, Dulce Elena Casarini, Antonio Carlos Cicogna, Patricia Chakur Brum and Carlos Eduardo Negrão

_Hypertension_. published online July 19, 2010;
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2010/07/19/HYPERTENSIONAHA.110.156141.citation

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/07/16/HYPERTENSIONAHA.110.156141.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
Supplemental Data

Exercise Training and Caloric Restriction Prevent Reduction in Cardiac Ca\textsuperscript{2+}-handling Proteins Profile in Obese Rats

Ellena Christina Paulino PhD\textsuperscript{1}, Julio Cesar Batista Ferreira PhD\textsuperscript{2}, Luiz Roberto Grassmann Bechara MS\textsuperscript{2}, Jeane Mike Tsutsui MD, PhD\textsuperscript{1}, Wilson Mathias Jr MD, PhD\textsuperscript{1}, Fabio Bessa Lima MD, PhD\textsuperscript{3}, Dulce Elena Casarini PhD\textsuperscript{4}, Antonio Carlos Cicogna MD, PhD\textsuperscript{5}, Patricia Chakur Brum PhD\textsuperscript{2}, Carlos Eduardo Negrão PhD\textsuperscript{1,2}

\textsuperscript{1}Heart Institute (InCor), University of São Paulo Medical School, São Paulo, Brazil; \textsuperscript{2}School of Physical Education and Sport, University of São Paulo, São Paulo, Brazil; \textsuperscript{3}Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil; \textsuperscript{4}Department of Medicine, Division of Nephrology, Federal University of São Paulo, São Paulo, Brazil; \textsuperscript{5}Department of Medicine and Cardiology, School of Medicine, UNESP-State University Júlio Mesquita Filho, Botucatu, São Paulo, Brazil.

Running title: Weight loss improves cardiac function in obesity

Author for correspondence:
Carlos Eduardo Negrão, PhD
Instituto do Coração (InCor)
Av. Dr. Enéas de Carvalho Aguiar, 44 – Cerqueira César – São Paulo – SP
CEP 05403-900- Brazil. Phone: (5511) 3069-5699 FAX: (5511) 3069-5043
E-mail: cndnegrao@incor.usp.br
Supplemental Methods

Diets. High-fat and sucrose diets consisted of five different diets. Hypercaloric diet 1 was prepared with standard chow (355g/kg), roasted peanuts (176g/kg), casein (123g/kg), corn oil 82 (g/kg), chocolate (88g/kg), corn biscuits (176g/kg); hypercaloric diet 2 was prepared with standard chow (439g/kg), roasted peanuts (218g/kg), casein (129g/kg), corn oil (61g/kg), French fried potatoes (153 g/kg); hypercaloric diet 3 consisted of commercial standard chow and water with sucrose (300g/L) added to drinking water; hypercaloric diet 4 was prepared with standard chow (371g/kg), roasted peanuts (185g/kg), casein (99g/kg), corn oil (68g/kg), instant noodles (185g/kg), grated cheese (92g/kg); and hypercaloric diet 5 was prepared with standard chow (359g/kg), roasted peanuts (179g/kg), casein 105(g/kg), corn oil (80g/kg), condensed milk (161g/kg), wafer biscuit (116g/kg) as previously described. The control diet consisted of standard chow (Purina®, Paulinia, SP, Brazil). The high-fat and sucrose diets were prepared from mixture of industrialized products and supplemented ingredients added to a previously triturated Purina rat chow. These diets had sufficient and similar amounts of vitamins, minerals and essential amino acids. The mixture was made into pellets, dried in a ventilated drying oven at 55±5ºC and stored at -20ºC. The composition of each diet expressed as % and the composition of fatty acids and carbohydrate of each diet are shown in Tables 1 and 2 as previously reported by one of us.

Blood Measurements. After 35th weeks all rats were killed by decapitation. Blood was collected, centrifuged at 3000 g for 15 min at 4 ºC, and then stored at -80 ºC. Serum leptin level was measured by radioimmunoassay (Linco Research Inc., USA). Plasma epinephrine and norepinephrine level were measured by HPLC using ion-pair reverse-phase chromatography coupled with electrochemical detection (0.5 V) as described by other investigators.

