Long-term obesity promotes alterations in diastolic function induced by reduction of phospholamban phosphorylation at serine-16 without affecting calcium handling

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Lima-Leopoldo AP, Leopoldo AS, da Silva DC, do Nascimento AF, de Campos DH, Luizotto RA, de Deus AF, Freire PP, Medeiros A, Okoshi K, Cicogna AC. Long-term obesity promotes alterations in diastolic function induced by reduction of phospholamban phosphorylation at serine-16 without affecting calcium handling. J Appl Physiol 117: 669–678, 2014. First published June 26, 2014; doi:10.1152/japplphysiol.00088.2014.—Few studies have evaluated the relationship between the duration of obesity, cardiac function, and the proteins involved in myocardial calcium (Ca2+) handling. We hypothesized that long-term obesity promotes cardiac dysfunction due to a reduction of expression and/or phosphorylation of myocardial Ca2+-handling proteins. Thirty-day-old male Wistar rats were distributed into two groups (n = 10 each): control (C; standard diet) and obese (Ob; high-fat diet) for 30 wk. Morphological and histological analyses were assessed. Left ventricular cardiac function was assessed in vivo by echocardiographic evaluation and in vitro by papillary muscle. Cardiac protein expression of sarcoplasmic reticulum (SR) Ca2+-ATPase (SERCA2a), calsequestrin, L-type Ca2+ channel, and phospholamban (PLB), as well as PLB serine-16 phosphorylation (pPLB Ser16) and PLB threonine-17 phosphorylation (pPLB Thr17) were determined by Western blot. The adiposity index was higher (82%) in Ob rats than in C rats. Obesity promoted cardiac hypertrophy without alterations in interstitial collagen levels. Ob rats had increased endocardial and midwall fractional shortening, posterior wall shortening velocity, and A-wave compared with C rats. Cardiac index, early-to-late diastolic mitral inflow ratio, and isovolumetric relaxation time were lower in Ob than in C. The Ob muscles developed similar baseline data and myocardial responsiveness to increased extracellular Ca2+. Obesity caused a reduction in cardiac pPLB Ser16 and the pPLB Ser16/PLB ratio in Ob rats. Long-term obesity promotes alterations in diastolic function, most likely due to the reduction of pPLB Ser16, but does not impair the myocardial Ca2+ entry and recapture to SR.

high-fat diet; obesity; cardiac dysfunction; Ca2+-handling proteins; PLB serine-16 phosphorylation

O B E S I T Y H A S B E C O M E T H E M O S T common metabolic and nutritional disorder in industrial countries, and represents one of the most prevalent risk factors for the development of common chronic metabolic diseases (22, 37). The association between obesity and the increased risk for the several chronic diseases affecting morbidity and mortality in the population, including cardiovascular disease, has been described in multiple epidemiological studies (7).

Obesity is associated with a high chronic cardiac workload, due to the need to supply more blood to peripheral tissues, and frequently leads to left ventricular (LV) dysfunction (69). Within this context, several authors have shown that cardiac dysfunction is associated with the degree and duration of obesity (1, 64). Studies suggest that early, subtle cardiomyocyte hypertrophy and interstitial matrix expansion may predate organ-level pathology. Long-term obese people exhibit a cardiometabolic profile, including LV hypertrophy, diastolic dysfunction, and increased vascular stiffness, which are precursors of future heart failure (1, 42, 54, 60). Although it is apparent that a variety of alterations or damage in cardiac performance, both in human and experimental models, occurs with elevations in adipose tissue content (1, 4, 32, 46, 56, 60), the mechanisms responsible for these abnormalities are not completely understood after long-term exposure to obesity. Therefore, this disease plays an important role in cardiovascular morbidity through multiple mechanisms, including intracellular calcium (Ca2+) handling (32, 50, 53, 56).

The literature reports that intracellular Ca2+-cycling proteins located in the sarcolemma and sarcoplasmic reticulum (SR), such as the L-type Ca2+ channel, SR Ca2+-ATPase (SERCA2a), phospholamban (PLB), and calsequestrin (CSQ), regulate Ca2+ homeostasis in cardiac muscle by modulating myocardial contraction and relaxation (8, 50). Previous studies have also shown that protein regulation of cardiac SR, a major regulator of Ca2+ handling during the cardiac excitation-contraction-relaxation cycle, is mediated mainly by the activity of SERCA2a (8, 21, 51, 68). SERCA2a activity is directly modulated by the natural inhibitory phosphoprotein PLB, which in its dephosphorylated state inhibits the apparent affinity of SERCA2a for Ca2+ (30, 66, 71). PLB can be phosphorylated at three distinct sites in vitro: serine-16 (Ser16) by cAMP- and cGMP-dependent protein kinases, threonine-17 (Thr17) by Ca2+-calmodulin-dependent protein kinase II (CaMKII), and Ser16 by protein kinase C (8, 14, 66). Upon phosphorylation of PLB through β-adrenergic stimulation and enhanced cAMP-dependent protein kinase A (PKA) activity, the inhibition of SERCA2a by PLB is relieved. PLB phosphorylation acts as a molecular switch that activates SERCA2a, which increases the calcium gradient across the SR during diastole, and accelerates relaxation (8, 29).
Thus it is necessary to establish the mechanisms of affecting transcription, translation, and/or posttranslational processes. Considering the lack of information regarding the mechanisms underlying the participation of Ca\textsuperscript{2+}-handling proteins in cardiac dysfunction during prolonged periods of obesity, the objective of this study was to test the hypothesis that an extended duration of obesity leads to cardiac dysfunction and that this damage is associated with a reduction in the expression and/or phosphorylation of protein levels. These alterations could contribute to reduced Ca\textsuperscript{2+} entry and SR Ca\textsuperscript{2+} reuptake; both are essential for normal cardiac function.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

