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Long-term high-fat diet-induced obesity decreases the cardiac leptin receptor without apparent lipotoxicity

André F. Nascimento [a,c,*], Renata A.M. Luvizotto [a,c], André S. Leopoldo [a], Ana P. Lima-Leopoldo [a], Fábio R. Seiva [a], Luís A. Justulin Jr. [b], Maeli Dal Pai Silva [b], Katashi Okoshi [a], Xiang-Dong Wang [c,d], Antonio C. Cicogna [a]

[a] Department of Medicine Clinical, Botucatu School of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil
[b] Department of Morphology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil
[c] Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, United States
[d] Department of Nutritional Science, Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA, United States

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ABSTRACT

Aims: Leptin resistance has been associated with cardiac lipotoxicity; however, whether leptin resistance is a risk factor associated with cardiac lipotoxicity at different time points in diet-induced obesity is unclear. The objective of this study was to evaluate this relationship.
Main methods: Male Wistar rats were fed a normal chow diet (12% from fat) or a high-fat diet (49% from fat) for 15 and 45 weeks, respectively. The adiposity index, body weight and co-morbidities were evaluated. Heart lipotoxicity was assessed by analyzing cardiac function and morphological changes as well as cardiac triglyceride, ceramide and lipid hydroperoxide accumulations. Cardiac apoptosis was examined using the TUNEL method. Leptin function was determined by examining plasma leptin levels, cardiac leptin receptors (OB-R) and related phosphorylations of AMP-activated kinase protein (AMPK) and Acetyl CoA carboxylase (ACC).
Key findings: The diet-induced obesity was characterized by an elevated adiposity index, body weight and leptin levels at both 15 and 45 weeks. There was no difference between groups in the cardiac triglyceride or lipid hydroperoxide levels. Interestingly, ceramide levels decreased in obese animals in both experimental periods. The cardiac morphological and functional parameters were not altered. Although down-regulation of OB-R has occurred in chronic obesity, it did not adversely affect AMPK or ACC phosphorylation.
Significance: The development of obesity via long-term feeding of a high-fat diet to rats does not result in cardiac lipotoxicity but promotes the down-regulation of OB-R. However, this does not result in altered levels of AMPK or ACC phosphorylations in this animal model.

Introduction

Obesity, which is defined as an excessive or abnormal accumulation of adipose tissue that may impair health (WHO, 2006), is considered to be a major cause of increased cardiovascular morbidity and mortality (Freedman et al., 1995) and an independent risk factor for the development of heart failure (Kenchaiah et al., 2002). The deterioration in cardiac function has been associated with the degree and duration of obesity (Pascual et al., 2003; Scaglione et al., 1992); however, the mechanisms responsible for this relationship are not completely understood. Under physiological conditions, 60% to 70% of the ATP consumed by the heart is from the oxidation of fatty acids (Stanley et al., 2005). Recent studies suggest that alterations in cardiac fatty acid metabolism may lead to the accumulation of products derived from non-oxidative metabolism, such as triacylglycerol, diacylglycerol and ceramide. This may lead to dysfunction and/or myocyte death and, eventually, cardiac function depression, a process known as lipotoxicity (Zhou et al., 2000; Chiu et al., 2001; Lopaschuk et al., 2007). Although this association has been noted in acyl-CoA synthase transgenic mice (Chiu et al., 2001) and genetically obese Zucker fa/fa rats (Zhou et al., 2000), its relationship with the development of long-term diet-induced obesity has not been reported.

Leptin, a hormone derived from adipose tissue, acts as a regulator of lipid metabolism, is anti-steatogenic and protects the heart from lipid overload (Unger et al., 1999; Lee et al., 2004). However, leptin resistance may produce myocardial lipotoxicity (Unger, 2002; Palanivel et al., 2006) by altering the phosphorylation of intracellular messengers, such as AMP-activated protein kinase (AMPK). Palanivel et al. (2006) showed that, when exposed to leptin for a short period, cardiomyocytes increased the fatty acid oxidation associated with a decrease in

* Corresponding author at: School of Medicine, São Paulo State University, Distrito de Rubião Jr., s/n, CEP 18618-970, Botucatu, SP, Brazil. Tel.: +55 14 38116339; fax: +55 14 38116424.
E-mail address: nascimentoaf@yahoo.com.br (A.F. Nascimento).

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intracellular lipid content. In contrast, there was a reduction in fatty acid oxidation and, consequently, intracellular lipid accumulation after 24 h of exposure to leptin. This result was associated with modulation of AMPK and ACC phosphorylation, which suggests that leptin plays a role in myocardial steatosis in the short term. However, this role does not occur in the leptin-resistant state.

