Caloric Restriction Overcomes Pre-Diabetic and Hypertension Induced by High Fat Diet and Renal Artery Stenosis

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Abstract

Background: Caloric restriction (CR) is a type of dietary intervention that is essential in weight loss through modulation of critical pathways of metabolic control; however, it is not yet clear what this intervention results to in association with renovascular hypertension.

Methods: Rats were divided into three groups: SHAM, and two groups that underwent surgery to clip the left renal artery and induce renovascular hypertension (OH and OHR). The SHAM diet was as follows: 14 weeks normolipidic diet; OH: 2 weeks normolipidic diet + 12 weeks hyperlipidic diet, both *ad libitum*; OHR, 2 weeks normolipidic diet + 8 weeks *ad libitum* high-fat diet + 4 weeks 40% calorie-restricted high-fat diet.

Results: Rats in the OHR group had decreased blood pressure, body weight, and glucose levels. Reductions in insulinemia and in lipid and islet fibrotic areas in the OHR group were observed, along with increased insulin sensitivity and normalization of insulin-degrading enzyme levels. The expression of nicotinamide phosphoribosyltransferase (NAMPT), insulin receptor (IR), sirtuin 1 (SIRT1), and complex II proteins were increased in the liver tissue of the OHR group. Strong correlations, whether positive or negative, were evaluated via Spearman's model between SIRT1, AMPK, NAMPT, PGC-1 α , and NNMT expressions with the restoration of normal blood pressure, weight loss, glycemic and lipid panel, and mitochondrial adaptation.

Conclusion: CR provided short-term beneficial effects to recover the physiological parameters induced by a high-fat diet and renal artery stenosis in obese and hypertensive animals. These benefits, even in the short term, can provide physiological benefits in the long term.

Key Points

- Caloric restriction overcomes pre-diabetes and hypertension.
- Caloric restriction regulated IR, SIRT1, NAMPT, and OXPHOS complex II protein expression in hypertensive rats.
- Caloric restriction activates mechanisms that mutually correlate with weight reduction, normalized biochemical and physiological parameters, and restores glucose homeostasis.

Introduction

Obesity and hypertension have become important global public health problems, resulting in morbidity and mortality. Together, these factors are responsible for the 5-fold increase in the possibility of developing type 2 diabetes and secondary cardiovascular diseases [31]. The main causes of obesity and hypertension are related to food intake, which has changed over the years with an increase in the content of fats and carbohydrates in the average diet. This, together with a lack of physical activity, provides the conditions conducive to the development of metabolic disorders such as diabetes [7].

The pancreas and liver are essential for carbohydrate and lipid homeostasis. Insulin is secreted by pancreatic β cells in response to increased post-prandial blood glucose levels. Insulin increases the rate of glucose uptake especially in the skeletal muscle and adipose tissue. An increase in insulin levels stimulates glycogen synthesis in hepatocytes through a reduction in GSK-3 levels, which increases glycogen synthase and promotes hepatic glycogen synthesis. Afterwards, excess glucose passes through pathways that lead to the synthesis of triglycerides for transfer to the adipose tissue. Insulin also reduces hepatic gluconeogenesis and hepatic glycogenolysis.

Therefore, one way of treating metabolic disorders is through caloric restriction (CR), involving administration of a diet with a 25–60% reduction in nutrients, in relation to the free diet, with a lower intake of calories derived from carbohydrates, fats, and/or proteins, without causing malnutrition [18]. CR has been proposed to reduce oxidative stress, decrease oxygen consumption, and stimulate intracellular signaling pathways that regulate the metabolism of glucose, lipids, and proteins [17]. In addition, CR regulates the expression of SIRT1 (regulator of the silent form of transcription 1), a molecule involved in the regulation of various pathological processes related to oxidative stress, anti-aging, cell survival, as well as stimulates the formation of autophagosomes, increasing autophagy under conditions of food restriction [17]. There is also evidence that SIRT1 regulates glucose and lipid metabolism [19]; however, there is still no evidence that demonstrated the antihypertensive effects of CR concomitantly with the activity of SIRT1.

SIRT1 requires nicotinamide adenine dinucleotide (NAD^+) for its enzymatic activity. Studies have shown that nicotinamide phosphoribosyltransferase (NAMPT) is a limiting enzyme in the biosynthetic pathway of NAD^+ in mammals and directly regulates the activity of SIRT1. NAMPT and NAD^+ levels in the tissues and organs are reduced due to the consumption of a high-fat diet and aging, contributing to type 2 diabetes [19]. Downregulation of NAMPT expression in both mRNA and protein levels were observed in rodent hearts in response to cardiac hypertrophy induced by pressure overload, while the overexpression of NAMPT in cardiomyocytes increased cellular levels of NAD^+ and ATP [28].

The NAD^+ -dependent deacetylase SIRT3, predominantly located in the mitochondria, is also involved in CR, as it increases the demand for NAD^+ in the mitochondria through NAMPT, protects against oxidative stress and cell death, and suppresses the generation of reactive oxygen species [27]. Protection against cellular aging is achieved by regulating glucose metabolism [22]. Taken together, these data also suggest that CR promotes, in addition to glucose homeostasis, an anti-aging action guided by increased autophagy and decreased oxidative stress, establishing itself as a beneficial experimental model.