Thirty-day-old male Wistar rats were obtained from the Central Animal House of Botucatu Medical School (São Paulo, Brazil) and randomly submitted to control (C, n = 10) or obese (Ob, n = 10) treatment. The C group was fed a standard diet (RC Focus 1765; Agroceres, Rio Claro, SP, Brazil), and the Ob group was alternately submitted to four palatable high-fat diets (RC Focus 2413, 2414, 2415, and 2416; Agroceres, Rio Claro, Brazil) for 33 wk. The high-fat diets were calorically rich (high-fat diet = 3.65 kcal/g vs. low-fat diet = 2.95 kcal/g) due to their higher fat energy (consisting of saturated and unsaturated fatty acids, which provided 20% and 80% of the fat-derived calories, respectively). Standard and high-fat diet components have been previously described (31).

All animals had free access to water and chow (50 g/day). After starting the experimental protocol, body weight was recorded weekly. Rats were individually caged and subjected to different dietary regimens. Animals were kept under standard environmental conditions of controlled light (12:12-h light-dark schedule; lights on at 6 AM), clean-air room temperature (23 ± 3°C), and relative humidity (60 ± 5%). All procedures involving animals were approved by the Ethics Committee of Botucatu Medical School (UNESP, SP, Brazil) under Study Number 573 and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 1996).

**Characterization of Obesity**

The adiposity index was measured to assess obesity. After animals had been anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (0.5 mg/kg), decapitated, and thoracotomized, the adipose tissue fat pads were dissected and weighed. The adiposity index was calculated with the following formula: adiposity index = [total body fat (BF)/final body wt] × 100. BF was measured from the sum of the individual fat pad weights as follows: BF = epididymal fat + retroperitoneal fat + visceral fat.

**Comorbidities Associated With Obesity**

**Systolic blood pressure.** At the end of the experiment (at 33 wk), the tail systolic blood pressure (SBP) was assessed in each animal with a semiautomated tail cuff device, Narco BioSystems PE 300 (International Biomedical, Austin, TX). Two pressure measurements were recorded.

**Metabolic profile.** At the end of the experimental period (at 33 wk), animals were fasted for 12–15 h, anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (0.5 mg/kg), and euthanized by decapitation. Blood samples were collected and the serum was separated by centrifugation at 3,000 g for 15 min at 4°C and stored at −80°C until further analysis. Serum was analyzed for levels of glucose, triglycerides (TG), total cholesterol (T-Chol), HDL, LDL, and hormones (insulin and leptin).

Serum concentrations of glucose, TG, T-Chol, HDL, and LDL were assayed using a commercial kit (CELIM, São Paulo, Brazil) and measured with an automatic enzymatic analyzer system (Technicon, RA-XTTM System; Global Medical Instrumentation, Ramsey, MN). Leptin and insulin levels were determined by ELISA using commercial kits (Linco Research, St. Louis, MO).

**Glucose tolerance test and homeostatic model assessment index.** After the end of the experimental protocol, all rats were fasted for 4–6 h prior to the glucose tolerance test (48). After fasting, a blood sample from the tip of the tail was collected. The basal blood glucose level of each animal was immediately determined using a handheld glucometer (Accuchek Advantage; Roche Diagnostics, Indianapolis, IN). Subsequently, an injection of glucose solution (2 g/kg body wt) dissolved in water was administered intraperitoneally, and blood glucose levels were measured after 15, 30, 60, 90, and 120 min. Glucose intolerance was evaluated by the area under the curve (AUC) for glucose. HOMA-insulin resistance (IR) was expressed as an index of insulin resistance and calculated using the following formula: HOMA-IR = [fasting glucose (mmol/l) × fasting insulin (μU/ml)]/22.5.

**Cardiac Remodeling**

Cardiac remodeling was measured by morphological analysis postmortem, histological study, echocardiographic evaluation, and papillary muscle function, as well as by intracellular Ca\textsuperscript{2+}-cycling protein analysis by Western blotting.