Obesity has been frequently associated with hyperleptinemia (Ashwin and Dilipbhai, 2007; Dong and Ren, 2007), which suggests a leptin-resistant state. If persistent leptin exposure causes myocardial lipid accumulation by altering the Leptin Receptor(OB-R)/AMPK/ACC/Malonyl-CoA axis, the hyperleptinemia associated with the development of diet-induced obesity will be a determining factor in cardiac leptin resistance and, consequently, lipotoxicity. Thus, the objective of this study was to evaluate the relationship between diet-induced obesity and cardiac lipotoxicity by assessing the OB-R/AMPK/ACC/Malonyl-CoA axis at different time points.

Materials and methods

Animals and the experimental model

This study was performed on male Wistar rats (6 weeks, 150 g), which were supplied by São Paulo State University Animal Center, UNESP, Botucatu/SP. Rats were fed either a normal diet (ND; RC Focus 1765, Agroceres®, Rio Claro, São Paulo, Brazil) or a high-fat diet (HD; RC Focus 2413, Agroceres®, Rio Claro, São Paulo, Brazil) for 15 or 45 weeks. The caloric composition of the diets is given in Table 1. The dietary regimen was adapted from previous results obtained in our laboratory (Nascimento et al., 2008; Lima-leopoldo et al., 2008).

The groups received water and food ad libitum. Food consumption was measured every day. Rats were housed in individual cages in a temperature- (24 ± 2 °C) and humidity- (55 ± 5%) controlled environment on a 12–12 h light–dark cycle. The study protocol was approved by Botucatu School of Medicine Research Ethics Committee, UNESP and followed the *Guide for Care and Use of Experimental Animals.*

Characterization of obesity model

Adiposity index

Adipose tissue was isolated and weighed from the epididymal, visceral and retroperitoneal fat pads. Total body fat was divided by body weight [(epididymal + visceral + retroperitoneal)/body weight × 100] to obtain an adiposity index.

Table 1
The calorie composition of the experimental diets.

Components	Diet	
	Normal	High-fat
Protein (%)	22	20
Carbohydrate (%)	43	26
Fat (%)	4	20
Others (%)*	31	34
% calories from protein	30	22
% calories from carbohydrate	58	29
% calories from fat	12	49
% calories from saturated fat	2.5	9.9
% calories from unsaturated fat	9.5	39.1
Calories (Kcal/g)	2.95	3.65
Fatty acid composition (%)		
Palmitic (C16:0)	16.56	15.09
Stearic (C18:0)	3.90	4.39
Oleic (C18:1n9c)	27.96	37.94
Linoleic (C18:2n6)	47.10	40.83
Others**	4.48	1.75

* Vitamins, minerals, cinders and water.
** Lauric (C12:0), miristic (C14:0), palmitoleic (C16:1), Linolenic (C:18:3n3).

Co-morbidities

Because obesity is characterized by numerous co-morbidities, in addition to an increase in body fat, we evaluated the following conditions: systemic insulin resistance, hyperinsulinemia, hyperleptinemia, hyperglycemia, dyslipidemia and elevated systolic arterial pressure.

Systemic insulin resistance

Systemic insulin resistance was analyzed using the insulin tolerance test (Carvalho-Filho et al., 2005). After the rats had fasted overnight, their blood was sampled for glucose analysis at 0 (before), 15, 30, 45, 60 and 90 min after administration of the insulin load (1.5 UI/kg/ip) using a handheld glucometer (ACCU-CHEK®GO, Roche Diagnostic, Mannheim, Germany). The insulin tolerance of all groups was determined according to the area under the glucose curve (0 to 90 min).

Hormone and metabolite determination

At the end of the treatments, all animals underwent a 12- to 15-hour fast, after which they were anesthetized with sodium pentobarbital (40 mg/kg/ip) and sacrificed by decapitation. Serum was separated by centrifugation at 1300 *g* for 10 min. An enzymatic colorimetric kit was used to measure glucose (CELM®, São Paulo, Brazil), triacylglycerol (CELM®, São Paulo, Brazil) and free fatty acid (WAKO®, WAKO Pure Chemical Industries Ltd, Osaka, Japan). Spectrophotometry was performed with the Technicon RA-XT™ System automatic spectrophotometer (Global Medical Instrumentation, Minnesota, USA). Serum insulin and leptin were measured by the Elisa method using assay kits from Linco Research Inc. (Missouri, USA).

Systolic arterial pressure

Caudal systolic arterial pressure was evaluated with the semi-automated tail cuff device Narco Bio-System® PE 300 (International Biomedical, Inc, Houston, TX, USA).

Characterization of cardiac lipotoxicity

Myocardial triacylglycerol and lipid hydroperoxide

Left ventricle samples of 200 mg were homogenized in 5 ml of a cold 0.1 M phosphate buffer (pH 7.4). Tissue homogenates were prepared in a motor-driven Teflon glass Potter Elvehjem tissue homogenizer (1 min, 100 *g*). The homogenate was centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was utilized to determine triacylglycerol (test kit CELM; Modern Laboratory Equipment, SP, Brazil) and lipid hydroperoxide (Jiang et al., 1991).