The liver and pancreas play a leading role in the central physiological response to the action of CR as they are central organs responsible for alterations in the metabolism of lipids and glucose in response to nutritional and hormonal signals. Here, we focused on a better understanding of how these organs act under conditions of caloric restriction and renovascular hypertension in obesity in animals, in order to elucidate the role of this dietary intervention in two pathologies that negatively interfere with glycemic and lipid homeostasis.

Materials And Methods

Chemicals and reagents

Bovine serum albumin (BSA, fraction V), tris-(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Triton X-100, Tween 20, aprotinin, and glycerol were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA). The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad® (Richmond, California, USA). PVDF membranes (BioRad®) and the SuperSignal West Pico Chemiluminescent Substrate western blotting analysis system were obtained from Thermo Fisher Scientific Inc. software (Rockford, IL, USA). Glucose was purchased from Fluka Biochemika® (Buchs, Switzerland) and NPH human insulin was purchased from Novolin® N (Novo Nordisk, Bagsvaerd, Denmark).

Animals

Male Wistar rats (50 days old each) were used for the *in vivo* experiments. The rats were randomly assigned into three groups by weight and age at the start of the experiment and in all experiments (n=5 per group). Animals were obtained from the Vivarium of the University Center of the Hermínio Ometto Foundation, where their maintenance and the experiments were carried out in accordance with the Institutional Guidelines for Ethics in Animal Experiments approved by the Center for Animal Research Ethics and Research (CEUA) of the Hermínio Ometto de Araras Foundation (protocol number 042/2016).

The animals were divided into three groups: SHAM, OH (hypertensive and obese), and OHR (hypertensive, obese, and with 40% caloric restriction). Renovascular arterial hypertension was induced by clipping the left renal artery using the Goldblatt 2K1C technique [12].

Diets and interventions

Some of the animals had access to a standard diet with commercial Nuvilab® food for rodents, while others had access to the Open Source Diets® high-fat diet. Throughout the experiment, the animals in the SHAM group received Nuvilab® normolipidic food *ad libitum* throughout the experiment, while the OH and OHR groups received this diet *ad libitum* only for 2 weeks. After this period, with the matching of body weight between the animals of the different groups verified, we started the obesity induction protocol using the Open Source Diets® hyperlipidic diet for 2 months. After this period, and the body weight matching verified, the OH group continued to receive a high-fat diet *ad libitum*, while the OHR group, in this same period, had a 40% less calorie consumption than the rats in the OH group. The composition of the diets is shown in Table 1.

Table 1
Nutritional composition of the diets.

	Hyperlipidic diet ^a	Normolipidic diet ^b
Protein	23.65%	22.23%
Carbohydrate	40.27%	60%
Fiber	5.80%	5.73%
Fat	23.60%	4.0%
Minerals	5.24%	9.0%
Vitamins/supplements	1.40%	*
Red dye	0.006%	*
Energy	4.72 kcal/g	3.97 kcal/g
Calcium	*	0.92%
Phosphorus	*	0.87%
Note: * value not determined.		
^a Source: Research Diets Inc.		
^b Source: Nuvilab CR1-Nuvital®.		

Body and blood pressure parameters

The animals were weighed on a conventional digital scale that was properly calibrated throughout the experimental period.

Blood pressure was analyzed through plethysmography of the tail. For this, a sleeve was coupled to a pressure transducer placed around the tail of an animal while it was awake, after being previously placed in a heating booth at 37°C. Pressure variants were captured in a PowerLab a/S analog-to-digital converter (AD Instruments Ltd., Castle Hill, Australia). Blood pressure was measured weekly during the experiment.

The Lee index was obtained as the ratio between the cubic root of the body weight and the nasoanal length of the animal [3], described by the formula:

$$\frac{\sqrt[3]{bodyweight (g)}}{nasoanallength (cm)}$$

Ip.GTT (intraperitoneal glucose tolerance test) and Ip.ITT (intraperitoneal insulin tolerance test)

The ip.GTT tests were performed after the animals fasted for 6 h (n=5 animals per group). The animals were intraperitoneally administered with glucose (2 g/kg body weight). Blood samples were collected from the tail before glucose overload (time zero) and at 30, 60, 90, and 120 min after glucose infusion.

Blood glucose was determined using reagent strips and a glucose meter (Abbott®, Chicago, IL, USA). The ip.ITT tests were also performed after the rats fasted for 6 h. The animals were intraperitoneally administered standard with crystalline insulin (1.5 U/kg body weight). Blood was collected at time zero (before insulin injection) and at 5, 10, 15, 20, 25, and 30 min.

After the blood glucose was determined using reagent strips and an Abbott® glucose meter, the glucose disappearance constant (K_{itt}) was calculated using the following formula: $\ln 2/t_{1/2}$. The serum glucose $t_{1/2}$ value was calculated from the slope of the minimum regression curve, considering the linear phase of the decrease in plasma glucose concentration.

