Obesity induces upregulation of genes involved in myocardial Ca\(^2+\) handling

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Obesity is a complex multifactorial disorder that is often associated with cardiovascular diseases. Research on experimental models has suggested that cardiac dysfunction in obesity might be related to alterations in myocardial intracellular calcium (Ca\(^2+\)) handling. However, information about the expression of Ca\(^2+\)-related genes that lead to this abnormality is scarce. We evaluated the effects of obesity induced by a high-fat diet in the expression of Ca\(^2+\)-related genes, focusing the L-type Ca\(^2+\) channel (Cacna1c), sarcolemmal Na\(^+\)/Ca\(^2+\) exchanger (NCX), sarcoplasmic reticulum Ca\(^2+\) ATPase (SERCA2a), ryanodine receptor (RyR2), and phospholamban (PLB) mRNA in rat myocardium. Male 30-day-old Wistar rats were fed a standard (control) or high-fat diet (obese) for 15 weeks. Obesity was defined as increased percent of body fat in carcass. The mRNA expression of Ca\(^2+\)-related genes in the left ventricle was measured by RT-PCR. Compared with control rats, the obese rats had increased percent of body fat, area under the curve for glucose, and leptin and insulin plasma concentrations. Obesity also caused an increase in the levels of SERCA2a, RyR2 and PLB mRNA (P < 0.05) but did not modify the mRNA levels of Cacna1c and NCX. These findings show that obesity induced by high-fat diet causes cardiac upregulation of Ca\(^2+\) transport-related genes in the sarcoplasmic reticulum.

Key words: Obesity; High-fat diet; Rat heart; Ca\(^2+\) cycling; mRNA; Ca\(^2+\)-related genes

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(Cacna1c), sarcolemmal Na+/Ca2+ exchanger (NCX), SERCA2a, ryanodine receptor (RyR2), and PLB genes in rats with high-fat diet-induced obesity.

Material and Methods

Animals and experimental design

All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (9) and were approved by the Botucatu Medical School Ethics Committee (UNESP, Botucatu, SP, Brazil).

Thirty-day-old male Wistar rats, provided by the Botucatu Animal Center of Botucatu Medical School, were randomly assigned to one of two groups: control (C; N = 13) and obese (Ob; N = 13). The control group was fed a standard rat chow containing 11.2% fat, 55.5% carbohydrate, and 33.3% protein; whereas the obese animals received a high-fat diet containing 45.2% kcal fat, 28.6% carbohydrate, and 26.2% protein. The high-fat diet was calorically rich (high-fat diet = 4.5 kcal/g vs standard diet = 3.3 kcal/g) due to the higher fat content.

All rats were housed in individual cages in an environmentally controlled clean-air room (23 ± 3°C; 60 ± 5% relative humidity) with a 12-h light/dark cycle (lights on at 6:00 am). Each group was fed the appropriate diet with free access to water and food for 15 consecutive weeks. Food consumption was measured daily; water intake and body weight were evaluated once a week. Weekly calorie intake was calculated by average weekly food consumption x caloric value of each diet. Feed efficiency, the ability to transform calories consumed into body weight, was determined by following the formula: mean body weight gain (g) / total calorie intake. Initial and final body weight (FBW), left ventricle weight (LVW), right ventricle weight (RVW), and LVW/FBW as well as RVW/FBW ratios were calculated.

Oral glucose tolerance test

After 15 weeks of feeding, rats fasted for 12-15 h were submitted to oral glucose tolerance test. Blood samples were drawn from the tip of the tail at baseline and after gavage administration of a glucose load (3 g/kg body weight) (10). Blood samples were then collected at 0, 60, 120, and 180 min. Glucose levels were determined using the ACCU-CHEK GO KIT glucose analyzer (Roche Diagnostic Brazil Ltda., Brazil). Glucose tolerance was determined by the area under the curve for glucose (0-180 min).

Determination of plasma hormones

At the end of the diet treatment, animals were submitted to a 12-15 h fast, anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and sacrificed by decapitation. Blood was collected in heparinized tubes, centrifuged at 3000 g for 15 min at 4°C, and then stored at -80°C. Plasma leptin and insulin concentrations were determined by ELISA (11) using commercial kits (Linco Research Inc., USA).

Body fat analysis

After the animals were decapitated and thoracotomized, the viscera were discarded leaving only the carcass. Carcasses were dried at 100 ± 5°C for 72 h in a ventilated Fanem® dryer (Fanem, Brazil). After drying, the carcass was wrapped in filter paper and the fat was extracted in a Soxhlet Extractor (Corning Incorporated Life Sciences, USA). The percentage of body fat in each carcass was calculated by the following formula: [(post-drying weight - dry weight after fat extraction) / pre-drying weight] x 100 (12).

