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Hyperphagia and hyperleptinemia induced by low-protein, high-carbohydrate diet is reversed at a later stage of development in rats

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Abstract: This study investigated whether increased food intake after 15 days of low-protein, high-carbohydrate (LPHC) and its normalization in the later period of development change the content of key proteins related to leptin or adiponectin signaling in the hypothalamus. Male rats were divided into five groups: Control groups received a control diet (17% protein, 63% carbohydrate) for 15 (C_{15}) or 45 (C_{45}) days; LPHC groups received an LPHC diet (6% protein, 74% carbohydrate) for 15 ($LPHC_{15}$) or 45 ($LPHC_{45}$) days; and Reverse group (R): received LPHC diet for 15 days followed by control diet for another 30 days. The $LPHC_{15}$ group showed increased adiposity index, leptin level, and adiponectin level, as well as decreased the leptin receptor (ObRb) and pro-opiomelanocortin (POMC) content in the hypothalamus compared with the C_{15} group. LPHC diet for 45 days or diet reversion (R group) rescued these alterations, except the adiponectin level in $LPHC_{45}$ rats, which was higher. In summary, LPHC diet reduced hypothalamic leptin action by diminishing ObRb and POMC levels, leading to hyperphagia and adiposity body. Medium-term administration of LPHC diet or reverting to control diet restored the levels of these proteins, thereby improving body lipid mass rearrangement in adulthood.

Key words: adiponectin, hypothalamus, low-protein high-carbohydrate diet, leptin.

INTRODUCTION

Living organisms have fuel storage mechanisms because an adequate supply of nutrients is essential for the maintenance of life and health. Consuming more calories than is required leads to the storage of energy as triacylglycerol (TAG) in white adipose tissues for later use. Uninterrupted ingestion of food, which is associated with sedentary life, has increased the prevalence of obesity worldwide in the last several decades (Ford et al. 2004, Fisberg et al. 2016). High content of body lipids has also been associated with low consumption of protein (Meyers & Hargus 1959, White et al. 2000a), and when the protein level does not deviate far from the minimum required amount, the elevation in lipid content is related to increased food intake (Peng et al. 1974, White et al. 1994, 2000b, Du et al. 2000, Aparecida de França et al. 2009).

Food intake is important for the maintenance of energy homeostasis. This physiologic function in higher animals is regulated by a complex system involving the neuro-endocrine system. Several brain areas participate in this process. The arcuate nucleus (ARC) in the hypothalamus is the primary site that transduces peripheral

clues into neural responses and regulates food intake and body weight (Timper & Brüning 2017). Leptin is one of the peripheral signals acting on hypothalamic neurons, and its level in plasma is proportional to body fat content (Kwon et al. 2016). The ARC contains a population of leptin-sensitive neurons, which express orexigenic peptides such as neuropeptide Y (NPY) and agouti-related protein (AgRP). Another population of neurons express peptides such as pro-opiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART), which suppress food intake. The binding of leptin to its receptor in neurons of the ARC stimulates POMC/CART neurons and inhibits NPY/AgRP neurons (Lanfray & Richard 2017)

Studies conducted in our laboratories with rats in the growing phase subjected to low-protein, high-carbohydrate diet (LPHC) for 15 days showed an increase in food intake associated with an increase in the absolute and/or relative body lipid content compared with rats treated with a balanced diet. The LPHC diet also induced a 100% increase in serum leptin and a decrease in postprandial insulin level (Aparecida de França et al. 2009). We have also investigated the profile of rats subjected to the LPHC diet for 45 days, and of rats provided with the LPHC diet for 15 days followed by a balanced diet for 30 days (Ceolin et al. 2019). The rats treated with LPHC diet for 45 days exhibited increases in food intake, body lipid content, and serum adiponectin levels. However, the leptin and fed insulin levels of these rats were similar to those of control diet-fed rats (Ceolin et al. 2019). Rats that had the LPHC diet replaced with a control diet showed hyperinsulinemia and increased food intake during the 45 days of treatment; however, their serum leptin and adiponectin levels were similar to those of rats fed a balanced diet for 45 days (Ceolin et al. 2019).

Studies showed that overfeeding in the early phases of life can lead to hyperinsulinism and interfere with the programming of the neuroendocrine system (Plagemann 2006, Bouret 2009, Reynolds et al. 2017). Acquired insulin and leptin resistance have also been suggested in these cases (Habbout et al. 2013). Elevated serum triiodothyronine or adiponectin can stimulate the AMP-Activated Protein Kinase (AMPK) pathway in the hypothalamus, inducing similar programming with an increase in food intake. AMPK can also mediate the fatty acid oxidation induced by leptin and adiponectin in skeletal muscles (Stark et al. 2013, Stern et al. 2016). Moreover, reduced AMPK activity in the hypothalamus can lead to reduced food intake and enhanced energy expenditure (López 2018).