Determination of hepatic and muscular glycogen content

The gastrocnemius muscles and the livers of the animals were dissected, and the hepatic and muscular glycogen concentrations were determined. Approximately 100 mg of tissue was placed in a test tube containing 30% KOH and incubated at 100°C for 15 min. Subsequently, 100% ethanol was added and incubated for 15 min at 70°C. The test tubes were then placed on ice for 20 min and then centrifuged for 10 min. The pellet was suspended in water, and glycogen levels were evaluated using a colorimetric method [21].

Euthanasia and sample collection

After the trial, the animals were euthanized via anesthesia with xylazine (10 mg/kg) and ketamine (90 mg/kg). Serum was collected for biochemical analyses. The liver and pancreas were removed for stereological, morphological, and biochemical analyses, as well as for protein quantification via western blotting.

The blood of the rats was collected during euthanasia via cardiac puncture after an 8-h fast. The blood was centrifuged for 10 min at 3500 rpm to obtain the serum. After centrifugation, the collected serum was frozen until analysis.

Biochemical analyses

Serum levels of aspartate aminotransferase and alanine aminotransferase, total cholesterol and its fractions, triglycerides, total protein, and glucose were measured using a Biotécnica® commercial colorimetric kit, following the manufacturer's instructions (Varginha, MG, Brazil). Insulin levels were determined via ELISA (Biotec Center, SPI-Bio and Ellipse Pharmaceuticals, France).

Determination of cholesterol and hepatic triglyceride levels

Approximately 100 mg of liver tissue was added to a 2:1 chloroform-methanol solution, crushed, and stirred for 1 min. Afterwards, methanol was added to the crushed sample, which was centrifuged for 10

min at 3000 rpm. The supernatant was then transferred to a conical tube containing chloroform and 0.73% NaCl solution. After centrifugation, the supernatant was discarded, and the precipitate was washed three times with Folch's solution (3% chloroform, 48% methanol, 47% water, and 2% NaCl). The lipid extracts were dried in a ventilated oven at 37°C and resuspended in 1 ml of isopropyl alcohol for the determination of cholesterol and triglycerides content using the commercial colorimetric kit Labtest®, following the manufacturer's instructions (Lagoa Santa, MG, Brazil) [9].

Western blotting

The liver tissue was homogenized in protein extraction buffer, and aliquots were prepared for protein quantification according to the Biuret method. The samples were treated with Laemmli buffer (0.1% bromophenol blue, 1 M sodium phosphate, 50% glycerol, and 10% SDS).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 50 µg of total protein. The proteins were transferred from the gel to polyvinylidene fluoride membranes (Immun-Blot, Bio-Rad®). After transfer, the membranes were blocked with a basal solution containing bovine albumin and then incubated with the primary antibodies (Table 2) overnight at 4°C under agitation.

Table 2
List of the antibodies used.

IR	Insulin Rβ (CT-3): sc-57342 (1:200) - Santa Cruz®
pAkt	Phospho-Akt (Ser 473): #9271 (1:1000) - Cell Signaling®
Akt	Akt Antibody #9272 (1:1000) - Cell Signaling®
Sirt1	Sirt1 (C14H4) - 1:1000 - Cell Signaling®
Nampt	PBEF (E-3) sc-393444 - 1:200 - Santa Cruz®
Sirt3	(C73E3) rabbit mAb - 1:200 - Santa Cruz®
NNMT	(G-4): sc-376048 - 1:200 - Santa Cruz®
pAMPKα	(Thr 172) - 1:1000 - Cell Signaling®
AMPKα	2532# 1:1000 - Cell Signaling®
PGC1α	(4A8): sc-517380 - 1:200 - Santa Cruz®
Foxo	(D-12): sc-48348 - 1:200 - Santa Cruz®
Oxphos	Cocktail ab123545 - 1:1000 - Abcam®

The membranes were then incubated with the corresponding secondary antibody (1:10000, Santa Cruz®), under agitation, followed by development with chemiluminescence reagents (Thermo Scientific®), and photodocumentation using G:BOX (Syngene®). The intensities of the bands were determined via densitometry using ImageJ® software (NIH, USA).

Histological analysis of liver tissue

After fixation in 10% formalin solution for 4 h, small fragments of liver tissue were embedded in paraffin using standard procedures. Sections of 4- μ m thickness with an inter-section distance of 20 μ m were stained with a hematoxylin-eosin solution for histological analysis. Images were captured using a Leica DM 2000® photomicroscope with the Las software (version 4.1). Ten images were captured per animal at 400 $\times$ magnification and analyzed using Image-Pro Plus® software (version 4.5.0.29).

Stereological analysis

For stereological analysis, in each of the ten images, a grid with 300 intersections was applied, for a total of 3000 intersections per animal. The intersections were classified into hepatocyte cytoplasm, mononuclei, binuclei, lipid deposits, and connective tissue elements (vessels, connective cells, and matrix). The frequencies (%) of each element are presented as the mean $\pm$ SEM.