Myocardial ceramide

Left ventricular fragments (100 mg) were crushed and shaken vigorously. The internal standard (1 ug of Cer17 ceramide/1 mL of methanol) was added to 50 µl ammonium hydroxide and 4 mL of hexane. The mixture was shaken for 60 min and centrifuged at 1700 *g* for 5 min to achieve phase separation. The organic layer was taken and evaporated to dryness under nitrogen gas. The dry residues were reconstituted with 0.4 ml of methanol and transferred to auto-injector microvials. The determination of palmitoylceramide (Cer16) in rat hearts was performed by chromatography with negative ion electrospray ionization coupled to tandem mass spectrometry (QUATTRO LC, Micromass, UK). The QUATTRO LC was set up for multiple reactions to monitor (MRM) for the following transitions: 550.4>293.5 for Cer17 and 536.4>237.5 for Cer16. The Cer16 (catalog number 860516P) and Cer17 (catalog number 860517P) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). This analysis was performed in the Cartesius Analytical Unit (Department of Pharmacology, State University of Campinas, UNICAMP, SP, Brazil).

TUNEL

To quantify the relative numbers of cells with DNA fragmentation, the TUNEL assay was performed on heart tissue sections using the FragEL DNA Fragmentation Detection Kit (Calbiochem), according to the manufacturer's protocol. Briefly, the slides were deparaffinized, and myocyte nuclei were stripped of proteins by incubation with 20 μg/mL proteinase K for 10 min. Following treatment with 3% H_2O_2 for 5 min, the slides were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme with TdT buffer and biotin-tagged nucleotides in a humidified chamber at 37 °C. Tagged nucleotides were detected using streptavidin-horseradish peroxidase (HRP). After washing, the sections were stained with diaminobenzadine (DAB)/H_2O_2 solution and counterstained with hematoxylin, then dehydrated and mounted.

Echocardiographic study

Echocardiography was performed at the end of experimental periods to evaluate left ventricular morphology and function using a commercially available Sonos 2000 echocardiograph (Hewlett-Packard Medical Systems, Andover, MA, USA) equipped with a 7.5-MHz phased array transducer. Imaging was performed at a 60° sector angle and 3 cm imaging depth. Rats were anesthetized by intraperitoneal injection with a mixture of ketamine (50 mg/kg) and xylazine (1 mg/kg). Two-dimensionally targeted M-mode echocardiograms were obtained using short-axis views of the LV at or just below the tip of the mitral valve leaflets; these images were recorded on a black-and-white thermal printer (Sony UP-890 MD, Somatechnology, Cheshire, CT, USA) at a sweep speed of 100 mm/s. All LV traces were manually measured with a caliper by the same observer according to the method approved by the American Society of Echocardiography (Sahn et al., 1978). Measurements are the mean of at least five consecutive cardiac cycles. The LV end-diastolic dimension (LVDD), posterior wall thickness (PWTd), and anterior wall thickness (AWTd) in diastole were measured at the maximum diastolic dimension. The LV end-systolic dimension (LVSD), posterior wall thickness (PWTs), and anterior wall thickness (AWTs) in systole were taken at the maximum anterior motion of the posterior wall. The left atrial dimension (LA), aortic dimension (AO), early peak transmitral flow velocity to late peak transmitral flow velocity ratio (mitral E/A), and heart rate (HR) were also measured. Relative wall thickness (RWT) was determined by PWT/LVDD. Left ventricular mass (LVM) was calculated using the following formula: $((LVDD + AWTd + PWTd)^3 - LVDD^3) \times 1.04$, where the value 1.04 indicates the specific density of the myocardium. Indices of left ventricular systolic function were assessed by calculating endocardial fractional shortening (FS) as follows: $FS\% = ((LVDD - LVSD)/LVDD \times 100)$; midwall $FS\% = ((LVDD + ½\ PWTd + ½\ AWTd) - (LVSD + PWTs + ½\ xAWTs)/(LVDD + ½\ PWTd + AWTd))$; and LV wall systolic stress = SBP × (LVSD/2)/PWTs, where SBP is systolic blood pressure.

Postmortem cardiac morphological study

The cardiac weight (HW), left ventricular weight (LVW), right ventricular weight (RVW) and atrium weight (AT) were measured. The cardiac weight/final body weight ratio (HW/FBW), left ventricular weight/final body weight ratio (LVW/FBW), right ventricular weight/final body weight ratio (RVW/FBW) and atrium weight/final body weight ratio (AT/FBW) were also calculated. Left ventricular fragments were used for total collagen quantification by Picrosirius Red.

Characterization of cardiac OB-R/AMPK/ACC axis

The metabolic functionality of the cardiac OB-R/AMPK/ACC axis was evaluated for gene and protein expression.