In the condition of overfeeding during the early phases of development, instead of a leptin deficiency, high circulating levels of the hormone are observed, which is associated with a loss of leptin responsiveness (Stefanidis & Spencer 2012, Castro et al. 2015). Based on these data and considering the results of our previous studies in rats fed an LPHC diet, we hypothesized that the alterations in food intake, which are associated with high body lipid accumulation, may be related with changes in the action of leptin, adiponectin, or both in the hypothalamus. Thus, our objective in the present study was to test this hypothesis. For this, we assessed the effects of LPHC diet for 15 and 45 days on plasma leptin and adiponectin hormone levels, white adipose tissue weight, and hypothalamic protein levels involved in the leptin and AMPK signaling pathways. Additionally, we investigated whether 15 days of LPHC diet followed by its replacement with a balanced diet in the later period of development can significantly modulate food intake and adiposity by altering the expression of proteins involved in the action of leptin and/or adiponectin in the hypothalamus.

MATERIALS AND METHODS

Animals and diets

The rats were treated with two types of diet: control (C) diet containing 17% of protein, 63% carbohydrate, and 7% lipid (AIN-93G) (Reeves et al. 1993); and LPHC diet containing 6% of protein, 74% carbohydrate, and 7% lipid. The diet composition details have been published previously (Aparecida de França et al. 2009). The amount of calories corresponding to the protein withdrawn from the LPHC diet was compensated by adding the same amount of calories in the form of carbohydrates. Thus, the diets were kept isocaloric (16.3 kJ g^{-1}) (Aparecida de França et al. 2009, Santos et al. 2012, Buzelle et al. 2010, Menezes et al. 2013, Pereira et al. 2017, Silva et al. 2018). Male Wistar rats aged 30 days and weighing 100 g were randomly divided into five groups: (i) C_{15}, rats fed the C diet for 15 days; (ii) $LPHC_{15}$, rats fed the LPHC diet for 15 days; (iii) C_{45}, rats fed the C diet for 45 days; (iv) $LPHC_{45}$, rats fed the LPHC diet for 45 days; (v) R, rats fed the LPHC diet for 15 days, followed by replacement with the C diet for the subsequent 30 days.

The rats were housed in individual metabolic cages in an environmentally controlled room with lights on from 6 a.m. to 6 p.m. and a temperature of 22 ± 1°C. They received water and food *ad libitum*. The body weight and food intake of each rat were recorded daily. All experiments were performed between 08:00 and 10:00 a.m., and the rats were euthanized on the 15^{th} (C_{15} and $LPHC_{15}$ groups) or 45^{th} (C_{45}, $LPHC_{45}$, and R groups) day of treatment. The rats were housed according to the Brazilian College of Animal Experimentation Rules, and the experiments were approved by the Ethics Committee of the Federal University of Mato Grosso (protocol no. 23108.109377/2015-15).

Sample collection

After the experiment, the rats were euthanized by decapitation. Blood samples were collected, and serum was obtained by centrifugation (3,000 rpm for 10 minutes) for measurement of leptin and adiponectin concentrations. Retroperitoneal, epididymal, and perirenal white adipose tissues were removed and weighed. The hypothalamus was collected to determine the content of the leptin receptor (ObRb), proteins of the leptin signaling pathway (JAK2, p-JAK2, STAT3, p-STAT3, SOCS3), neuropeptides (NPY, POMC), AMPK, and p-AMPK via western blotting analyses.

Adiposity index

The adiposity index was determined from the sum of the weight of retroperitoneal, epididymal, and perirenal white adipose tissues. The results were expressed as percentage of the total body weight.

Hormonal analysis

Serum leptin concentration was measured using a rat leptin Elisa kit (limit of detection: 50 pg/mL of sample; Société de Pharmacologie et d'Immunologie – BIO, Montignyle Bretonneux, France). Serum adiponectin was measured using a rat adiponectin Elisa kit (limit of detection: 0.4 ng/mL of sample; R&D Systems, Minneapolis, MN, USA).

Western blotting protein analysis

Hypothalamus samples were homogenized in ice-cold lysis buffer pH 6.4 (50 mM HEPES, 1 mM $MgCl_2$, 10 mM EDTA, Triton X-100 1%, 5 µg mL^{-1}aprotinin, 1 µg mL^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium orthovanadate, 100 mM sodium fluoride, and 10 mM tetrasodium pyrophosphate). Homogenates were centrifuged at 4°C and 10,000 rpm for 5 min. Protein concentration was determined via Bradford assay (1976). Protein samples were

denatured in sample buffer (50 mM Tris·HCl pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and heated at 95°C for 5 min.