Cell quantification and morphometry

In each of the ten images, all hepatocytes and lipid drops present in the area were counted. Only fully delimited cells were considered. Morphometry involved defining the diameter of the hepatocytes and their nucleus for a total of 30 cells per animal. The values for all cells are presented as the mean $\pm$ SEM.

Histological analysis of pancreatic tissue

After fixation, small fragments of the pancreas were embedded in paraffin using standard procedures, which were dehydrated with increasing concentrations of alcohol. Sections (5- μ m thick) were stained with hematoxylin-eosin, Perls (for analysis of iron deposits), and Gomori's trichrome (for analysis of collagen fibers). Two sections were used for each animal in the SHAM, PH, and OHR groups, for a total of 12 sections per animal group. Images were acquired using a Leica DM 2000® photomicroscope operated with a LAS v.4.1 software.

Qualitative histological evaluations of the Perls and Gomori staining results were performed by two observers, considering the presence and absence of staining. The evaluations included the entire area of the section using images acquired at 400 $\times$ magnification which were analyzed using Image-Pro Plus® (v.4.5.0.29) software. For each animal, the diameters were obtained for all islets that were fully delineated in the images.

Detection of IDE mRNA expression via RT-PCR

Total RNA was isolated from approximately 100 mg of rat liver using TRIzol® reagent (Invitrogen®, Carlsbad, CA, USA), including the digestion of contaminating DNA with amplification grade DNase I (Invitrogen®), following the manufacturer's instructions. RNA purity and concentration were determined spectrophotometrically using a Thermo Fisher Scientific Evolution™ 249 300 UV-Vis spectrophotometer. RNA concentration was determined at OD₂₆₀ and purity was verified if the OD₂₆₀/OD₂₈₀ > 1.6. cDNA was synthesized using 2 μ g of RNA in the presence of dithiothreitol, dNTP, random primers, RNaseOUT, and SuperScript™ II Reverse Transcriptase (Invitrogen®), for a final volume of 20 μ L. The mRNA levels of the

insulin-degrading enzyme (IDE) genes were investigated using semi-quantitative RT-PCR. The primer sequences used in the PCR reactions were chosen based on the sequences available in GenBank. IDE gene amplification was performed using sense (5'-AGGAAATGTTGGCTGTGGACGCA-3') and antisense (5'-CCTGGCAAGAACGTGGACGGATA-3') primers to amplify a predicted amplicon 62 bp in length ($T_m = 57^\circ\text{C}$). The amplified products were separated using a 2.0% agarose gel stained with ethidium bromide, and the gel was photographed using LPix-Touch[®] (Loccus Biotechnologia[®]). The signal intensities of the bands were obtained using G:Box (Syngene[®]), with densitometric measurements performed using Scion Image[®] software (Scion Corp., Frederick, MD, USA). Each reported value was calculated as the mean of three densitometric readings. The results were expressed as the average ratios of the relative expression of transcripts normalized using β -actin as the control housekeeping gene. β -actin gene amplification was performed using forward (5'-AGAGGGAAATCGTGCGTGACA-3') and reverse (5'-CGATAGTGATGACCTGACCGTA-3') primers to amplify a predicted amplicon 138 bp in length ($T_m = 57^\circ\text{C}$).

Different networks of animal markers stratified into groups

Data from marker networks in obese and hypertensive animals, diagnosed with or without restricted dietary intake, together with the SHAM group, were analyzed using special Spearman correlations at $P < 0.05$. Correlation analysis of all groups was performed using Cytoscape[®] 3.0.4 software (Cytoscape Consortium, San Diego, CA, USA). The data were represented by connecting edges to highlight positive (strong, $r \geq 0.68$, thick continuous line; moderate, $0.36 \leq r \leq 0.68$, thinner continuous line; weak, $r < 0.35$, thin continuous line) and negative (strong, $r \leq -0.68$, thick connected line; moderate, $-0.68 \leq r \leq -0.36$, thinner initiated line; weak, $r > -0.36$, thin initiated line thin) correlations, as previously proposed [13]. The absence of a line indicates the absence of a relationship.

Statistical analyses

For comparison of multiple groups, we performed one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. The differences between any two groups were evaluated using a two-tailed Student's *t*-test. All calculations were performed using GraphPad Prism 9.0 software (GraphPad[®], San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$. The results were expressed as the mean $\pm$ standard error of the mean (SEM).