**Morphological Analysis Postdeath**

The rats were euthanized and after thoracotomy, the heart, ventricles, and tibia were separated, dissected, weighed, and measured. Cardiac remodeling at the macroscopic level, which identifies the presence or absence of hypertrophy, was determined by analyzing the following parameters: heart weight, LV weight, and their relation with tibia length.

**Histological Study**

LV transverse sections of animals from each group were fixed in 10% buffered formalin and embedded in paraffin (49). Thick sections of 5 μm were cut from the tissue block and stained by routine Gomori’s reticulin silver staining technique (5). After the silver staining was developed, slides were mounted and visualized under light microscopy to determine the myocyte cross-sectional area (CSA), which was determined for at least 100 myocytes per slide and used as an indicator of cell size.

The interstitial collagen fraction (CF) was determined for the entire picrosirius red-stained cardiac section using an automatic image analyzer (Image-Pro Plus 3.0). The components of the cardiac tissue were identified according to color level as follows: red for collagen fibers, yellow for myocytes, and white for interstitial space. The CF was calculated as the sum of all connective tissue areas divided by the sum of all connective tissue and myocyte areas. On average, 35 microscopic fields were analyzed using a 20× lens. Perivascular collagen was excluded from this analysis.

**Echocardiographic Evaluation**

One week before euthanasia, echocardiographic evaluation was performed using a commercially available echocardiograph (Philips HDL-5000) equipped with a 5- to 12-MHz electronic transducer. Rats were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). A two-dimensional parasternal short-axis
view of the LV was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral valve leaflets and at the level of the aortic valve and left atrium (49). M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All measurements were obtained by the same observer using the method recommended by the American Society of Echocardiography (60).

The following LV structural variables were measured: left atrium (LA) and aortic (AO) diameters, LV end-diastolic (LVEDD) and LV systolic (LVSD) dimensions, LV diastolic posterior wall thickness (PWT), LV diastolic septal wall thickness (SWT), and relative wall thickness (RWT). LV function was assessed by the following parameters: heart rate, endocardial and midwall fractional shortenings, posterior wall shortening velocity (PWSV), early and late diastolic mitral inflow (E-wave and A-wave, respectively), early-to-late diastolic mitral inflow ratio (E/A ratio), E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT).

Papillary Muscle Function

Isolated papillary muscles from LV were evaluated as previously described (31). To determine the mechanism by which obesity induces negative inotropic effects on contractile function, the papillary muscles were evaluated under the baseline condition of 2.5 mM Ca2+ and after elevations of extracellular Ca2+ concentration. Inotropic responses were recorded 5 min after the addition of each dose of extracellular Ca2+ (0.5, 1.0, 1.5, 2.0, and 2.5 mM) to the bathing solution.

Myocardial L-type Ca2+ Channel and SERCA2a Activity

To determine the activity or performance of L-type Ca2+ channels and SERCA2a during the myocyte contractile cycle, L-type Ca2+ channel and SERCA2a blockers were employed. All drugs were obtained from Sigma-Aldrich (St. Louis, MO).

The evaluation of L-type Ca2+ channel activity was performed using a specific inhibitor, diltiazem hydrochloride (10−4 M), in the presence of cumulative Ca2+ concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mM). Twenty minutes after diltiazem addition to the solution, each concentration of Ca2+ was separately added to the bathing solution for 10 min, and muscle function was evaluated. The result was expressed as mean percent of inhibition. The solution of diltiazem was prepared and diluted using double-distilled water.

To evaluate the role of SERCA2a, we used the highly specific blocker, cyclopiazonic acid (CPA; 30 μM), in the presence of cumulative Ca2+ concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mM). Sixty minutes after CPA addition to the solution, each Ca2+ concentration was separately added to the bathing solution for 10 min, and the papillary muscle response was analyzed. The data are expressed as mean percent of inhibition (%).

At the end of the study, the parameters used to characterize the papillary muscle were length (mm), weight (mg), and CSA (mm²). The CSA was calculated from the length and weight of papillary muscle, assuming uniformity and a specific gravity of 1.0. The muscle length at Lmax was measured with a catheterometer (Gartner Scientific, Chicago, IL), and the muscle between the two clips was blotted dry and weighed.