The supernatants were analyzed by the SDS-PAGE method using 8%, 10%, or 12% polyacrylamide gel, with 30 µg or 50 µg of total proteins in each slot of the gel. Next, the gel was electroblotted in a nitrocellulose membrane (Hybond P ECL membrane; Amersham Pharmacia Biotech, Sweden). The membrane was then incubated in Tris Buffered Saline (TBS) containing 5% of nonfat dry milk for 60 min to block nonspecific binding sites. Next, the membrane was washed with TBS and incubated overnight with the following primary antibodies (Santa Cruz Biotechnology, USA.): anti-Ob-R (1:200); anti-phosphorylated (p) JAK2 (1:500); anti-JAK2 (1:200); anti-STAT3 (1:500); anti-phosphorylated (p) STAT3 (1:500); anti-SOCS3 (1:500); anti-β actin (1:500); POMC (1:200); (Sigma Aldrich, USA.): NPY (1:3000); (Cell Signaling, USA.): anti-phosphorylated (p) anti-AMPK (1:1000); and AMPK (1:1000). Furthermore, the membrane was washed and incubated with the appropriate secondary antibodies conjugated to biotin (0.5% nonfat dry milk diluted in TBS; Santa Cruz Biotechnology) for 1 h at 25°C. The membrane was subsequently washed and incubated for 1 h at 25°C with streptavidin horseradish peroxidase-conjugated secondary antibody (1:5000 or 1:10000; Zymed, California). Immunoreactive proteins were visualized by horseradish peroxidase substrate (ECL-plus; Amersham Pharmacia Biotech) and then exposed to an X-ray film. Finally, the area and density of the protein bands were quantified by using the Image J Program (Wayne Rasband, National Institutes of Health, New York, NY)

The band intensity of phosphorylated JAK2, phosphorylated STAT3, and phosphorylated AMPK was normalized to that of the total JAK2, STAT3, and AMPK, respectively. For the other proteins, the band intensity was normalized to that of β-actin.

Statiscal analyses

The test results were expressed as mean ± standard error of the mean. Data were subjected to statistical analyses using the Statistics for Windows program (StatSoft, USA). Levene's test for homogeneity of variances was initially used to determine whether the data complied with the assumptions of parametric analysis of variance. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test or Student's *t*-test for independent samples. Differences were considered significant at $p>0.05$.

RESULTS

The energy intake (daily and total) of the $LPHC_{15}$ group was 28% higher than that of the C_{15} group (Table I). The $LPHC_{15}$ group showed an approximately 74% increase in body weight over 15 days, whereas the C_{15} group showed an approximately 104% increase (Table II), compared with the respective initial weights. The energy intake (daily and total) of the $LPHC_{45}$ and R groups was 22% and 11% higher, respectively, than that of the C_{45} group (Table I). Despite the increased energy intake in the $LPHC_{15}$ and $LPHC_{45}$ groups, the total protein intake (g/100g b.w.) was approximately 55% lower than that of their respective controls. The R group showed increased protein intake (g/100g b.w.) compared with the $LPHC_{45}$ group, but in the same period, the protein intake was 24% lower than that of the C_{45} group (Table I).

The daily body mass gain in rats subjected to LPHC diet ($LPHC_{15}$ and $LPHC_{45}$ group) was lower than that in their respective control groups (C_{15} and C_{45}, respectively). These differences resulted

Table I. Relative values of daily and total energy intake and total protein intake in the C_{15}, $LPHC_{15}$, C_{45}, $LPHC_{45}$, and R groups.

	Groups				
	C_{15} n=10	$LPHC_{15}$ n = 9	C_{45} n=10	$LPHC_{45}$ n= 10	R n=10
Daily energy intake (kJ/day)	173.2±4.5[a]	222.1±4.5[b]	135.0±3.3[a]	166.0±4.5[b]	150.3±3.4[c]
Total energy intake (kJ)	2627.6±83.2[a]	3334.4±77.8[b]	6076.2±147.9[a]	7433.1±205.6[b]	6763.3±151.0[c]
Total protein intake (g/100g b.w.)	27.1±0.7[a]	12.3±0.2[b]	63.3±1.4[a]	27.5±0.8[b]	47.8±1.1[c]

Data are expressed as mean ± standard error of the mean (M ± SEM), n = number of animals. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences ($p<0.05$; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R.

in the lower final body mass of the $LPHC_{15}$ (12%) and $LPHC_{45}$ (20%) groups than that of the C_{15} and C_{45} groups, respectively.

In the R group, after the LPHC diet was substituted with the C diet, the daily body mass gain increased. Moreover, after 5 days, the body mass of the R group was similar to that of the C_{45} group (Table II).

$LPHC_{15}$ rats had significantly increased adiposity index (39%) and higher retroperitoneal (27%) and epididymal (22%) white adipose tissue weight than the control rats (Table III). There was no difference in perirenal white adipose tissue weight after 15 days between the diets. The diet also did not alter white adipose tissue weight and adiposity index after 45 days (Table III).