Results

Caloric restriction promotes restoration of blood pressure and body weight and recovers glycemic homeostasis in obese and hypertensive animals

After being allocated and paired, the animals were monitored throughout the experiment, and the groups and the different dietary interventions are represented in a graphic summary (Figure 1A). The rats were subjected to renovascular arterial hypertension using the Goldblatt 2K1C technique and were fed a diet that was high in fat (HFD) or remained on a normal chow diet. After 10 weeks on the HFD, a subset of the HFD group underwent CR for 4 weeks (OHR). After 10 weeks, HFD feeding induced a significant increase

in the body weight and Lee index of mice compared with the SHAM and OHR groups (Figure 1B and 1F). Rats in the OHR group had a significant reduction in body weight and blood pressure compared with the OH group (Figure 1B and C), in agreement with the literature [10, 15]. As shown in Figure 1D, despite being offered food *ad libitum*, ingestion of a diet rich in lipids resulted to a lower consumption, suggesting that it provided satiety after intake of a smaller quantity and/or that the animals in the OH group did not find it pleasant to eat. Despite the decreased ingestion, the animals that received the lipid-rich feed showed increases in body weight and Lee index values (Figure 1B and 1F) due to lipid/calorie overload and a nutritional imbalance of the food. In the OHR group, despite their excessive lipid consumption, the animals consumed less food due to CR; therefore, their body weight was similar to that of the SHAM group, as reported previously [14]. There was no difference in the serum concentrations of proteins (data not shown), indicating that the animals were not malnourished. As expected [16], CR decreased serum glucose levels and improved the lipid profile of the rats (Figure 1E and Figure 2A, 2C, 2D, 2E, 2I). In Figure 1G, the GTT and the area under the curve (AUC) values revealed intolerance to glucose in the OH group and reestablishment of glycemic control in the OHR group, when compared to the SHAM group (OH: 26409 ± 2039 ; OHR: 22950 ± 291.2 ; SHAM: 19766 ± 1467). In Figure 1H, the ITT indicated greater insulin sensitivity in the OHR group, compared to the SHAM and OH groups, which was reflected in the K_{itt} values shown in Figure 1H (OHR: $1.58 \pm 0.14\%/min$; SHAM: $1.24 \pm 0.09\%/min$; OH: $0.78 \pm 0.10\%/min$). HDL (Figure 2B) and hepatic cholesterol (Figure 2H) levels were significantly elevated in the OHR group. Figure 2J shows that there was increased IDE gene expression in the livers of rats in group OH, compared to the SHAM group (1.2 ± 0.09 and 0.81 ± 0.04 , respectively), while those between the OHR and SHAM groups were similar (0.92 ± 0.06 and 0.81 ± 0.04 , respectively). According to the results we obtained in the GTT and ITT, IDE can preserve peripheral insulin sensitivity. Figure 2F shows that hepatic glycogen levels (in g per 100 g of tissue) were lower in the OHR group than in the SHAM group (0.32 ± 0.045 g and 0.52 ± 0.04 g, respectively), while the levels were similar for the OH and SHAM groups. Reduced glycogen stores in the OHR animals strengthen the adaptive physiological mechanisms of weight loss. Calorie-restricted animals initially have reduced or depleted glycogen stores, together with decreased insulin secretion, both of which are required to meet the energy needs of the brain [24]. There was no difference in muscle glycogen levels (Figure 2G) between the groups.

Caloric restriction preserves pancreatic structures, reverses islet fibrosis, but causes an increase in the number of lipid drops in hepatocytes

In Figure 3A, representative images of liver and pancreas sections show the slides stained using the H&E, Perls, and Trichrome Gomori techniques. No differences were observed in aspartate aminotransferase and alanine aminotransferase levels (Figure 3B and 3C) as well as the diameters of the islets from the H&E staining (Figure 3F). Perls staining (Figure 3G) showed an absence of iron deposits (hemosiderin) for the three groups (SHAM, OH, and OHR). The slides stained using Gomori's trichrome (Figure 3H) showed a higher amount of collagen in the islets of rats from the OH group than in the OHR and SHAM groups. Fibrotic pancreatic islets have been observed in human type 2 diabetes, as well as in rodent models [6, 8]. Our results showed that CR contributes to the functional preservation of the islet cells,

characterized by the absence of collagen associated with increased insulin levels (Figure 3E). According to the histological data summarized in Table 3, the diameter of the hepatocytes, area occupied by conjunctive elements, and the number of binuclear and mononuclear cells were similar in all animal groups, suggesting the maintenance of cellular integrity. The increase in the area of hepatocyte nuclei of rats in the OHR group (23.81%) compared to the SHAM (4.76%) and OH (4.79%) groups may indicate the elevated proliferative capacity of cells in the liver, an organ with a high degree of cell regeneration. Thus, it is suggested that the nucleus occupies space in the hepatocyte cytoplasm, which was reduced in the OH (26.54%) and OHR (26.82%) groups versus the SHAM group (46.19%). The hepatocytes showed no signs of inflammation or fibrosis in any of the groups, suggesting the maintenance of hepatic integrity. The OHR group had an increase in the number of lipid drops in the liver compared to the SHAM group. Histological analysis of the liver revealed hepatic adaptation to caloric restriction without causing harm to the hepatocytes, as confirmed by the consistent levels of hepatic transaminases (Figure 3B and 3C).

Table 3
Stereological and morphometric profiles of the hepatic tissues of the Sham, HO, and HOR group animals.