Western Blot Analysis

At the end of the protocol, the levels of SERCA2a, CSQ, phospholamban (PLB), PLB Ser16 phosphorylation (pPLB Ser16), PLB Thr17 phosphorylation (pPLB Thr17), and L-type Ca2+ channel were determined by Western blot analysis. Briefly, LV samples were frozen in liquid nitrogen from C (n = 6) and Ob (n = 6) rats and homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM DTT, 1 mM EDTA (pH 8.0), 0.3 mM PMSF, 10 mM NaF, and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). Samples were subjected to SDS-PAGE in 8–12% polyacrylamide gels, depending on the molecular weight of the protein. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Equal loading of samples (50 mg) and transfer efficiency were monitored with 0.5% Ponceau S staining of the 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 2 h at room temperature and then incubated overnight at 4–8°C with specific antibodies against SERCA2a (1:2,500; ABR, Affinity BioReagents, Golden, CO), CSQ (1:2,500; Upstate, Lake Placid, NY), PLB (1:500; ABR, Affinity BioReagents), pPLB Ser16 (1:5,000; Badrilla, United Kingdom), pPLB Thr17 (1:5,000, Badrilla), and L-type Ca2+ channel alpha 1C (1:100; Chemicon International, Temecula, CA). Binding of the primary antibody was detected with peroxidase-conjugated secondary antibodies (rabbit or mouse, depending on the protein, for 2 h at room temperature), developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and detected by autoradiography. Quantification analysis of blots was performed by Scion Image software (Scion based on NIH image). Targeted bands were normalized to the expression of β-actin using an antibody (1:1,000) obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis

Data on general characteristics, comorbidities, cardiac remodeling, echocardiographic evaluation, papillary muscle function, and analysis of key intracellular Ca2+ cycling proteins were reported as the means plus minus standard deviation. Comparisons between groups were performed using Student’s t-test for independent samples. A repeated-measures two-way ANOVA was utilized to evaluate the glucose tolerance test and papillary muscle function; when significant differences were found (P < 0.05), the Bonferroni or Student-Newman-Keuls post hoc test for multiple comparisons was carried out. The level of significance considered was 5% (α = 0.05).

RESULTS

General Characteristics and Comorbidities

The general characteristics of rats after 30 wk are displayed in Table 1 and Fig. 1. The long-term exposure to a high-fat diet promoted a substantial elevation of final body weight, epididymal, retroperitoneal, and visceral fat pads, body fat and

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<tbody>
<tr>
<td>IBW, g</td>
<td>153 ± 7</td>
</tr>
<tr>
<td>FBW (g) at Week 3</td>
<td>294 ± 12</td>
</tr>
<tr>
<td>FBW (g) at 30 wk</td>
<td>489 ± 33</td>
</tr>
<tr>
<td>Epididymal fat, g</td>
<td>6.1 ± 2.5</td>
</tr>
<tr>
<td>Retroperitoneal fat, g</td>
<td>8.5 ± 2.5</td>
</tr>
<tr>
<td>Visceral fat, g</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td>Body fat, g</td>
<td>19.1 ± 5.4</td>
</tr>
<tr>
<td>Adiposity index, %</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>HW, g</td>
<td>1.14 ± 0.09</td>
</tr>
<tr>
<td>LVW, g</td>
<td>0.81 ± 0.07</td>
</tr>
<tr>
<td>HW/Tibia, g/cm</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>LVW/Tibia, g/cm</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10 animals). C, control; Ob, obese; IBW, initial body weight; FBW, final body weight; HW, heart weight; LVW, left ventricle weight; *P < 0.05 vs. C. Student’s t-test for independent samples.
adiposity index. There was no significant difference between the groups for initial body weight. Ob rats had a body fat content (126.7%) and an adiposity index (82.1%) that was significantly greater than in C rats. Epididymal, retroperitoneal, and visceral fat pads were increased by 95%, 128%, and 166% significantly greater than in C rats. Figure 1 and Table 1 illustrate that a significant separation of body weight between the two diet groups was observed from Week 3 that continued throughout the rest of the study (30 wk), determining the initial moment of obesity.

The comorbidities associated with obesity are summarized in Table 2. Long-term high-fat diet-induced obesity caused significant metabolic and hormonal alterations. The glucose, AUC, insulin, HOMA-IR, TG, and leptin levels were higher in Ob rats than in C rats. Furthermore, the glucose tolerance test (data not shown) and HOMA-IR revealed compromised glucose tolerance and insulin resistance in the Ob rats. The other parameters including SBP, T-Chol, HDL, and LDL were similar between the groups.

**Cardiac Remodeling**

Figure 2 and Table 3 show the influence of obesity on the structural cardiac remodeling process. Absolute heart weight, LV weight, heart-to-tibia length ratio, and LV-to-tibia length ratio were significantly elevated in Ob rats compared with those in the C group. Additionally, the histological analysis revealed that cardiomyocyte CSA was higher in Ob rats than in C rats (Fig. 2A). Moreover, the long-term diet-induced obesity, after 30 wk, did not promote alterations in the LV interstitial collagen fraction (Fig. 2B). Echocardiography revealed significantly greater values of LVSD, LVDT, SWT, PWT, and RWT in Ob rats compared with C rats. LVDD, LA and AO diameters, LA/AO ratio, and LV mass were similar between the groups (Table 3).

LV functional data are shown in Table 4. Echocardiographic evaluation showed that Ob rats had increased fractional shortening (FS) endocardial, FS midwall, PWSV, and A-wave compared with C rats. Cardiac index, E/A ratio, and IVRT were lower in Ob rats than in C rats. Heart rate, cardiac output, E-wave, and EDT did not differ between the groups.