Serum leptin level was 100% higher in the $LPHC_{15}$ group than in the C_{15} group, but similar between the R and C_{45} groups (Figure 1). Serum adiponectin was 120% higher in the $LPHC_{15}$ group than in the C_{15} group (C_{15}= 5.3 ± 0.5 µg/mL and $LPHC_{15}$= 11.9 ± 0.3 µg/mL; (n=8 rats in both groups); $p<0.0001$). Serum adiponectin levels in the $LPHC_{45}$ and R groups have been previously presented by Ceolin et al. 2019. The authors showed that plasma adiponectin concentration in $LPHC_{45}$ rats was two-fold higher than that in C_{45} and R rats, but similar between the C_{45} and R groups.

ObRb content was reduced by 40% after 15 days of treatment with the LPHC diet compared with that in the control (C_{15}) group (Figure 2a). The content of proteins in leptin signaling, namely JAK2, basal p-JAK2, STAT3, basal p-STAT3 (Figure 2), and SOCS3 (Figure 3a), in the hypothalamus was not altered in the $LPHC_{15}$ group compared with that in the C_{15} group. The diet did not alter orexigenic NPY peptide content (Figure 3b). However, POMC content was reduced by approximately 30% in (Figure 3c) after 15 days of the LPHC diet. Furthermore, both the $LPHC_{45}$ and R groups showed no alteration in the content of ObRb (Figure 2b), NPY (Figure 3b), POMC (Figure 3c), and proteins of leptin signaling (Figure 2b) compared with the control group (C_{45}). The levels of AMPK and basal p-AMPK proteins, which are involved in the regulation of food intake, were also unaltered between the groups (Figure 4a and 4b). Figure 5 shows the schematic representation of leptin signaling in the hypothalamus of rats subjected to LPHC diet for 15 days.

Table II. Initial and final body mass as well as body mass gain in the C_{15}, $LPHC_{15}$, C_{45}, $LPHC_{45}$, and R groups.

Variables	Groups				
	C_{15} (n=10)	$LPHC_{15}$ (n=9)	C_{45} (n=11)	$LPHC_{45}$ (n=10)	R (n=10)
Initial body mass (g)	93.4±3.6[a]	95.5±3.3[a]	93.9±3.3 [a]	95.8±2.9 [a]	93.6±2.0 [a]
Final body mass (g)	190.7±7.6[a]	166.2±7.6 [b]	343.3±11.0[a]	273.9±10.5[b]	338.9±6.2[a]
Body weight gain (g/day)	6.2±0.3[a]	4.6±0.5 [b]	5.5±0.2[a]	4.0±0.2[b]	5.4±0.2[a]

Data are expressed as mean ± standard error of the mean (M ± SEM), n = number of animals. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences (p<0.05; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R.

Table III. White adipose tissue weight and adiposity index of the C_{15}, $LPHC_{15}$, C_{45}, $LPHC_{45}$, and R groups.

Variables	Groups				
	C_{15} (n=10)	$LPHC_{15}$ (n=10)	C_{45} (n=9)	$LPHC_{45}$ (n=9)	R (n=8)
Retroperitoneal WAT (g)	1.70±0.14[a]	2.15±0.13[b]	7.53±0.55 [a]	7.03±0.48 [a]	7.33±0.57 [a]
Epididymal WAT (g)	1.91±0.12[a]	2.32±0.15 [b]	7.31±0.56 [a]	7.14±0.46 [a]	7.77±0.78 [a]
Perirenal WAT (g)	0.32±0.05[a]	0.34±0.04[a]	1.43±0.16 [a]	1.21±0.13 [a]	1.32±0.12 [a]
Adiposity index (%)	2.04±0.09[a]	2.84±0.12[b]	4.89±0.39 [a]	5.76±0.45 [a]	4.90±0.46 [a]

Data are expressed as mean ± standard error of the mean (M ± SEM), n = number of animals. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences (p<0.05; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R.