Gene expression studies

Cacna1c, NCX, SERCA2a, RyR2, and PLB mRNA were measured by semiquantitative RT-PCR (13). 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 (14), 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, Amersharm 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 the 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. The cDNA (1.5 µL) was then amplified using 10 µM of each primer, 10X PCR buffer, DEPC water, 50 mM MgCl2, 10 mM dNTPs and 2 units Taq polymerase® (Invitrogen) in a final volume of 25 µL. Transcript levels for the constitutive housekeeping gene product cyclophilin were measured in each sample and used to normalize the transcript data obtained. The data were expressed as change relative to control values.

The primer sequences used were: PLB: S 5’TACC
TTACTCGCTCGGCTATC3’ and AS 5’CAGAAGCATCA
CAATGATGCAG3’; SERCA2a: S 5’ATGAGATCACAGCT
ATGACTGGTG3’ and AS 5’GCATTGCACATCTCTAT
GGTGACTAG 3’; RYR2: S 5’GAATCAGTGAGTTACT
GGGCATGG3’ and AS 5’CTGGTCTCTGAGTTCTC
CTTITTC3’ and AS 5’TGCCTTCTTTCACCTTCC3’ as pro-
pussed by Mirit et al. (15) and Coussin et al. (16). Primer
pairs for Cacna1c, and NCX were determined using the
Primer 3 software available on-line at http://frodo.wi.mit.edu/
cgi-bin/primer3/primer3_www.cgi. To determine specific-
ity, sequences were compared with GenBank using the
Blast program available at the National Central Biotechnol-
gy Information website (http://www.ncbi.nlm.nih.gov/). The
following primers were used: Cacna1c: S 5’TCTGCTCTG
CCTGACTCTGA3’ and AS 5’GAGATACTCCACCC
GTTCCA3’; NCX: S 5’GGCAGAAACAGGAGGAAATG3’
and AS 5’AGCGGACACAACACAGATGG3’. Preliminary
experiments were conducted with each gene product to
determine the number of PCR cycles that provided a linear
range of amplification. cDNA from each sample for both
the control and obese groups was amplified simultaneously
using aliquots from the same PCR mixture. After PCR
amplification, 10 µL of each reaction was electrophoresed
on 1% agarose gels stained with ethidium bromide. Im-
ages were captured and bands corresponding to each
gene were quantified by densitometry by the Labworks™
Analysis Software 3.0 (UV/White Darkroom, UVP Labora-
tory Products, USA). PCR products were run in triplicate on
gels different for each gene and results were averaged.
The size (number of base pairs) of each band corresponds
to the size of processed mRNA. The target genes were
normalized to housekeeping gene cyclophilin (17).

Statistical analysis
Data are reported as means ± standard deviation. Comparisons between groups were performed using the Student t-test for independent samples. The mean weekly body weight and the glucose profile of the groups were compared by ANOVA for repeated measures. When signif-
nicant differences were found (P < 0.05), the post hoc
Bonferroni multiple comparisons test was carried out (18).
The level of significance was considered to be 5%.

Results
The control and obese groups started with similar body
weight at week 0 of the study. However, a significant
difference in body weight between the groups was ob-
served at week 2 and thereafter (Figure 1). Table 1 shows
the influence of obesity on the general and nutritional
characteristics of the animals. Although the obese group
ingested less food than the control group, the calorie
intake, feed efficiency, and final body weights of the obese
rats after 15 weeks were greater than the control rats by
approximately 23%. Furthermore, the percentage of car-
cass body fat was markedly higher for the obese group (C
= 9 ± 1 vs Ob = 17 ± 7%, P < 0.05). Water consumption was
similar for both groups. LVW and RVW were higher in
obese animals than in controls, but no statistical difference

Table 1. Effect of high-fat diet-induced obesity on the general and nutritional characteristics of rats.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control (N = 13)</th>
<th>Obese (N = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBW (g)</td>
<td>106 ± 10</td>
<td>111 ± 10</td>
</tr>
<tr>
<td>FBW (g)</td>
<td>439 ± 26</td>
<td>539 ± 52*</td>
</tr>
<tr>
<td>LVW (g)</td>
<td>0.85 ± 0.09</td>
<td>0.99 ± 0.09*</td>
</tr>
<tr>
<td>RVW (g)</td>
<td>0.22 ± 0.03</td>
<td>0.28 ± 0.04*</td>
</tr>
<tr>
<td>LVW/FBW (mg/g)</td>
<td>1.93 ± 0.20</td>
<td>1.84 ± 0.19</td>
</tr>
<tr>
<td>RVW/FBW (mg/g)</td>
<td>0.52 ± 0.03</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>FC (g/day)</td>
<td>22.2 ± 1.2</td>
<td>16.4 ± 1.4*</td>
</tr>
<tr>
<td>WC (mL/day)</td>
<td>40.9 ± 4.7</td>
<td>41.8 ± 3.7</td>
</tr>
<tr>
<td>CI (g kcal⁻¹·day⁻¹)</td>
<td>73.2 ± 3.8</td>
<td>85.2 ± 6.7*</td>
</tr>
<tr>
<td>FE (g/kcal)</td>
<td>0.043 ± 0.002</td>
<td>0.048 ± 0.003*</td>
</tr>
<tr>
<td>FAT (%)</td>
<td>9 ± 1</td>
<td>17 ± 7*</td>
</tr>
<tr>
<td>AUC</td>
<td>20247.7 ± 1416.8</td>
<td>23596.2 ± 3023.4*</td>
</tr>
</tbody>
</table>