**Papillary Muscle Function Evaluation**

Figure 3 summarizes the mechanical properties of isolated papillary muscle from control and obese rats at baseline and after Ca\(^{2+}\) stimulation. Long-term obesity did not promote contractile dysfunction at basal condition and after inotropic intervention. Maximum developed tension (DT) was similar between the C and Ob rats under basal conditions (C: 5.87 ± 1.0 g/mm\(^2\) and Ob: 5.93 ± 1.05 g/mm\(^2\)) and after maneuver (Fig. 3, A and D). Additionally, long-term obesity failed to elicit any significant effect on +dT/dt and −dT/dt at baseline and after maneuver, suggesting that intracellular Ca\(^{2+}\) entry and resquestration were not compromised in Ob rats (Fig. 3, B, E, C, and F).

Figure 4 shows the myocardial L-type Ca\(^{2+}\) channel and SERCA2a activity from C and Ob rats. The myocardial dysfunction induced by long-term obesity was not related to L-type Ca\(^{2+}\) channel impairment and damage on SERCA2a performance. The maximal inhibition of DT in response to diltiazem was 84 ± 5% and 88 ± 3% at a Ca\(^{2+}\) exposure of 0.5 mM in C and Ob rats, respectively, but without significant effects between the groups (Fig. 4A). At the same time, diltiazem displayed similar negative inotropic behavior on +dT/dt without significant changes between the groups (Fig. 4B). This result suggests that long-term exposure obesity (30 wk) did not impair L-type channel activity and myocardial Ca\(^{2+}\) entry.

Figure 4, C–D, shows the influence of the SERCA2a inhibitor (CPA; 30 μM) in the presence of cumulative Ca\(^{2+}\) con-

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Table 2. **Comorbidities and hormones associated with obesity**

<table>
<thead>
<tr>
<th>Variables</th>
<th>C</th>
<th>Ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>127 ± 8</td>
<td>127 ± 16</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>91 ± 4</td>
<td>103 ± 4*</td>
</tr>
<tr>
<td>AUC, mg·dl(^{-1})·min(^{-1})</td>
<td>14,937 ± 3,029</td>
<td>20,631 ± 4,096*</td>
</tr>
<tr>
<td>Insulin, mg/dl</td>
<td>0.81 ± 0.10</td>
<td>1.18 ± 0.18*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>13 ± 2</td>
<td>21 ± 3*</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>54 ± 9</td>
<td>93 ± 19*</td>
</tr>
<tr>
<td>T-Chol, mg/dl</td>
<td>71 ± 9</td>
<td>73 ± 16</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>25 ± 4</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>15 ± 3</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>2.98 ± 0.78</td>
<td>8.16 ± 2.71*</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10 animals). C, control; Ob, obese; SBP, systolic blood pressure; AUC, area under the curve for glucose; HOMA-IR, homeostatic model assessment index; TG, triglycerides; T-Chol, total cholesterol. *P < 0.05 vs. C. Student’s t-test for independent samples.
centrations on DT and $-dT/dr$ in LV papillary muscle from C and Ob rats. Although this drug produced negative inotropic effects on DT in both groups, alterations were observed under low concentrations of Ca$^{2+}$ (0.5, 1.0, and 1.5 mM; Fig. 4C), with significant effects between the groups (maximal inhibition of DT; C > Ob). Additionally, CPA produced a similar response on $-dT/dr$ between groups, despite significant change in Ca$^{2+}$ concentration (0.5 mM; Fig. 4D). This result suggests that long-term obesity (30 wk) did not depress SERCA2a activity and cardiac sarcoplasmic reticulum Ca$^{2+}$ transport.

**Intracellular Ca$^{2+}$ Cycling Proteins**

The levels of intracellular Ca$^{2+}$ cycling proteins SERCA2a, CSQ, PLB, pPLB Ser16, pPLB Thr17, and L-type Ca$^{2+}$ channel were assessed to determine the mechanism for obesity-induced changes on cardiac function. These results are summarized in Figs. 5 and 6. Figure 5 shows that obesity did not change the protein levels of SERCA2a, PLB, CSQ, and L-type Ca$^{2+}$ channel. Furthermore, the SERCA2a/PLB ratio was also similar between groups (Fig. 5). Additionally, as shown in Fig. 6, protein levels of pPLB Ser16 (C: 1.00 ± 0.25 vs. Ob: 0.66 ± 0.12; P < 0.02) and the pPLB Thr17/PLB ratio (C: 1.00 ± 0.17 vs. Ob: 0.62 ± 0.20; P < 0.005) were significantly diminished in Ob rats. Protein levels of pPLB Thr17 and pPLB Thr17/PLB were similar between the groups (Fig. 6).