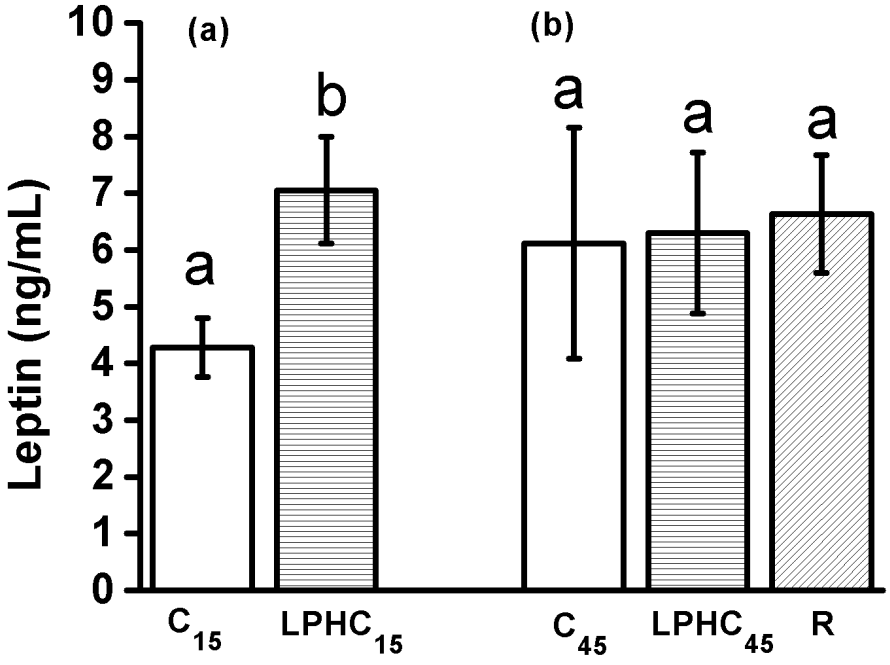

Figure 1. Serum leptin of (a) the C_{15} and $LPHC_{15}$ groups as well as (b) the C_{45}, $LPHC_{45}$, and R groups. Data are expressed as mean ± standard error of the mean (M ± SEM) of 7-10 rats per group. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences (p<0.05; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R.

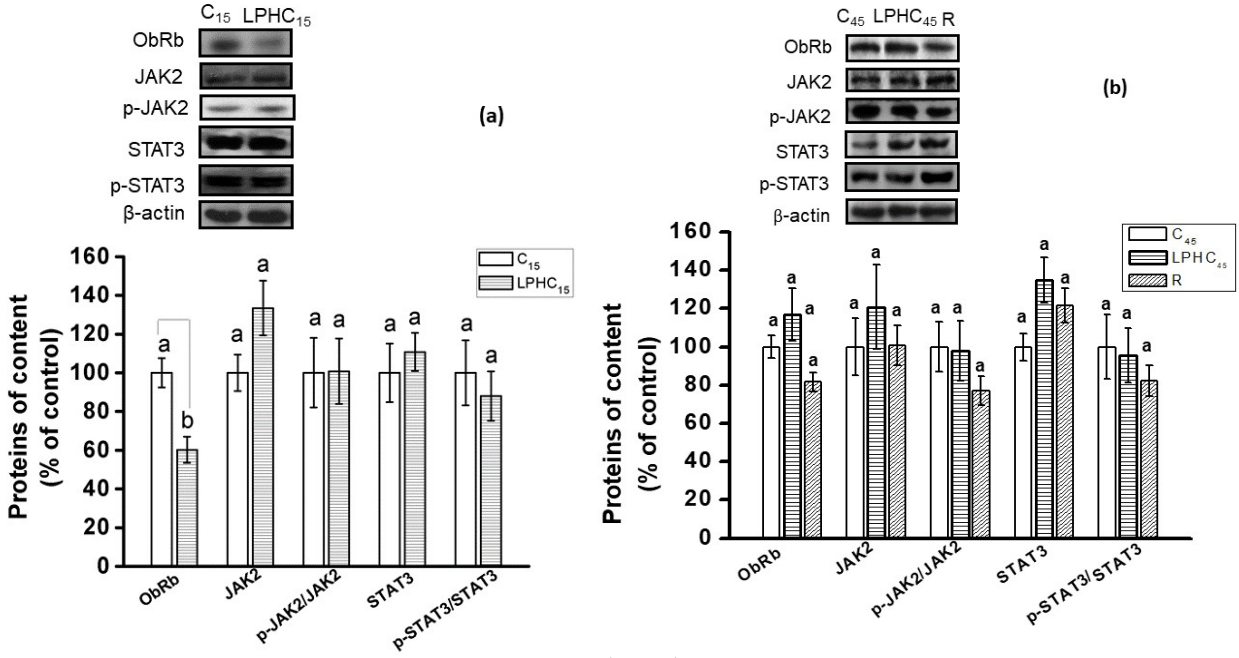

Figure 2. Content of the long form of the leptin receptor (ObRb), JAK2, p-JAK2/JAK2, STAT3, and p-STAT3/STAT3 in the hypothalamus of the C_{15} and $LPHC_{15}$ groups (a) as well as the C_{45}, $LPHC_{45}$, and R groups (b). Data are expressed as mean ± standard error of the mean (M ± SEM) of 6-8 rats per group. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences ($p<0.05$; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R.