Gene expression by real-time PCR

The real-time reverse transcriptase polymerase chain reaction (PCR) method with an Assay-on-Demand Gene Expression Product (Applied Biosystems, Foster City, CA, USA) utilized unlabeled PCR primers and a TaqMan MGB probe (FAM dye-labeled) optimized to work with the TaqMan Universal PCR Master Mix (P/N 4304437) in a StepOnePlus PCR system (Applied Biosystems). This method was used to quantitatively measure OB-R (Rn 00561465-m1), AMPK (Rn 00576935-m1), ACC (Rn 00573474-m1) and cyclophilin (Rn 00690933-m1) messenger RNA (mRNA) expression. All samples were assayed in triplicate. The mRNA contents were normalized to cyclophilin mRNA levels, and the differences in expression were determined by the Ct method described in the ABI user's manual (Applied Biosystems).

Protein concentration by western blotting

Cardiac tissues were homogenized and incubated in extraction Whole Cell Lysate Buffer (25 mM HEPES, 300 mM NaCl, 1.5 MgCl2, 0.2 mM EDTA, 0.05% Triton X-100, and 20 mM b-glycerophosphate, and a mixture of protease inhibitors, 1 mM PMSF, 1 ug/ml leupeptin, aprotinin, pepstatin) with agitation at 4 °C for 15 min. Samples were then centrifuged at 2000 and 100,000 *g* at 4 °C for 30 min and 60 min, respectively, and the supernatant was collected. Protein concentrations were determined using a Coomassie Plus protein assay kit (Pierce, Rockford, IL, USA). Cardiac protein extract (100 μg) was added to a solution containing 50 mM Tris–HCl (pH 6.8), 200 mM 2-Mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol, then boiled for 5 min before being run on SDS-PAGE gel electrophoresis. The protein was transferred to a polyvinylidene difluoride membrane. Membranes were incubated overnight with 5% non-fat milk in Tris- buffered saline containing 0.01% Tween 20 (TBS-T 0.01%). After blocking the membrane, immunoblotting was performed according to the manufacturer's instructions for each primary antibody against OB-R (Santa Cruz Biotechnology, CA, USA; catalog number sc 8391), phospho-AMPKα (Thr172) (Cell Signaling, Beverly, MA, USA; catalog number 2531) and phospho-ACC (Ser79) (Cell Signaling, Beverly, MA, USA; catalog number 3661). A positive control for AMPK and ACC was used (Cell Signaling, Beverly, MA, USA; catalog number 9158). Membranes were then incubated with secondary rabbit or mouse antibodies (Bio-Rad Laboratory, Hercules, CA). Anti-GAPDH antibody (Chemicon International, CA) was used as an internal control. The blots were developed using the ECL SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA, catalog number 34080) and analyzed by using a densitometer (GS-710 calibrated imaging densitometer, Bio-Rad lab, CA, USA).

Statistical analysis

Data are reported as means ± standard deviation. Comparisons between groups were performed using two-way ANOVA for independent groups and completed using the *post hoc* Tukey test. To evaluate the association between leptin and the leptin receptor, Pearson's correlation coefficient was determined. A 5% significance level was adopted.

Results

Characterization of obesity model

Obese animals ingested less food overall than the control group in both experimental periods. In contrast, total caloric consumption between the groups was similar at 15 weeks (O15 = C15) and elevated at 45 weeks (O45 > C45). Body weight, adiposity index and serum leptin increased in a time-dependent manner in obese and control animals but were higher in the obese group (O15 > C15; O45 > C45). Serum insulin levels were affected only in the long-term obesity group (O45 > C45) (Table 2). High-fat diet-induced obesity did not influence the levels of serum glucose, NEFA, and triglycerides as well as the area under the glucose curve and arterial blood pressure (data not shown).

Table 2
General features of the control and diet-induced obese groups at the end of the experimental periods (15 and 45 weeks).

Variable	15 weeks		45 weeks	
	Control (n = 9)	Obese (n = 10)	Control (n = 7)	Obese (n = 7)
Food intake (g/day)	28 ± 1.4	23 ± 1.1*	26 ± 1.3#	22 ± 1.2*
Caloric intake (Kcal/day)	84 ± 4.2	83 ± 4.0	77 ± 3.9#	81 ± 4.4*
Body weight (g)	472 ± 27	507 ± 29*	550 ± 46#	663 ± 55*,#
Adiposity index (%)	4.0 ± 0.4	6.3 ± 1.2*	5.6 ± 0.9#	8.5 ± 1.0*,#
Leptin (ng/dL)	2.6 ± 0.8	5.6 ± 1.2*	6.0 ± 2.3#	11.3 ± 3.1*,#
Insulin (ng/dL)	0.9 ± 0.3	1.3 ± 0.3	1.2 ± 0.4	2.5 ± 0.8*,#

Data are expressed as means ± SD. Comparisons between groups were performed using two-way ANOVA for independent groups and completed using the *post hoc* Tukey test.
* $p<0.05$ vs control.
\# $p<0.05$ vs 15 weeks.