**DISCUSSION**

The adverse effects of obesity have been extensively studied in experimental models (13, 31, 50, 53, 56). Interestingly, although several mechanisms have been postulated to identify obesity-induced cardiac dysfunction, little information is available on the relationship between the duration of obesity, 

Table 3. Cardiac structural parameters analyzed by echocardiogram

<table>
<thead>
<tr>
<th>Variables</th>
<th>C</th>
<th>Ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD, mm</td>
<td>8.66 ± 0.50</td>
<td>8.51 ± 0.44</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>4.74 ± 0.36</td>
<td>4.13 ± 0.36*</td>
</tr>
<tr>
<td>LVDT, mm</td>
<td>1.51 ± 0.04</td>
<td>1.58 ± 0.05*</td>
</tr>
<tr>
<td>SWT, mm</td>
<td>1.52 ± 0.04</td>
<td>1.59 ± 0.04*</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>2.71 ± 0.15</td>
<td>2.96 ± 0.18*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.17 ± 0.01</td>
<td>0.19 ± 0.01*</td>
</tr>
<tr>
<td>LA, mm</td>
<td>5.74 ± 0.50</td>
<td>5.78 ± 0.44</td>
</tr>
<tr>
<td>AO, mm</td>
<td>4.13 ± 0.21</td>
<td>4.17 ± 0.16</td>
</tr>
<tr>
<td>LA/AO</td>
<td>1.39 ± 0.12</td>
<td>1.39 ± 0.11</td>
</tr>
<tr>
<td>LV mass</td>
<td>0.99 ± 0.12</td>
<td>1.02 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SD. LV, left ventricle; LVDD, LV end-diastolic dimension; LVSD, LV end-systolic dimension; LVDT, diastolic thickness of the left ventricle; SWT, diastolic septal wall thickness; PWT, LV diastolic posterior wall thickness; RWT, relative wall thickness; LA, left atrium diameter; AO, aortic diameter. *P < 0.05 vs. C. Student’s t-test for independent samples.

Table 4. Left ventricular functional data analyzed by echocardiogram

<table>
<thead>
<tr>
<th>Variables</th>
<th>C</th>
<th>Ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>290 ± 28</td>
<td>308 ± 44</td>
</tr>
<tr>
<td>Cardiac index, ml·min$^{-1}$·g$^{-1}$</td>
<td>332 ± 61</td>
<td>276 ± 38*</td>
</tr>
<tr>
<td>FS endocardial, %</td>
<td>45.3 ± 2.5</td>
<td>51.5 ± 3.3*</td>
</tr>
<tr>
<td>FS midwall, %</td>
<td>28.4 ± 2.3</td>
<td>31.9 ± 2.4*</td>
</tr>
<tr>
<td>PWSV, mm/s</td>
<td>34.8 ± 2.9</td>
<td>39.7 ± 2.2*</td>
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<tr>
<td>E-wave, cm/s</td>
<td>73.1 ± 7.8</td>
<td>77.1 ± 8.6</td>
</tr>
<tr>
<td>A-wave, cm/s</td>
<td>47.4 ± 8.6</td>
<td>66.6 ± 14.3*</td>
</tr>
<tr>
<td>E/A</td>
<td>1.59 ± 0.34</td>
<td>1.20 ± 0.26*</td>
</tr>
<tr>
<td>EDT, ms</td>
<td>48.9 ± 9.8</td>
<td>49.5 ± 7.8</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>31.3 ± 3.4</td>
<td>25.8 ± 2.9*</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10 animals). HR, heart rate; bpm, beats per minute; FS, fractional shortening; PWSV, posterior wall shortening velocity; E/A, early-to-late diastolic mitral inflow ratio; EDT, E-wave deceleration time; IVRT, isovolumetric relaxation time. *P < 0.05 vs. C. Student’s t-test for independent samples.
cardiac function, and Ca\(^{2+}\) protein regulation. The major finding in the current study was that cardiac dysfunction in Ob rats, after long-term obesity, was related to the reduction of PLB phosphorylation at Ser16 without affecting cardiac calcium influx and recapture.

In this study, the chronic feeding of a diet rich in unsaturated fat induced obesity in rats. The high-fat diet used was of sufficient intensity and duration to promote obesity in the experimental time period of 30 wk. According to the literature, fat-enriched diets have been used for decades to model obesity in rodents (11). The development of obesity was characterized by significant differences in body weight, body fat, fat pads, and adiposity index compared with control rats. The animal models in this study also presented almost all the features of metabolic syndrome, such as central obesity, glucose intolerance, hypertriglyceridemia, and insulin resistance; but systolic

![Fig. 3. Basal condition (A, B, and C) and effects of increasing extracellular Ca\(^{2+}\) concentration (D, E, and F) in papillary muscles from control (open bars) and obese (closed bars) rats after 30 wk. DT, maximum developed tension; +dT/dt, peak of positive tension derivatives; −dT/dt, peak of negative tension derivatives. All parameters normalized per cross-sectional area. Data are means ± SD. *P < 0.05 vs. control. Student’s t-test for independent samples (A, B, and C) and repeated-measures two-way ANOVA (D, E, and F); Student-Newman-Keuls post hoc test.](image)