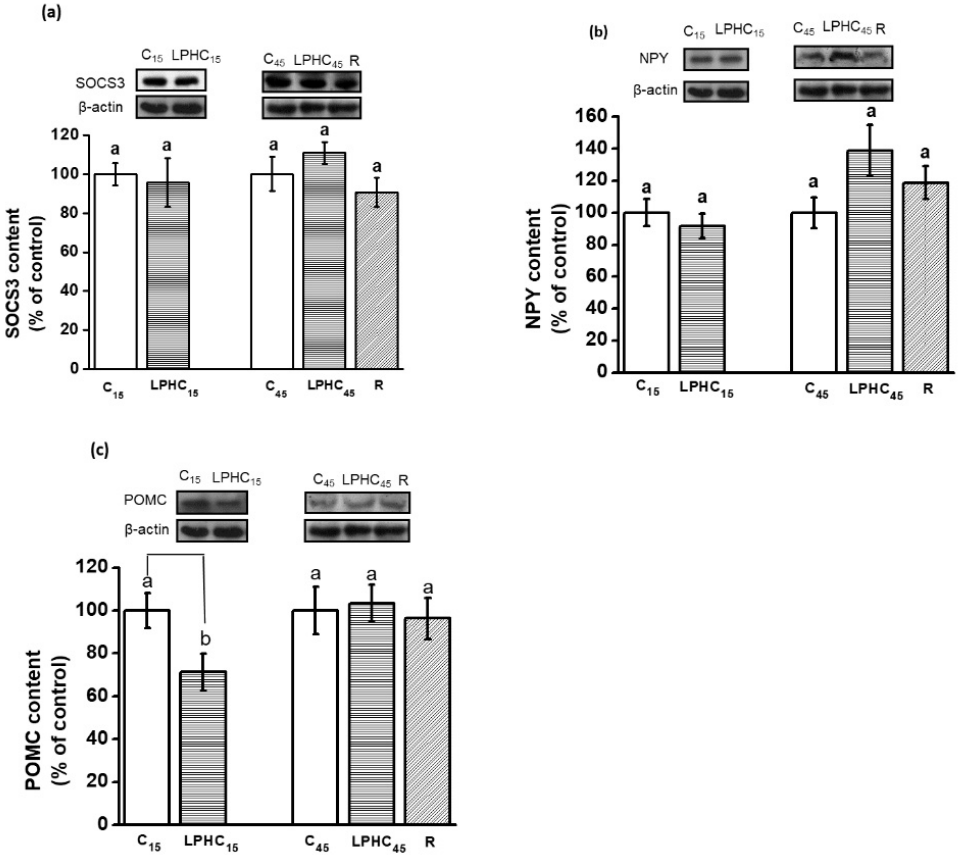

Figure 3. Content of SOCS3 (a), NPY (b), and POMC (c) in the hypothalamus of the C_{15} and $LPHC_{15}$ as well as the C_{45}, $LPHC_{45}$, and R groups. Data are expressed as mean ± standard error of the mean (M ± SEM) of 6-8 rats per group. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment. Different letters represent significant differences ($p<0.05$; C_{15} vs $LPHC_{15}$; C_{45} vs $LPHC_{45}$ vs R).

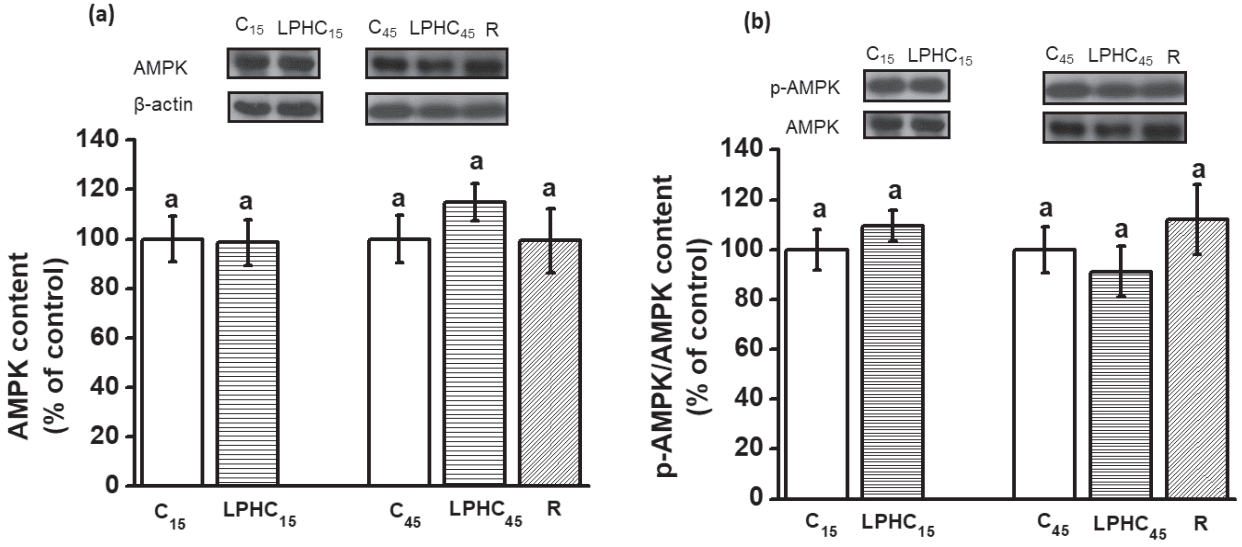

Figure 4. AMPK (a) and p-AMPK/AMPK (b) content in the hypothalamus of the C_{15} and $LPHC_{15}$ groups as well as the C_{45}, $LPHC_{45}$, and R groups. Data are expressed as mean ± standard error of the mean (M ± SEM) of 6-8 rats per group. One-way ANOVA test for 45 days of treatment and Student's *t*-test for 15 days of treatment.