Histological Analysis. Forty-eight hours after the last exercise training session, all rats were killed and their tissue harvested. The left ventricle was isolated of both atria and of the right ventricle. For morphometric analysis, left ventricle samples obtained from the free wall, at the level of papillary muscle, were fixed in 4% buffered formalin and embedded in paraffin, cut in 4 μm sections and subsequently stained with hematoxylin and eosin. Two randomly selected sections from each animal were visualized by light microscopy using an objective with a calibrated magnification (400 x). Myocytes with visible nucleus and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocyte displayed on a viewing screen was manually traced across the middle of the nuclei with a digitizing pad and determined by a computer assisted image analysis system (Quantimet 520; Cambridge Instruments, UK). For each animal approximately 30 cardiomyocytes were analyzed.

Quantification of left ventricular fibrosis was achieved by Sirius red staining. Two randomly selected sections from each animal were visualized by light microscopy using an objective with a calibrated magnification of 200 x. Interstitial collagen area was quantified by a computer assisted image analysis system (Quantimet 520; Cambridge Instruments, UK). For each animal approximately five visual fields were analyzed.
Western Blot. At the end of the protocols, the levels of sarcoplasmic reticulum Ca$^{2+}$-related proteins were determined by Western blot analysis. Briefly, samples were subjected to SDS-PAGE in polyacrylamide gels (8-12%) depending on protein molecular weight. After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (BioRad Biosciences; Piscataway, NJ, USA). Equal loading of samples and transfer efficiency were monitored with 0.5% Ponceau S staining of the blot membrane. The blotted membrane was blocked (5% nonfat dry milk, 10 mM Tris-HCl (pH = 7.6), 150 mM NaCl, and 0.1% Tween 20) for 2h at room temperature and then incubated overnight at 4°C with specific antibodies against SERCA2 (1:2,500), NCX (1:2,000), PLN (1:500), and RyR (1:5,000) polyclonal antibodies (Affinity Bioreagents, CO), α-tubulin (1:1,000) polyclonal antibody (Santa Cruz Biotechnology, CA), phospho-Ser$^{2808}$-RyR2 (1:2,000), phospho-Ser$^{16}$-PLN (1:5,000), and phospho-Thr$^{17}$-PLN (1:5,000) polyclonal antibodies (Badrilla, UK), PKA (1:1,000), phospho-Ser/Thr-PKA substrate (1:1,000), CaMKII (1:1,000) and phospho-Thr$^{286}$-CaMKII (1:1,000) (Cell Signaling Technology, Inc., MA). Binding of the primary antibody was detected with peroxidase-conjugated secondary antibodies (rabbit or mouse, depending on the protein, for 2h at room temperature) and developed using enhanced chemiluminescence (Amersham Biosciences, NJ, USA) detected by autoradiography. Quantification analysis of blots was performed by Scion Image software (Scion based on NIH image). Samples were normalized to relative changes in α-tubulin.