Characterization of cardiac lipotoxicity

The influence of the duration of high-fat diet-induced obesity on the progression of cardiac lipotoxicity was assessed by analyses of cardiac triglyceride, lipid hydroperoxide, ceramide and apoptosis in relation to cardiac morphology and function. Ceramide levels (C-16) were decreased in the obese group at both experimental moments (O15<C15, O45<C45) but was not influenced by time. Lipid hydroperoxide and triglyceride did not differ by experimental group or time (Fig. 1). Myocardial apoptosis, as evaluated by TUNEL, was also unaffected (data not presented).

Right ventricular and total heart weights were elevated along with obesity duration, suggesting cardiac remodeling in the obese group at 45 weeks. The decrease in total heart/BW ratio in the control and obese groups at 45 weeks when compared with the respective control at 15 weeks was due to higher body weight in relation to total heart weight. The obesity and experimental time did not influence total collagen levels (Table 3). There was no evidence of systolic or diastolic dysfunction in long-term high-fat diet-induced obesity (Table 4). There was improvement in the posterior wall shortening velocity in obese animals at 45 weeks in relation to the control, which may be due to the cardiac remodeling that occurred in the long-term obesity group.

Characterization of cardiac OB-R/AMPK/ACC pathway

The possible role of the cardiac OB-R/AMPK/ACC pathway in lipotoxicity was also evaluated. Different periods of high-fat diet-induced obesity were accompanied by decreased gene expression in leptin receptors (O15>C15; O45>C45). However, protein expression was decreased only with long-term obesity (O45>C45). This did not influence the gene and protein expressions of AMPK or ACC, which were unaltered between the obese and control groups at both experimental moments (Fig. 2). High serum leptin levels were accompanied by decreased cardiac leptin receptor expression ($r=-0.60$, $p=0.0048$), indicating a down-regulation mechanism in the leptin pathway.

Discussion

Consistent with our previous investigation (Nascimento et al., 2008), the high-fat diet used in this study was effective in promoting obesity, which was demonstrated by an increased adiposity index in association with a higher body weight. This condition was exacerbated by long-term feeding with a high-fat diet. These results were evident even in the absence of an increase or a slight increase in caloric intake between the obese and control groups at 15 and 45 weeks, respectively (Table 2). The

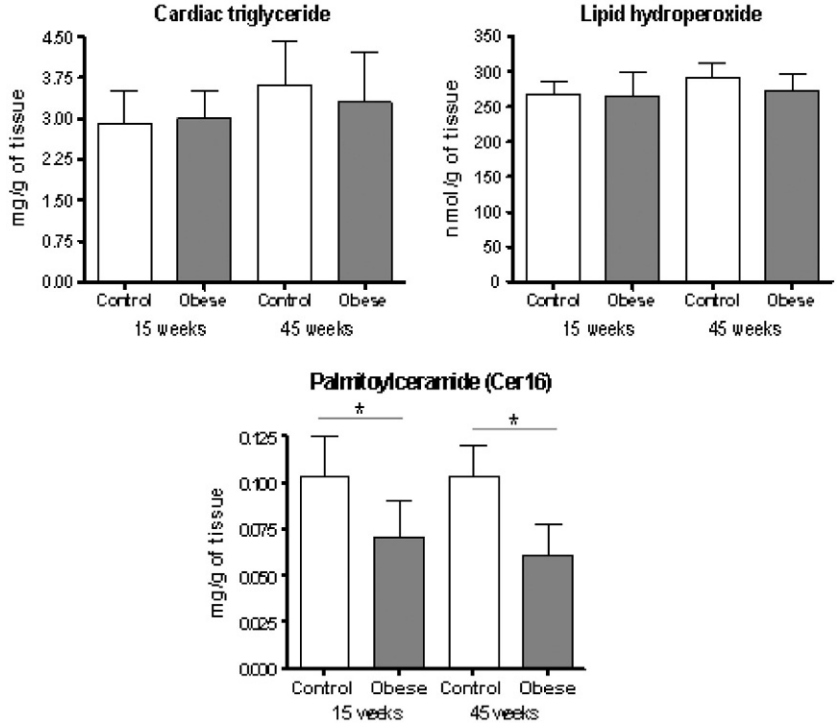

Fig. 1. Cardiac ceramide, lipid hydroperoxide and triglycerides in the control (C) and diet-induced obese (O) groups at the end of the 15- and 45-week experimental periods. Data are expressed as means ± SD (C15 = 9, O15 = 10, C45 = 7 and O45 = 7 animals/group). * Significant difference ($p<0.05$, Two-way ANOVA, *post hoc* Tukey Test).

Table 3
Post mortem cardiac morphology in control and diet-induced obese groups at the end of the experimental periods (15 and 45 weeks).