DISCUSSION

Previous studies by our group have indicated that after the administration of an LPHC diet for 15 days ($LPHC_{15}$ group), rats showed higher serum leptin concentrations and absolute and relative food intake and energy intake than the control rats (C_{15}). Despite the increase in food and energy intake, $LPHC_{15}$ rats had decreased final body weight (Aparecida de França et al. 2009). In the present work we also observed the same results in the $LPHC_{15}$. However, the $LPHC_{15}$ group showed increase (74% in body weight over 15 days), when compared with the respective initial weight. Despite, this a reduction in daily body mass gain (12%) was observed in $LPHC_{15}$ group. We believe that, although the energy intake is greater in the $LPHC_{15}$ group when compared with the C_{15} group, the excess calories ingested are dissipated as heat. This was evidenced previously by the increase in mass and sympathetic flow in the brown adipose tissue (Aparecida de França et al. 2009) as well as the increase in the tissue temperature (Aparecida de França et al. 2014). In addition, our results previously also showed a reduction in the tissue protein synthesis both in the soleus (Batistela et al. 2014) and extensor digitorum longus muscles (Dos Santos et al. 2016). All of these factors together contribute to the reduction in body mass gain.

We also observed that rats subjected to medium-term LPHC diet ($LPHC_{45}$ group) and rats treated with LPHC diet for 15 days followed by control diet for 30 days (R group) showed higher total relative food intake than control (C_{45}) rats (Ceolin et al. 2019).

In the present study, we confirmed hyperleptinemia in $LPHC_{15}$ rats. Additionally, $LPHC_{15}$ rats exhibited higher adiposity index than C_{15} rats. There is an evidence that hyperleptinemia and leptin resistance are directly associated with elevated adiposity and food intake (Maffei et al. 1995, Silva-Bertani et al. 2020, Gruber et al. 2021). Thus, it is possible that the hyperphagia observed in $LPHC_{15}$ rats was attributed to dysfunctional leptin signaling or leptin resistance in the hypothalamus, and not to leptin deficiency. To assess this possibility, we investigated the content of proteins related

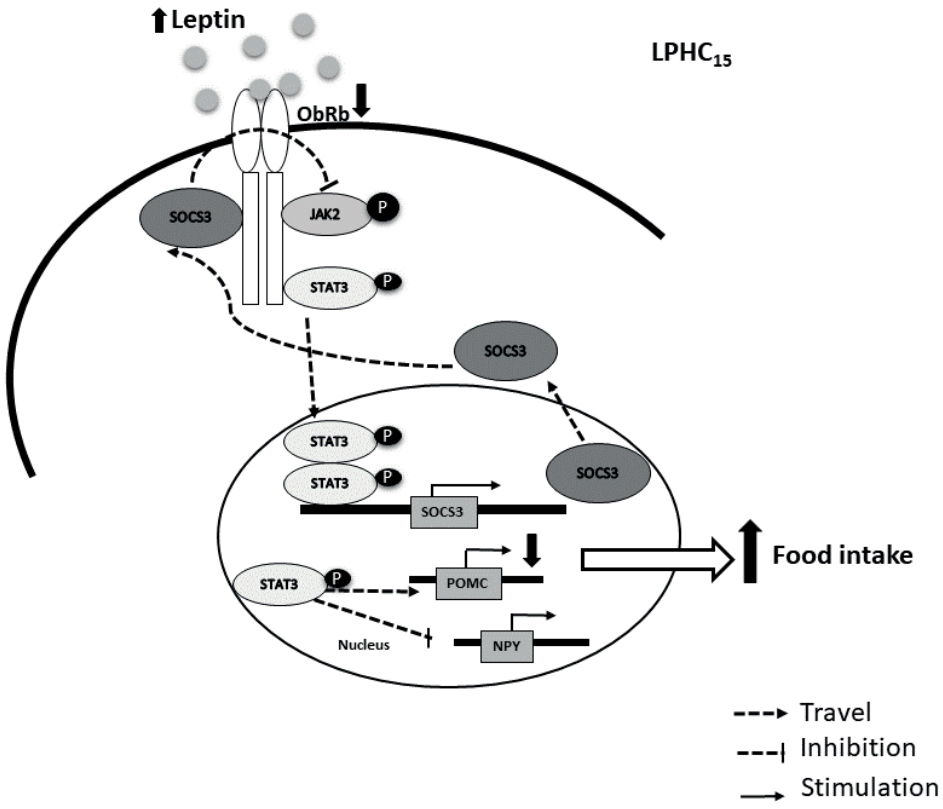

Figure 5. Schematic representation of leptin signaling in the hypothalamus of rats subjected to LPHC diet for 15 days.

to leptin signaling in the hypothalamus in basal conditions.