Parameters	Sham	HO	HOR
Mononucleate (%)	18.55	17.58	17.78
Binucleate (%)	4.45	5.27	5.84
Lipid (%)	16.99	35.54*	38.09**
Cytoplasm (%)	46.19	26.54*	26.82**
Conjunctive element (%)	13.49	13.65	11.38
Number of cells (n)	183	192	226
Cell nucleus diameter	4.76	4.79	23.81***
Hepatic cell diameter (µm)	13.53	13.90	13.61
ANOVA with the Tukey post-test: *p<0.05 Sham <i>versus</i> HO; **p<0.05 Sham <i>versus</i> HOR; +p<0.05 HO <i>versus</i> HOR; mean ± SEM (n=6).			

Oxidative stress is reversed during caloric restriction, mediated by SIRT1, SIRT3, and NAMPT, establishing a strong correlation with lipid metabolism

As shown in Figure 4A, there was a higher expression of insulin receptor (IR) in the OHR group (14.92 ± 0.53) than in the SHAM group (10.64 ± 1.98) and OH group (7.31 ± 1.30). The expression of SIRT1 (Figure 4D) was higher in the OHR group (7.02 ± 0.88) than in the SHAM group (4.48 ± 0.91) and the OH group (3.24 ± 0.87). NAMPT expression was higher in the OHR group (12.62 ± 0.87) than in the SHAM (3.34 ± 0.32) and OH groups (2.61 ± 0.34) (Figure 4G). Both IR and SIRT1 regulate glucose metabolism and improve GTT and ITT results in the rats of the OHR group. Given that NAMPT directly regulates the activity of SIRT1, our data seem to establish metabolic hepatic regulation in OHR, consistent with that

reported in the literature [14]. The values obtained for the other proteins (Figure 4B, 4C, 4E, 4F, and 4H) were similar for all groups. Figure 4I shows that the expression of complex II was low in the OH group (5.355 ± 0.79) but was significantly higher in the OHR group (11.08 ± 1.53). Given the importance of mitochondrial complex II [2, 13] in the maintenance of cellular integrity, our results indicate the presence of mitochondrial modifications due to diet-related effects by preserving the integrity of OXPHOS in the livers of obese/ hypertensive/calorie-restricted animals.

Shown in Figures 4J, 4K, and 4L are the correlation data. The degree of significance is represented by the thickness of the lines shown. The Spearman test was performed and P values were used to classify weak ($r \leq 0.35$, $P < 0.05$), moderate ($r = 0.36-0.67$, $P < 0.01$), or strong ($r \geq 0.68$, $P < 0.001$) correlations. The absence of a line indicates the absence of a relationship. To assess the relationship between the markers SIRT1, SIRT3, NNMT, NAMPT, IR, pAKT/AKT, PCG-1 α , FOXO, pAMPK/AMPK, OXPHOS, Body Weight (BW), Blood Pressure (BP), Cholesterol, HDL, LDL, VLDL, Triglycerides, Insulin, Glucose, K_{itt} , GTT, and IDE, a series of correlation analyses was performed (Figure 4J, 4K, 4L). When comparing the interactions between the SHAM group profiles, OH, and OHR. In the SHAM group (Figure 4J), we observed that interactions occurred mainly between SIRT1 and pAKT/AKT (moderate), SIRT1 and OXPHOS C.II (moderate), OXPHOS C.II, and HDL and cholesterol between pAMPK/AMPK. LDL and K_{itt} have moderate interactions, similar to VLDL and triglycerides. In the OH group (Figure 4K), SIRT1 and SIRT3 did not interact with the other studied markers. NNMT was moderately positively correlated to triglycerides, VLDL, and IR. NAMPT was also moderately positively correlated with triglycerides and VLDL. pAKT/AKT had a moderately positive correlation with OXPHOS C.I, as well as FOXO, HDL, and OXPHOS C.III. Insulin and blood pressure correlated positively and moderately with insulin, and glucose had a moderately positive correlation with K_{itt} , as expected. Also as expected, we detected a moderately positive correlation between VLDL and triglycerides. In the animals of the OHR group (Figure 4L), there was a moderate interaction between SIRT1 and IDE as well as between SIRT3 and insulin. NNMT correlated moderately and positively with HDL, but only moderately with IR and pAKT/AKT; NAMPT interacted moderately and positively with PGC-1 α ; pAMPK/AMPK had a moderately positive correlation with VLDL and OXPHOS C.I, but was negatively correlated with cholesterol. C.II correlated negatively with blood pressure; however, C. III had a positive correlation of the same strength with K_{itt} , as well as C.IV and its correlation with body weight. Cholesterol had a moderately positive correlation with K_{itt} and IDE. Finally, GTT was negatively correlated with glucose.

Discussion

Glucose and lipid homeostasis requires the continuous control of blood sugar levels and body weight to minimize the risk of metabolic dysfunctions. Thus, the pancreatic islets and the liver are critical tissues involved in this control. Pancreatic islets release insulin, which stimulates the entry of glucose into the skeletal muscles, liver, and adipose tissue via special transporters, thus controlling glucose homeostasis. The removal of insulin from the system is dependent on the levels of IDE present in various tissues, predominantly in the liver [34].