Variable	15 weeks		45 weeks	
	Control (n = 9)	Obese (n = 10)	Control (n = 7)	Obese (n = 7)
Left ventricular weight (g)	0.79 ± 0.06	0.77 ± 0.10	0.80 ± 0.06	0.87 ± 0.03[#]
Right ventricular weight (g)	0.32 ± 0.07	0.38 ± 0.08	0.39 ± 0.16	0.52 ± 0.06[*,#]
Atrium weight (g)	0.06 ± 0.02	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.02
Total heart (g)	1.16 ± 0.08	1.21 ± 0.13	1.22 ± 0.16	1.45 ± 0.07[*,#]
Total heart/body weight (g/g)	2.46 ± 0.17	2.39 ± 0.16	2.22 ± 0.17[#]	2.20 ± 0.14[#]
Total collagen (%)	2.2 ± 0.8	1.8 ± 0.8	2.3 ± 0.5	1.9 ± 0.6

Data are expressed as means ± SD. Comparisons between groups were performed using two-way ANOVA for independent groups and completed using the *post hoc* Tukey test.
[*] $p<0.05$ vs control.
[#] $p<0.05$ vs 15 weeks.

obese state may have been due to a high feeding effectiveness ratio in the hyperlipidic diet. There is evidence that high-fat consumption is not accompanied by a proportional increase in its oxidation. This phenomenon favors the deposition of lipids such as triacylglycerol in adipose tissue, leading to an increase in body weight (Tentolouris et al., 2008; Schrauwen and Westerterp, 2000).

Obesity has been associated with numerous co-morbidities in association with body weight and fat including the following: systemic insulin resistance, hypertension, hyperleptinemia, diabetes mellitus, dyslipidemia and hyperinsulinemia (Woods et al., 2003; Dourmashkin et al., 2005). The majority of these co-morbidities did not occur during the obese state in this study; however, there were increased leptin levels in both periods, and increased insulin levels were the result of long-term high-fat feeding. Because leptin is synthesized and secreted by adipose tissue and has been strongly associated with increased fat deposits (Frederich et al., 1995), the increase in leptin hormone may be due to higher body fat (Table 2). The increase in fasting insulin concentrations in the obese animals in the 45-week group may have occurred to maintain carbohydrate homeostasis and serum lipids, as insulin plays a fundamental role in the metabolic regulation of these two substrates (Khan and Pessin, 2002). In the present study, the fact that adipose tissue expansion, fasting normoglycemia and the area under the glucose curve were unchanged in the insulin tolerance test supports the hypothesis that the increase in insulin was effective in maintaining the homeostasis of serum carbohydrate and lipid levels. The normolipidemia in this study, as assessed by normal serum levels of free fatty acids and triglycerides, also supports this hypothesis. These facts were evident even in the presence of some level of systemic insulin resistance, as demonstrated by the combination of elevated circulating insulin levels and normal glucose levels.

The relationship between different periods of obesity and cardiac lipotoxicity was not evident in our results. This finding is corroborated by normal heart function in association with the absence of alterations in triacylglycerol, lipid hydroperoxide and apoptosis of cardiomyocytes. Interestingly, the normality in these lipotoxicity markers was accompanied by a decrease in the ceramide concentration among obese and control groups in both experimental time spans. The cardiac lipotoxicity concept is derived from the hypothesis that an excessive fatty acid supply to the heart may cause a greater concentration of products derived from its non-oxidative metabolism, such as triacylglycerol, diacylglycerol and de novo formation of ceramide (Zhou et al., 2000; Chiu et al., 2001; Lopaschuk et al., 2007; Park et al., 2008). Additionally, the excessive oxidation of fatty acids could lead to an increase in ROS production (Borradaile and Schaffer, 2005) and, consequently, lipid peroxidation. Together, the normal triglyceride concentration (a non-oxidative pathway marker) and lipid hydroperoxide (an indicator of lipid peroxidation from excessive catabolism) shown in the present study suggest a balance among the input, storage and oxidation of cardiac fatty acids. This notion is reinforced by normal serum fatty-acid and TG levels, which suggest that lipid delivery to the heart is normalized. The de novo formation of ceramide requires the incorporation of a saturated fatty acid, such as 16:0 palmitic acid (Summers, 2006). The small concentration of this lipid, which was 15.09% in the high-fat diet used, in association with the reduced dietary intake in the obese animals (Table 2), may be responsible for lower ceramide levels. These data are in concordance with the results of Okere et al. (2006), who showed that supplementation with unsaturated fatty acids reduced ceramide formation in the myocardium of rats throughout a twelve-week diet of unsaturated fatty acid. To the best of our knowledge, our data are the first to explore long-term exposure to a high-fat diet. The decrease in ceramide concentration, and the normal production of free radicals may explain the absence of apoptosis in our study, as these products have been linked with programmed cell death activation (Zhou et al., 2000; Chiu et al., 2001; Lopaschuk et al., 2007; Park et al., 2008; Borradaile and Schaffer, 2005; Summers, 2006). In addition, this absence may explain the maintenance of normal cardiac function in obese rats during long-term feeding. There is little information concerning the relationship between different periods of obesity induced by a high-fat diet and cardiac lipotoxicity. Wilson et al. (2007) studied rats subjected to one of three diets: low fat, Western or high fat (10%, 45% and 60% calories from fat, respectively) for periods from one day to 48 weeks. They found cardiac dysfunction in animals subjected to the Western diet after 48 weeks, which was not associated with lipotoxic markers.