Gene Expression. SERCA2a, RyR2, and PLB mRNA were measured by Quantitative Real Time RT-PCR. Total RNA was extracted from rat left ventricles in each experimental group using Trizol reagent (Invitrogen, Life Technologies, Brazil), which is based on the guanidine thiocyanate method, according to manufacturer recommendations. Total muscle RNA (100 mg) was homogenized mechanically on ice in 1 mL ice-cold Trizol reagent. RNA was solubilized in RNase-free H2O and quantified by spectrophotometry (GeneQuant™ RNA/DNA Calculator, Amersham Pharmacia Biotech, USA) at 260 nm. The ratio of absorbance at 260 to 280 nm was >1.8 for all samples. Degradation of RNA samples was monitored by observation of appropriate 28S to 18S ribosomal RNA ratios as determined by ethidium bromide staining of the agarose gels. One microliter of RNA (1000 ng/μL) was reverse transcribed with random hexamer primers and Superscript II RT, according to standard methods (Invitrogen). Negative control RT reactions were carried out in which the RT enzyme was omitted. The negative control RT reactions were PCR amplified to ensure that DNA did not contaminate RNA. All primers were synthesized by Taqman. The cDNA (1.5 μL) was then amplified using 10 μM of each primer, 10X PCR buffer, DEPC water, 50 mM MgCl$_2$, 10 mM dNTPs and 2 units Taq polymerase® (Invitrogen) in a final volume of 20 μL. Transcript levels for the constitutive housekeeping gene product beta-actin were measured in each sample and used to normalize the transcript data obtained. The results were quantified as Ct values, where Ct is defined as the threshold cycle of the polymerase chain reaction at which the amplified product is first detected. The data were expressed as change relative to control values.
**PKA Activity.** Cardiac PKA activity was determined using a commercially available kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s recommendations. Briefly, 15μL of cardiac lysate were incubated with 40μL PKA reaction mixture at 30°C for 60 min and probed with primary (phosphospecific substrate antibody) and secondary antibodies (anti-rabbit IgG:HRP conjugate). PKA activity was measured at 450 nm.

**Redox Status.** As reactive oxygen species are produced in cardiac muscle and related to decrease cardiac performance\(^4\), we evaluated reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in cardiac homogenate of all mice studied. GSH/GSSG ratio was determined by high performance liquid chromatography (HPLC) as described elsewhere. In brief, cardiac muscle samples were homogenized in cold buffer (1:20 w/v) containing 0.32 mol/L sucrose, 10 mmol/L HEPES, 1mmol/L EDTA at pH 7.4, and immediately centrifuged at 10,000 g for 20 min at 4 °C. Crude homogenates were mixed with ice-cold 10% (w/v) meta-phosphoric acid, incubated for 30 min on ice and centrifuged for 20 min at 14,000 g at 4 °C. Supernatants were transferred to autosample vials and injected into a HPLC system, where samples were separated using a reverse phase C18 column (5 μm; 4.6×150 mm). Following HPLC, GSH and GSSG were detected with an electrochemical detector (ESA Coulochem III, USA) equipped with a two-channel analytical cell. Redox status was described as calculated GSH/GSSG ratio.

**References**

## Supplemental Tables

### Table S1. Components of each diet.

<table>
<thead>
<tr>
<th>Components</th>
<th>Standard chow</th>
<th>HFD1</th>
<th>HFD2</th>
<th>HSD3</th>
<th>HFD4</th>
<th>HFD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>27.2</td>
<td>28.9</td>
<td>31.3</td>
<td>27.2</td>
<td>30.5</td>
<td>28.9</td>
</tr>
<tr>
<td>Carbohydrates (%)</td>
<td>46.1</td>
<td>35.6</td>
<td>26.5</td>
<td>46.1</td>
<td>32.6</td>
<td>28.9</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>3.5</td>
<td>20.4</td>
<td>23.6</td>
<td>3.5</td>
<td>20.5</td>
<td>24.6</td>
</tr>
<tr>
<td>Others (%)</td>
<td>23.2</td>
<td>15.1</td>
<td>18.6</td>
<td>23.2</td>
<td>16.4</td>
<td>17.6</td>
</tr>
<tr>
<td>Calories (Kcal/g)</td>
<td>3.2</td>
<td>4.4</td>
<td>4.4</td>
<td>4.8</td>
<td>4.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

HFD: High fat diet; HSD: High sucrose diet. Diet 3: Standard chow and water with sucrose, which increased caloric intake by 1.2 Kcal per 1 mL of drinking water.
Table S2. Fatty Acid and Carbohydrate Compositions.