Rats in the OH group became overweight, developed hypertension and pre-diabetes, as shown by their glucose intolerance, reduction in glycogen stores, peripheral insulin resistance, mild hyperglycemia and hyperinsulinemia, increased hepatic IDE expression, and increased pancreatic collagen areas. Islet inflammation in type 2 diabetes was likely the cause of fibrosis and collagen deposition [6]. Feeding with a HFD was previously reported to increase the number of macrophages in the islets of non-human primates [25]. In fact, the injured islets of the rats fed with a HFD showed similar fibrosis, suggesting a possible link between pre-diabetic features. The absence of glucose homeostasis in the OH animals, together with an increase in their hepatic IDE levels, was suggestive of an imbalance in insulin clearance, which corroborates the results of our correlation study. Previously, it was found that animals subjected to a cafeteria diet [3] showed increased IDE activity in the liver, corroborating the present results [4].

The hyperlipidic diet followed by a 40% CR for four weeks reestablished the control of glucose metabolism by preventing pancreatic fibrosis, ensuring insulin sensitivity, and reducing hepatic IDE activity, while simultaneously reducing arterial blood pressure [17]. The blood pressure recovery observed in the OHR group was in agreement with the literature showing that the administration of NAD⁺ in mice or primary cardiomyocytes exposed to hypertrophic agonists led to an improvement of the hypertrophic phenotype [27, 28]. CR has been shown to reverse endothelial alterations in a diet-induced obesity model that reduced vascular oxidative stress in Otsuka Long Evans Tokushima Fatty (OLETF) rats and reduced arterial blood pressure in spontaneously hypertensive rats [15]. In contrast to the increased liver lipids after one month of caloric restriction can be explained by the restriction time. This is due to the fact that 40% caloric restriction for 12 weeks in db / db mice reversed insulin resistance, hepatic steatosis, body weight and adiposity [16].

In addition, as previously reported, reduced mRNA expression of IDE as compared to OH could explain the reduced serum insulin levels in the OHR group [33]. This mechanism increased peripheral insulin sensitivity through increased expression of IR in the liver, suggesting that it was associated with decreased insulin clearance. In addition, we observed an increase in IDE levels in the OHR group compared to that in the SHAM group, which was associated with increased protein expression of IR, NAMPT, SIRT1, and reduced levels of circulating cholesterol and triglycerides, suggesting a mechanism of compensation of circulating glucose and insulin clearance in the OHR group. The pre-diabetic features observed in the OH group described above may interfere with insulin clearance, as previously reported [33].

Our findings suggest that the hepatic mechanism for the physiological changes in the OHR group increased the expression of the hepatic proteins IR and NAMPT, with a subsequent increase in the levels of SIRT1 and complex II of the electron transport chain, as well as the mitochondrial adaptation of OXPHOS. In the OHR group, CR led to the activation of SIRT1 by NAMPT, as reported previously [5]. In addition, after a partial withdrawal of food, the SIRT1 protein binds to and represses the genes responsible for fat storage, and its overexpression attenuates adipogenesis ultimately leading to lipolysis [26]. Due to food restrictions, it is likely that fat provides an energy source for the maintenance of cellular metabolism. SIRT1 provides protection against diseases related to insulin resistance, such as metabolic

syndrome and diabetes mellitus [20, 30]. In mice, the knockout of NAMPT resulted in lethality in homozygotes, while heterozygotes showed glucose intolerance [29]. Other studies found that NAMPT mRNA expression levels were decreased in the livers of obese mice fed a diet rich in fat [36]. In contrast, fasting was found to increase hepatic NAMPT levels, NAD⁺ biosynthesis, and hepatic metabolic function, leading to a reduced accumulation of triglycerides [35, 36]. In contrast, transgenic mice with enzymatically inactivated NAMPT presented increased accumulation of triglycerides, insulin resistance, and a phenotype of non-alcoholic fatty liver disease [32].

An important finding in this study was that levels of the NNMT protein were similar among the groups (SHAM, OH, and OHR), since this protein can inhibit SIRT1 [19]. However, a previous study suggested that increased NNMT expression could stabilize the SIRT1 protein, resulting in metabolic benefits [14]. Knockdown of NNMT in white adipose tissue and the liver can protect against diet-induced obesity [19]. Modulation of complex II in the animals of the OH and OHR groups was suggestive of mitochondrial adaptation as a result of the imposed diets [24]. Levels of the Akt, AMPK, PGC1 α , and FoxO3 proteins showed no alterations in the groups studied, despite the benefits reported in the literature [23].

In addition, in our correlation study, the OHR group showed the strong interaction of IR with AKT, NAMPT, PGC1, K_{itt}, lipid profile, and OXPHOS C.III. In addition, there was a correlation between OXPHOS C.I and VLDL, and body weight with OXPHOS C.IV, corroborating our suggestion that caloric restriction recovers the pre-diabetic metabolic profile of hypertensive animals fed a fat-rich diet. It is possible that if the CR protocol was modified, such as with a longer duration and/or greater restriction, the expression of these proteins might be increased, thereby affecting metabolism. Studies concerning CR vary considerably in terms of duration, animal age, and dietary composition, but a commonly used protocol is a 40% restricted diet (moderate CR), compared to the control, for four weeks [10].