Another interesting finding from this study is that the cardiac leptin receptor, but not the AMPK/ACC axis, exhibited the down-regulation in

Table 4
Cardiac morphology and function assessed by transthoracic echocardiography in the control and diet-induced obese groups at the end of the experimental periods (15 and 45 weeks).

Variable	15 weeks		45 weeks	
	Control (n = 9)	Obese (n = 10)	Control (n = 7)	Obese (n = 7)
Left ventricular mass (g)	0.96 ± 0.09	1.02 ± 0.12	0.99 ± 0.10	0.99 ± 0.09
Left ventricular mass relative (mm)	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.02
PWSV (mm/s)	44 ± 4.0	44 ± 3.9	37 ± 3.2[#]	42 ± 6.6[*]
Endocardial fractional shortening (%)	50 ± 3.5	49 ± 4.1	47 ± 6.1	52 ± 5.3
E/A	1.52 ± 0.22	1.46 ± 0.26	1.23 ± 0.15	1.39 ± 0.69

Data are expressed as means ± SD. Comparisons between groups were performed using two-way ANOVA for independent groups and completed using the *post hoc* Tukey test.
[*] $p<0.05$ vs control.
[#] $p<0.05$ vs 15 weeks.

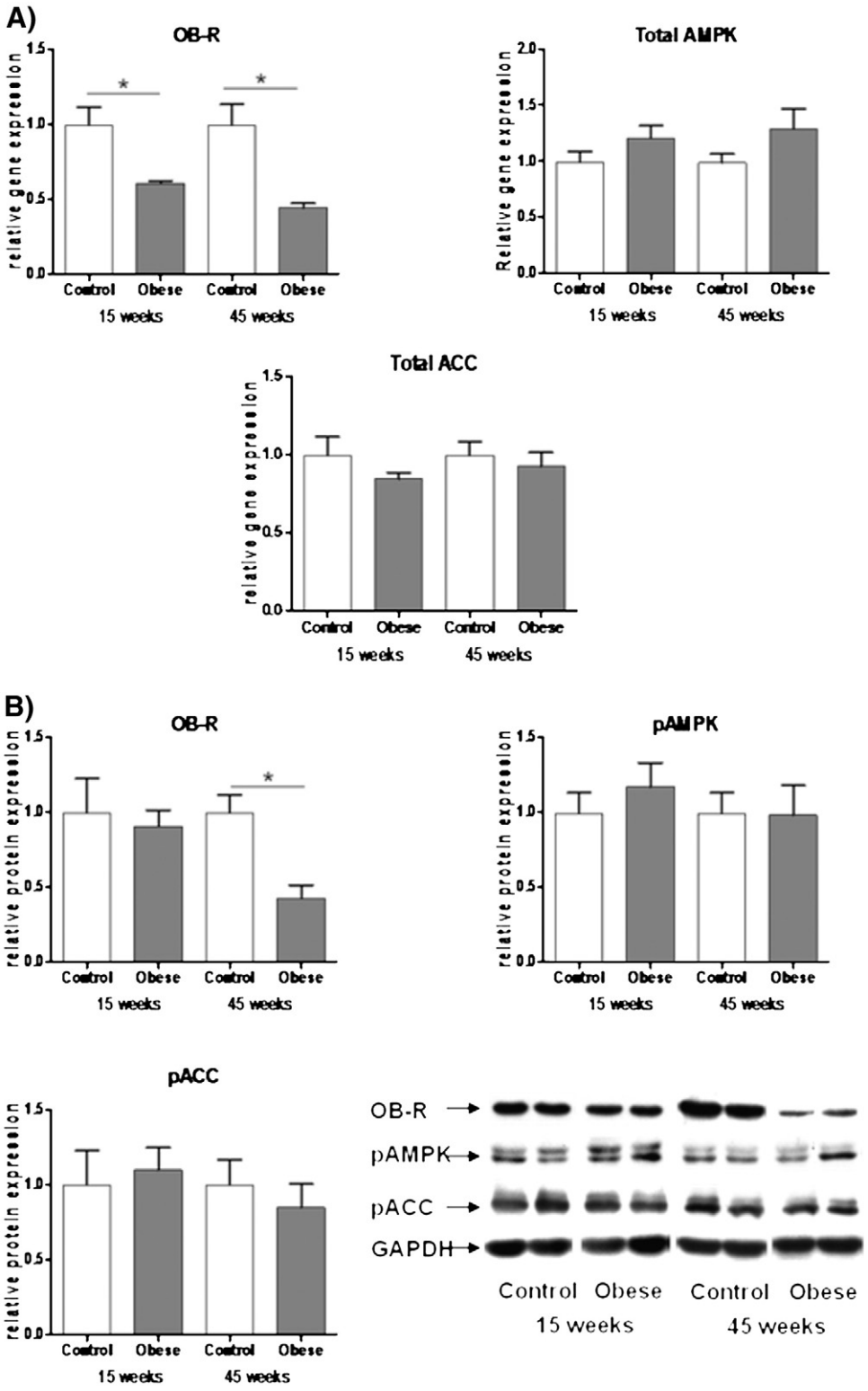

Fig. 2. Cardiac relative gene (A) and protein (B) expression of leptin receptor (OB-R), AMP-activated kinase protein (AMPK) and acetyl CoA carboxylase (ACC) in the control (C) and diet-induced obese (O) groups at the end of the 15- and 45-week experimental periods. The phosphorylation sites of AMPK and ACC were Thr172 and Ser79, respectively. Data are expressed as means ± SD (Sample number = five animals/group). * Significant difference ($p<0.05$, Two-way ANOVA, *post hoc* Tukey Test).

long-term obesity. It is known that leptin exerts various actions on different organs (Ahima and Flier, 2000; Friedman, 2002). Leptin receptors have been identified in heart cells, suggesting that leptin has direct effects on the heart, i.e., playing a role in regulating fatty-acid metabolism, thus protecting the heart from lipid overload (Unger et al., 1999; Lee et al., 2004). In contrast, leptin resistance may lead to cardiac intracellular lipid accumulation (Unger, 2002; Palanivel et al., 2006). Our results, namely the absence of cardiac lipotoxicity in obese animals, suggest the maintenance of leptin action on cardiac tissue. The decrease in leptin receptor levels could be due to a negative feedback mechanism, as hyperleptinemia was evident in the obese rats (Table 2) and was negatively correlated with cardiac leptin receptors. Despite the fact that

cardiac leptin receptor gene expression also decreased during short-term administration of a high-fat diet (Fig. 2), there was no difference in protein expression. These divergent data may be due the poly A tail, which influences the transduction process (Seraphim et al., 2007). The down-regulation of the cardiac leptin receptor may indicate a protective adaptation via the leptin axis in the heart during the hyperleptinemic state. To the best of our knowledge, our findings are the first to demonstrate the down-regulation of the cardiac leptin receptor during different periods of diet-induced obesity. This fact was not associated with alterations in AMPK/ACC