Fatty Acid Composition (g/100g of fat)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Standard</th>
<th>HFD1</th>
<th>HFD2</th>
<th>HSD3</th>
<th>HFD4</th>
<th>HFD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total saturated</td>
<td>21.16</td>
<td>16.94</td>
<td>22.67</td>
<td>21.16</td>
<td>24.37</td>
<td>23.36</td>
</tr>
<tr>
<td>Total monounsaturated</td>
<td>28.02</td>
<td>40.43</td>
<td>40.89</td>
<td>28.02</td>
<td>40.43</td>
<td>38.15</td>
</tr>
<tr>
<td>Total polyunsaturated</td>
<td>50.82</td>
<td>42.63</td>
<td>36.44</td>
<td>50.82</td>
<td>35.20</td>
<td>38.49</td>
</tr>
<tr>
<td>Total unsaturated</td>
<td>78.84</td>
<td>83.06</td>
<td>77.33</td>
<td>78.84</td>
<td>75.63</td>
<td>76.64</td>
</tr>
</tbody>
</table>

Carbohydrate Composition (g/100g of sugar)

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Standard</th>
<th>HFD1</th>
<th>HFD2</th>
<th>HSD3</th>
<th>HFD4</th>
<th>HFD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose</td>
<td>1.74</td>
<td>0.50</td>
<td>0.67</td>
<td>1.74</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.07</td>
<td>0.73</td>
<td>3.63</td>
<td>1.07</td>
<td>3.13</td>
<td>1.73</td>
</tr>
<tr>
<td>Free glucose</td>
<td>0.82</td>
<td>0.73</td>
<td>0.74</td>
<td>0.82</td>
<td>1.76</td>
<td>0.62</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.65</td>
<td>0.41</td>
<td>1.24</td>
<td>0.65</td>
<td>0.61</td>
<td>2.63</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.17</td>
<td>0.09</td>
<td>0.17</td>
<td>0.17</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>Arabinose</td>
<td>5.55</td>
<td>2.91</td>
<td>4.50</td>
<td>5.55</td>
<td>3.78</td>
<td>2.97</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.55</td>
<td>2.20</td>
<td>3.93</td>
<td>4.55</td>
<td>2.88</td>
<td>3.17</td>
</tr>
<tr>
<td>Glucose</td>
<td>46.58</td>
<td>43.83</td>
<td>49.21</td>
<td>46.58</td>
<td>50.22</td>
<td>35.37</td>
</tr>
<tr>
<td>Xilose</td>
<td>5.47</td>
<td>2.45</td>
<td>3.89</td>
<td>5.47</td>
<td>3.42</td>
<td>2.50</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.22</td>
<td>-</td>
<td>0.20</td>
<td>0.22</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.79</td>
<td>1.33</td>
<td>2.55</td>
<td>2.79</td>
<td>2.77</td>
<td>2.19</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.83</td>
<td>29.22</td>
<td>9.13</td>
<td>8.83</td>
<td>7.34</td>
<td>32.69</td>
</tr>
<tr>
<td>Free fructose</td>
<td>0.62</td>
<td>1.03</td>
<td>0.57</td>
<td>0.62</td>
<td>1.04</td>
<td>0.72</td>
</tr>
<tr>
<td>Fructose</td>
<td>20.93</td>
<td>14.56</td>
<td>19.57</td>
<td>20.93</td>
<td>22.23</td>
<td>14.76</td>
</tr>
</tbody>
</table>

HFD: High fat diet; HSD: High sucrose diet.
Figure S1. (A): SERCA 2, (B): PLB and (C): RyR mRNA levels after 10 weeks of exercise training and/or caloric restriction in obese rats (35th week). Groups are divided in lean (L), high-fat and sucrose diet (O), high-fat and sucrose diet and exercise training (OT), caloric restriction (OR), and exercise training and caloric restriction (OTR). All data were normalized against cyclophilin.
Figure S2: (A): Representative blots, (B): PKA expression, (C): PKA activity, (D): p-PKA-substrate and (E): p-CAMKII\textsubscript{Thr286}/CAMKII ratio after 10 weeks of exercise training and/or caloric restriction in obese rats (35\textsuperscript{th} week). Groups are divided in lean (L), high-fat and sucrose diet (O), high-fat and sucrose diet and exercise training (OT), caloric restriction (OR), and exercise training and caloric restriction (OTR). All data were normalized against α-tubulin.