Conclusion

It could be concluded that short-term CR provided benefits in terms of glucose and lipid homeostasis, protecting the pancreatic islets and hepatic cells against fibrosis, as well as improving arterial blood pressure in obese and hypertensive animals. Although our rodent models of obese/hypertension do not always perfectly reproduce the human pathology of obesity and hypertension, it has helped better understand the cellular, metabolic, and molecular pathways of lipid and glucose homeostasis. The critical proteins activated by CR, such as NAMPT, SIRT1, and OXPHOS, are involved in many metabolic defects associated with obesity and hypertension. Therefore, a better understanding of the regulation of proteins that control the effects of CR may assist in the development of therapeutic approaches for this metabolic disease in the future.

Declarations

Author's contributions

MECA, CAO, and BFT conceived and performed the experimental design of the project. CAO performed renal artery stenosis to induce hypertension. VEP, MCB, JVH, and NTH performed dietary interventions and observations throughout the study period. VEP, MCB, JVH, NTH, and BFT performed the processing and histological analyses. VEP and MSSNF performed the biochemical tests. CAO, VEP, MSSNF, and MECA performed the molecular assays. PVSN and VEP performed the study of correlations and created the figures. MECA, VEP, and MSSNF have edited the final manuscript. All authors read and approved the final version of the manuscript.

The authors declare that all data were generated in-house, and no paper mill was used.

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Conflicts of interest

The authors declare that this research was performed without any conflicts of interest or commercial or financial gains.

Additional information

Available in the Supplementary Appendix

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Figures

Figure 1

Establishing hypertension and caloric restriction reversal of obesity. (A) Timeline to show application of diets in three rat groups. One group consumed a standard rodent diet (SHAM, normalipidic diet). The second group, OH, hypertensive and consumed a high-fat-diet (HFD), and third group, OHR, received an HFD and then 60% of the HFD food, corresponding to a moderate caloric restriction of 40% of the HFD. Graphs showing in (B) Body Weight, (C) Blood pressure, (D) Ingestion, (E) Fasting blood glucose, (F) Lee index, (G) time course of intraperitoneal glucose tolerance test (ipGTT) following 14 weeks of high-fat

diet feeding, after CR and area under curve of ipGTT, (H) time course of intraperitoneal insulin tolerance test (ipITT) following 14 weeks of high-fat diet feeding, after CR and KITT for ITT. Data expressed for SHAM, OH, and OHR groups. The bars represent the mean $\pm$ SEM (n=5), $p < 0.05$ indicates a significant difference based on one-way ANOVA, Tukey post hoc test.

Figure 2

Serum biochemical parameters and tissue characteristics of the SHAM, OH and OHR animal groups. (A) Cholesterol, (B) HDL, (C) LDL, (D) VLDL, (E) Triglycerides, (F) Hepatic Glycogen, (G) Muscle glycogen, (H) Hepatic cholesterol, (I) Hepatic triglycerides, (J) Liver IDE mRNA. The bars represent the mean $\pm$ SEM (n=5), $p < 0.05$ indicates a significant difference based on one-way ANOVA, Tukey post hoc test.

Figure 3

Photomicrographs, histological analyzes of liver and pancreas of animals of the SHAM, OH and OHR animal groups. (A) Representative images from liver and pancreas it can be seen that the slides stained by the H&E, Perls and Trichrome Gomori technique; (B, C) aspartate aminotransferase, alanine aminotransferase levels; (D) liver lipid drops; (E) insulin levels; (F) determination of pancreatic islet size; (G) presence of hemosiderin and (H) presence of collagen using the H&E, Perls, and Gomori's trichrome staining techniques, respectively. Magnification: 40x. The bars represent the mean $\pm$ SEM ($n=5$), $p<0.05$ indicates a significant difference based on one-way ANOVA, Tukey post hoc test.

Figure 4

Protein expression in the liver tissues of animals of the SHAM, OH, and OHR groups. Hepatic: (A) IR; (B) pAkt/Akt; (C) PGC1 α ; (D) SIRT1; (E) SIRT3; (F) Foxo; (G) Nampt; (H) NNMT; (I) OXPHOS. The representation of the bands is given next to the graphic. In right bands of expression of proteins, in left bands of the bands of proteins of complexes V, III, IV, and II of the electron transport chain. The bars represent the mean $\pm$ SEM (n=4), $p < 0.05$ indicates a significant difference based on one-way ANOVA, Tukey post hoc test. An interaction network between body, biochemical and protein parameters of interest in the (J) SHAM groups; (K) OH; (L) OHR. Network layouts for these personalized markers were configured to identify a relevant association between different groups of animals with or without dietary intervention. Each connection line is an express correlation between a pair of markers. Continuous lines represent positive correlations, while dashed lines represent negative correlations ($p < 0.05$). More information in the Supplementary Data Values in arbitrary units.

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