phosphorylations. This result differs from Palanivel et al. (2006) who showed a negative association between long-term leptin exposure and AMPK/ACC phosphorylations, with the final result of increased triglyceride accumulation in myocytes. This accumulation might be seen as a protective mechanism, as incorporation into neutral triglycerides has been demonstrated to protect cardiomyocytes (Listenberger et al., 2003) from lipotoxicity by buffering FFA accumulation. One difficulty with the in vivo interpretation of the OB-R/AMPK/ACC axis in this study is the lack of a clear causal link between OB-R and AMPK/ACC. It is known that circulating levels of multiple signaling factors (e.g., ghrelin, adiponectin, IL-6, thyroid hormone and others) change during the development of high-fat diet-induced obesity (Tataranni and Ortega, 2005). Several of these circulating endocrine signals are known to not only play a role in the control of energy metabolism but also to participate in the regulation of AMPK (Steinberg and Kemp, 2009).

Diet composition may be considered a factor in the absence of cardiac lipotoxicity in this study. It been has shown that a diet high in palmitate, a saturated fatty acid, provides a higher cardiac cell death rate than a diet rich in unsaturated fatty acids (Okere et al., 2006; Sparagna et al., 2001). These results are consistent with data from studies of cell cultures derived from neonatal cardiomyocytes; they demonstrate that oleate incubation prevents palmitate-induced apoptosis (Hickson Bick et al., 2000; Miller et al., 2005). Furthermore, recent studies found that a diet rich in fructose (Sharma et al., 2007; Chess et al., 2007) and sucrose (Sharma et al., 2008) exacerbates left ventricular dysfunction and increases mortality. It has also been shown that supplementation with fish oil, a polyunsaturated lipid, reverses lipotoxicity and changes glucose metabolism in rats whose hearts are dyslipidemic and insulin-resistant, by feeding them a sucrose-rich diet (D'Alessandro et al., 2008). The present study extends these findings, showing that high intake of unsaturated fatty acids in association with the lower intake of simple sugars might generate protective effects on the heart, even in the presence of chronic obesity. Additional studies are needed to elucidate the effects of chronic obesity on substrates other than a non-unsaturated fatty acid on cardiac lipotoxicity and the role of the OB-R/AMPK/ACC/Malonyl-CoA axis.

Conclusion

The results of this study demonstrate that the development of obesity by the long-term administration of a high-fat diet does not cause cardiac lipotoxicity. This absence may be due, in part, to the lack of alteration in the AMPK/ACC axis, even in the presence of a decrease in leptin receptors. The presence of a diet composed of high-unsaturated fatty acids may be a mechanism that protects the heart, even in the presence of obesity.

Conflict of interest statement

The authors declare they have no conflicts of interest.

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