![Fig. 4. L-type Ca\(^{2+}\) channels (A and B) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a; C and D) activity in papillary muscles from control (open bars) and obese (closed bars) rats after 30 wk; diltiazem and cyclopiazonic (CPA) experiment: 10 rats per group. All parameters normalized per cross-sectional area. DT and negative tension derivatives normalized per cross-sectional area. Data are mean percent of inhibition (% ± SD). *P < 0.05 vs. control. Repeated-measures two-way ANOVA; Student-Newman-Keuls post hoc test.](image)
blood pressure was not affected by long-term obesity. Consistent with previous investigations, the high-fat diet used in this study was effective at promoting numerous comorbidities associated with long-term obesity (19, 24, 33, 34, 43).

Morphological analysis postdeath, histological study, and echocardiographic assessment can identify relevant pathophysiological changes in structure and cardiac function during the progression of cardiovascular diseases associated, for example, with obesity. The present study showed that Ob rats developed cardiac hypertrophy compared with C rats. This structural alteration was visualized by higher total heart and LV weights; elevated cardiomyocyte CSA; and greater values of LVDD, LV diastolic thickness, LV diastolic SWT, PWT, and RWT. In the present investigation, obesity did not lead to an increase in interstitial collagen deposition in the LV. The mechanisms underlying the accumulation of collagen in Ob animals remain unknown; Brands et al. (10) suggested that the higher collagen concentration is linked to abnormalities in insulin metabolism, and other authors reported that it is associated with elevation of cytokines, endothelin, and renin-angiotensin-aldosterone (52, 59). These findings suggest that, in the current study, there was a remodeling of cardiac processes after a long period of obesity, without alterations in the levels of collagen. Rider et al. (57) proposed that cardiac remodeling is an adaptive characteristic of obesity.

Obesity-induced changes in cardiac structure may be elicited directly, by obesity-induced increases in cardiac loading conditions (preload and afterload), or indirectly, by obesity-induced cardiometabolic abnormalities such as dyslipidemia and insulin resistance and/or diabetes (1, 73). Dhanasekaran et al. (18) reports that insulin resistance induced by obesity with associated hyperinsulinemia could promote cardiac remodeling via the growth-promoting properties of insulin or by attenuating the antiapoptotic signaling of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway elicited by insulin receptor activation. Our data are in agreement with previous findings that have shown cardiac remodeling in long-term obesity (34, 43, 53).

For cardiovascular function, the cardiac remodeling induced by long-term obesity promoted significant functional changes after 30 wk compared with C rats. The main findings were visualized by measuring endocardial and midwall FS, posterior wall shortening velocity, A-wave, E/A ratio, and IVRT. Within this context, although Ob rats did not present an increase in LV end-systolic volume (data not shown), there was an improvement of LV ejection as evaluated by endocardial and midwall FS and posterior wall shortening velocity; these data corroborate the reduction of the LV end-systolic dimension. These parameters depend on the following factors: contractility, load variations, and heart rate; therefore, these parameters are influenced by the hormonal and autonomic nervous systems (45).

An explanation for the elevated systolic function may be related to decreased afterload and LV contractility improvement. Our data differ from some experimental studies that found decreased or unchanged systolic function by echocardiography (13, 20, 32).

The literature shows that studying LV diastolic function is very important because changes in the filling of this cavity may precede systolic dysfunction (28). In this study, long-term obesity increased the A-wave and promoted a reduction of the E/A ratio, indicating impairment of cardiac filling. These data suggest that obesity caused mild alterations in diastolic function due to the absence of changes in the E-wave, EDT, and decreased IVRT. According to some authors, IVRT prolongation is the most consistent diastolic abnormality observed in obese individuals (6, 41, 74). Furthermore, a higher A-wave and a lower E/A ratio suggest the presence of diastolic dysfunction (25, 26, 41). Diastolic filling depends on the active relaxation and passive properties of cardiac muscle, while the former is associated with Ca$^{2+}$ handling (16, 55); the latter depends on myocardium viscoelastic properties (8, 9, 72). Diastolic dysfunction in obesity models are not clarified (27, 61), but may be associated with insulin resistance, hyperinsulinemia, hyperglycemia, hemodynamic changes, and/or Ca$^{2+}$-handling homeostasis. Impaired insulin metabolic signaling and increased generation of reactive oxygen species play important roles in maladaptive myocardial remodeling (1, 63, 67).