Obese rats received a high-fat diet (45.2% fat, 28.6% carbohy-
drate) and controls received a standard diet (11.2% fat and 55%
carbohydrate) for 15 weeks. Data are reported as mean ± SD
for 13 rats in each group. *P < 0.05 vs control (ANOVA for repeated measures and post hoc
Bonferroni test).

Figure 1. Weekly body weight control of obese and control rats. Data are reported as means ± SD for 13 rats in each group. *P < 0.05 vs control (ANOVA for repeated measures and post hoc Bonferroni test).
between groups was observed after normalization to FBW (Table 1).

The area under the curve for glucose (C = 20247.7 ± 1416.8 vs Ob = 23596.2 ± 3023.4; P < 0.05) (Table 1), plasma leptin (C = 2.31 ± 0.53 vs Ob = 4.82 ± 1.34; P < 0.05) and insulin levels (C = 0.45 ± 0.19 vs Ob = 1.48 ± 0.67; P < 0.05) were higher in the obese group than in the control group, which indicates that the obese rats were hyperinsulinemic, hyperleptinemic and glucose intolerant.

The measurement of gene expression by RT-PCR revealed that the cardiac mRNA expression of SERCA2a, PLB and RyR2 was enhanced in the obese group (C = 1.00 ± 0.11 vs Ob = 1.18 ± 0.17, P < 0.05; C = 1.00 ± 0.05 vs Ob = 1.58 ± 0.24, P < 0.05; C = 1.00 ± 0.06 vs Ob = 1.83 ± 0.08, P < 0.05, respectively). However, Cacna1c and NCX mRNA levels were not different (C = 1.00 ± 0.10 vs Ob = 1.03 ± 0.08, C = 1.00 ± 0.05 vs Ob = 1.01 ± 0.04, P > 0.05, respectively; Figure 2).

**Discussion**

The results of the present study show that rats fed a high-fat diet for 15 weeks had a 22.8% increase in final body weight and an 89% increase in body fat compared with control animals. The difference in body weight between groups was observed after two weeks of obesity induction (Figure 1). Although the obese group ingested less food, the higher weight gain exhibited by these animals was most likely due to their increased calorie intake and feed efficiency. In addition, the obese rats developed metabolic abnormalities that are typically associated with obesity, e.g., glucose intolerance, hyperinsulinemia and hyperleptinemia. These findings are consistent with other studies that have reported that diet-induced obesity displays several characteristics commonly related to human and experimental obesity (7,19).

Our major findings suggest that obesity induced the

![Figure 2](image-url)
upregulation of the gene expression of proteins related to Ca²⁺ transport, SERCA2a, RyR2 and PLB, but did not cause changes in sarcosomal Ca²⁺ genes, NCX and Cacna1c. This result confirms Relling et al. (7) who reported enhanced protein expression of SERCA2a and PLB in obese rats. These investigators have suggested that the increased protein expression of SERCA2a may reflect a compensatory mechanism to restore impairedCa²⁺ handling. On the other hand, other investigators have observed unchanged SERCA2a, RyR2 and PLB protein expression levels in non-obese rats fed a high-fat diet (6). This result could indicate that only a high-fat diet without obesity does not affect expression of Ca²⁺-related genes.

The mechanisms responsible for changes in transcriptional factors that regulate the expression of Ca²⁺-related genes in obesity are still unknown. Given that most studies on obesity report increased triiodothyronine levels (T3) (20-22), and that this hormone is related to SERCA2a expression (23,24), the T3 elevation may be responsible for the increase in SERCA2a mRNA. However, since obesity has been associated with elevated insulin, leptin, cytokines, endothelin and renin, angiotensin and aldosterone levels, as well as sympathetic nerve activity (19,25-30), which stimulate different transcriptional signaling pathways, it is possible that one or more of these factors may be involved in the overexpression of the SERCA2a, RyR2 and PLB.

Our findings showed that obesity induced by high-fat diet causes cardiac upregulation of Ca²⁺ transport-related genes in the sarcoplasmic reticulum. Further studies are necessary to determine if changes in the mRNA expression are accompanied by alterations of protein expression and the mechanisms responsible for changes in myocardial Ca²⁺-related genes in obese rats.

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