We observed a reduction in ObRb content in $LPHC_{15}$ rats, which suggested a loss of leptin responsiveness in the hypothalamus. High fat-fed rats also showed leptin resistance with low ObRb content. In these rats, these effects appeared to be post-transcriptional, as equivalent changes were not observed in the expression of the long and short form mRNAs of the leptin receptor (Madiehe et al. 2000).

Leptin in the hypothalamus stimulates neurons that express anorexigenic peptides and inhibits orexigenic neurons. We measured the content of the orexigenic peptide NPY and anorexigenic peptide POMC in the hypothalamus of $LPHC_{15}$ rats. NPY peptide content was not different between the $LPHC_{15}$ and C_{15} groups, but the content of POMC, which is a target for the programming of obesity (Stevens et al. 2011), was lower. There is also an evidence that maternal undernutrition can lead to epigenetic changes in the gene promoter of POMC in the fetus (Stevens et al. 2011). Studies in rodents have shown that hypothalamic programming can occur in both fetal and early postnatal lives (Coupé et al. 2010). In our experimental model, the LPHC diet was introduced when the rats were approximately 1 month old (after weaning). DNA methylation or histone modification by acetylation or methylation (Ho & Tang 2007) are epigenetic modifications that transcriptionally silence or activate genes, thereby altering the structure of chromatin. DNA methylation is associated with gene silencing, and its effects are thought to be permanent. The obese human phenotype is associated with POMC gene methylation and reducted POMC expression, resulting in elevated food intake. Thus, the results of the present study suggested that the reduction in POMC concentration in the $LPHC_{15}$ group may be caused by impairment of leptin signaling, which

negatively modulated hypothalamic POMC gene expression.

We also determined whether medium-term LPHC diet ($LPHC_{45}$ group) or replacement of the 15-day LPHC diet with control diet for 30 days (R group) can reverse this dysfunction and normalize the content of proteins involved in leptin signaling in the hypothalamus. The levels of leptin in the serum as well as ObRb and POMC in the hypothalamus of the $LPHC_{45}$ and R groups were similar to those of the C_{45} group, indicating that the loss leptin responsivity induced by 15 days of LPHC diet was not maintained until the 45th day of this diet nor after reverting to a balanced diet (R group). Despite this improvement of leptin sensitivity, the $LPHC_{45}$ and R groups did not display a significant reduction in food intake. However, the white adipose tissue mass and adiposity index of R and $LPHC_{45}$ rats were similar to those of C_{45} rats. Therefore, this enhancement of leptin sensitivity induced by the LPHC diet may improve the body lipid mass rearrangement which, in turn, can improve obesity outcomes.

Intriguingly, the R group did not show increased body mass gain after the diet was changed (Table II), despite the lower duration of the control diet treatment. This fact showed that the limited increase in body mass in the LPHC group was not caused by calory intake but was certainly caused by the low protein content of the diet.

To evaluate the possible participation of adiponectin in the normalization of the relative food intake and POMC content in adult rats ($LPHC_{45}$ and R groups), AMPK and p-AMPK content in the hypothalamus was determined. Adiponectin stimulates food intake by activating AMPK (Kubota et al. 2007). Serum adiponectin concentration in the R group was the same as that in the C_{45} group on the 45th day (Ceolin et al. 2019), and the relative daily food intake was the same since the 20th day of treatment, as noted previously by Ceolin et al. (2019). However, adiponectin concentration in the LPHC group was higher than that in the C_{45} and R groups (Ceolin et al. 2019). Adiponectin levels and relative food intake in the $LPHC_{15}$ group were also higher than those in the C_{15} group. In the R group, which showed similar adiponectin concentrations to those of the C_{45} group, the daily relative food intake decreased more rapidly than C_{45} group (since the 20th day of treatment) (Ceolin et al. 2019). We believe that in $LPHC_{45}$ rats, despite the high adiponectin concentration, adiponectin signaling in the hypothalamus was not altered because there were no changes in AMPK, p-AMPK, and p-STAT3 levels between the groups. Therefore, the increased food intake in the $LPHC_{45}$ group was not due to a loss in the hypothalamic signaling of leptin or adiponectin (considering that the content of key proteins of both signaling pathways was preserved), but occurred as an attempt to fulfill the protein requirement of the animals. We believe that high adiponectin concentration is an adaptation process contributing to the restoration of adiposity index when the central leptin sensibility was normalized in $LPHC_{45}$ rats. However, further experiments are required to elucidate the exact role of adiponectin in this process.

In conclusion, 15 days of LPHC diet treatment induced alterations in content of ObRb and POMC in the hypothalamus, causing hyperphagia, hyperleptinemia, and adiposity body. Treatment with medium-term LPHC diet or diet replacement with control diet normalized the hypothalamic leptin action and restored ObRb and POMC content, thus improving body lipid mass rearrangement in adulthood.

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