Cardiac function was also evaluated in vitro by studying LV isolated papillary muscle. Our results showed that long-term

![Graph 1](http://jap.physiology.org/)

![Graph 2](http://jap.physiology.org/)
obesity did not promote contractile dysfunction after Ca\(^{2+}\) stimulation. In addition, long-term obesity failed to cause any significant effect on \(+\delta t/dt\) and \(−\delta t/dt\), suggesting that intracellular Ca\(^{2+}\) entry and resequestration were not compromised in Ob rats even with downregulation of cardiac pPLB Ser\(^{16}\). Alterations in intracellular Ca\(^{2+}\) handling have been described in obese rodents (31, 35, 50, 53), but there are few studies that verified these changes after long-term obesity. In the current study, although there was damage in the PLB phosphorylation at Ser\(^{16}\) after long-term obesity, the absence of changes in SERCA2a suggests that there was a compensatory mechanism in the Ca\(^{2+}\) handling.

The absence of changes after long-term obesity may be related to the type of high-fat diet utilized in this study, which was rich in unsaturated fatty acids. Increasing the time of exposure to an unsaturated high-fat diet may have caused a protective effect on the heart, unlike what was seen earlier by Leopoldo et al. (31). Several studies have shown that dietary fat intake plays a significant role in the development of cardiovascular disease (15, 17, 23, 44, 70). Although some controversy still exists as to the role of high-fat diets in human and animal health and disease, it is well accepted that dietary saturated fatty acid is positively associated with cardiovascular risk and, consequently, to damage of cardiac function (23, 36, 44, 70). In contrast, high-fat diets consisting of polyunsaturated fatty acid or those rich in monounsaturated fatty acid have beneficial effects on plasma lipids, fatty acid tissue composition, blood pressure, and myocardium structure and function in rats (3, 17, 36, 44, 58), and have been associated with low cardiovascular mortality (15).

Our data revealed impaired regulation of intracellular Ca\(^{2+}\) protein following long-time exposure to unsaturated high-fat diet intake, manifested by a reduction of PLB phosphorylation at Ser\(^{16}\). PLB phosphorylation of Ser\(^{16}\) and Thr\(^{17}\) by PKA or CaMKII is physiologically relevant for controlling SERCA2a activity (2, 38, 62). Ser\(^{16}\) and Thr\(^{17}\) phosphorylation is believed to be part of a sequential model that requires the phosphorylation of Ser\(^{16}\) before Thr\(^{17}\) (2, 38, 62). However, studies have shown that an individual and independent phosphorylation may occur at the two sites (12, 14). PLB phosphorylation/dephosphorylation is regulated by a pool of kinases upstream and by a pool of phosphatases and other regulatory proteins downstream. The major stimulus for controlling PLB activity is related to the \(\beta\)-adrenergic system. Activation of cardiac \(\beta\)-adrenergic receptors induces PLB phosphorylation at Ser\(^{16}\) and Thr\(^{17}\) via the PKA and CaMKII pathways, although PKA-dependent Ser\(^{16}\) phosphorylation is considered to be prevalent (12). Overall, our results indicate that PLB dephosphorylation at Ser\(^{16}\) contributes to the alterations in diastolic function observed after 30-wk diet-induced obesity. A possible explanation for the impairment of PLB phosphorylation at Ser\(^{16}\) in obese rats may be related to downregulation of the \(\beta\)-adrenergic system.

Another important feature of PLB phosphorylation at Ser\(^{16}\) is that this site is more physiologically important than Thr\(^{17}\) due mainly to the lower level of phosphorylation at Thr\(^{17}\) (12, 40). Phosphorylation of PLB at Thr\(^{17}\) must be potentiated by Ser\(^{16}\) phosphorylation, and Thr\(^{17}\) phosphorylation has a negligible effect after Ser\(^{16}\) has been phosphorylated (2, 38). Thus, although alteration in PLB phosphorylation at Thr\(^{17}\) was not observed, the damage to phosphorylation at Ser\(^{16}\) visualized in obese rats could impair phosphorylation at Thr\(^{17}\). On the other hand, phosphorylation of PLB at Thr\(^{17}\) does not appear to affect significantly interactions with SERCA, suggesting that the mechanism of releasing the inhibitory effect is different between Thr\(^{17}\)-phosphorylated and Ser\(^{16}\)-phosphorylated PLB (62).

In disagreement with our hypothesis, obesity did not affect SERCA2a, CSQ, and L-type Ca\(^{2+}\) channel protein levels. One explanation for such a result is that the mechanisms responsible for modulating these proteins, directly or indirectly, were not changed by obesity. Leopoldo et al. (31) showed that, despite the lack of changes in the level of L-type Ca\(^{2+}\) channel protein, the myocardial dysfunction caused by obesity after 15 wk was related to L-type Ca\(^{2+}\) channel activity impairment, suggesting that the \(\beta\)-adrenergic system was damaged. Although there was damage to PLB phosphorylation at Ser\(^{16}\) in this study, the absence of changes in L-type Ca\(^{2+}\) channel activity and expression suggests that other mechanisms are involved after long-term exposure to obesity.

In conclusion, our study shows that long-term exposure to obesity promotes alterations in diastolic function due to reduction of PLB phosphorylation at Ser\(^{16}\) but does not impair cardiac Ca\(^{2+}\) influx and recapture.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


