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ORIGINAL ARTICLE

# A Low-Protein, High-Carbohydrate Diet Stimulates Thermogenesis in the Brown Adipose Tissue of Rats via ATF-2

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**Abstract** The aim of this study was to evaluate thermogenesis in the interscapular brown adipose tissue (IBAT) of rats submitted to low-protein, high-carbohydrate (LPHC) diet and the involvement of adrenergic stimulation in this process. Male rats (~100 g) were submitted to LPHC (6 %-protein; 74 %-carbohydrate) or control (C; 17 %-protein; 63 %-carbohydrate) isocaloric diets for 15 days. The IBAT temperature was evaluated in the rats before and after the administration of noradrenaline (NA) (20 μg 100 g b $w^{-1}$ $min^{-1}$). The expression levels of uncoupling protein 1 (UCP1) and other proteins involved in the regulation of UCP1 expression were determined by Western blot (Student's *t* test, $P \leq 0.05$). The LPHC diet promoted a 1.1 °C increase in the basal temperature of IBAT when compared with the basal temperature in the IBAT of the C group. NA administration promoted a 0.3 °C increase in basal temperature in the IBAT of the C rats and a 0.5 °C increase in the IBAT of the LPHC group. The level of UCP1 increased 60 % in the IBAT of LPHC-fed rats, and among the proteins involved in its expression, such as β3-AR and α1-AR, there was a 40 % increase in the levels of p38-MAPK and a 30 % decrease in CREB when compared to the C rats. The higher sympathetic flux to IBAT, which is a consequence of the administration of the LPHC diet to rats, activates thermogenesis and increases the expression of UCP1 in the tissue. Our results suggest that the increase in UCP1 content may occur via p38 MAPK and ATF2.

**Keywords** Brown adipose tissue · Thermogenesis · Low-protein, High-carbohydrate diet · Rats

## Introduction

Brown adipose tissue (BAT) was initially recognized as the site of non-shivering thermogenesis associated with the adaptation of homeothermic animals to cold conditions [1, 2]. Subsequently, its role in the thermogenesis induced by diet (diet-induced thermogenesis) has also been demonstrated [3–5], as it is an important component of energy balance, particularly in small rodents. Studies have shown that the impairment in the thermogenesis in BAT is an important factor in the etiology of obesity in animal models [6]. Recently, with the discovery of metabolically active BAT in adult humans [7–10] and the possibility of a relationship between BAT and human obesity, there has been a growing interest in the elucidation of the mechanisms of the activation of this tissue [11].

Brown adipocytes are cells with multilocular lipid deposits and a great number of mitochondria, and uncoupling protein 1 (UCP1) is present in the inner mitochondrial membrane. When activated, UCP1 allows the flow of protons across the inner membrane without passing

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through ATP synthase, dissipating the energy of the proton gradient as heat [12]. Thermogenesis is mainly activated by the sympathetic nervous system in which noradrenaline (NA) released by free nerve endings interacts with β adrenergic receptors (AR). β3-AR is associated with the activation of different signaling pathways in IBAT, including thermogenesis [13]. This receptor, which is coupled to a G-protein, activates adenylate cyclase, increasing the level of cyclic AMP (cAMP) in adipocytes. This amplifies the signal for protein kinase A (PKA), which releases its catalytic sub-unit to phosphorylate target proteins. The activation of PKA leads to increased lipolysis, releasing free fatty acids (FFA) [14]. These FFA are transported into the mitochondrial matrix for beta-oxidation. FFA can also act as activators of UCP1. PKA can also increase the uncoupling capacity of the tissue by activating the cAMP response element-binding protein (CREB) through phosphorylation. Phosphorylated CREB binds to response elements in the promoter region of many genes, including the gene for UCP1 [15]. CREB can also be activated by adrenergic stimulation via the protein kinase C (PKC)-mediated receptor α1 [16]. When activated, CREB can also regulate deiodinase 2 (DIO-2), an enzyme for thyroid hormones [17] that is responsible for the conversion of thyroxine (T4) thyroid hormone into its bioactive form triiodothyronine (T3). T3 binds the promoter region of the UCP1 gene and thus promotes its expression [18]. The p38 mitogen-activated protein kinases (MAPK) are another class of proteins affected by PKA regulation [19]. When activated, p38 MAPK phosphorylates the transcription factor ATF-2 (activation transcription factor-2), which binds to the promoter region of the peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and transactivates the expression of UCP1. Despite the fact that numerous factors modulate the expression of UCP1 [20, 21], little is known about the molecular mechanisms involved in this regulation system.

Our laboratory has investigated the effects of a low protein, high-carbohydrate (LPHC) diet in rats [22–29] because the nutrition of children, after weaning, across the Western world is rich in carbohydrates and poor in protein content [30, 31]. The administration of this diet to young rats (soon after weaning) promotes an increase in their food and calorie intake. This diet also augments the total energy gain with an increase in the body lipid content and a reduction in the body weight when compared to rats treated with control diet. These changes were accompanied by an increase in the energy expenditure, which may have contributed to the fact that these animals present with adiposity without being considered obese. The IBAT of LPHC-fed rats have a higher weight and lipid content, and they have increased sympathetic outflow and increased lipogenesis, suggesting the activation of thermogenesis in this tissue [22, 27]. Therefore, our goal in this study was to confirm a greater thermogenic capacity of the tissue *in vivo* and to examine the involvement of adrenergic stimulation in this activation by evaluating the protein expression of the intermediate adrenergic signaling pathway in Wistar male rats that were growth stage-adapted to an LPHC diet introduced for 15 days after weaning.

## Materials and Methods

### Animals and Treatment

Male Wistar rats (4–8 animals) with an initial weight of approximately 100 g were randomly divided into two groups: the control (C) group and the LPHC group. The control rats received a diet consisting of 17 % protein, 63 % carbohydrate and 7 % fat (AIN-93G) [32], while the LPHC rats received a diet composed of 6 % protein, 74 % carbohydrate and 7 % fat for 15 days [22]. The reduction in dietary protein was counterbalanced by an increase in carbohydrates to keep the diets isocaloric (16.3 kJ $g^{-1}$) (Table 1). The rats were kept in individual metabolic cages at 22 ± 1 °C with a light-dark cycle of 12 h and water and diet *ad libitum*. The body weight and food intake of each rat were recorded daily. All of the experiments were performed between 8–10 AM, and all of the rats were euthanized on day 15 of treatment. The rats were housed according to the Brazilian College of Animal Experimentation Rules, and the Ethics Committee of the Federal University of Mato Grosso (Protocol no. 23108.041931/13-6) approved the experiments.

### Surgical Procedure and Telemetric Measurement of Temperature IBAT

Seven days before the end of the experiment, the animals were anesthetized with a mixture of ketamine and xylazine

**Table 1** The compositions (g $kg^{-1}$) of the control and low-protein, high-carbohydrate (LPHC) diets

| Ingredient | Control diet (17 %) | LPHC diet (6 %) |
|---|---|---|
| Casein (84 % protein) | 202 | 71.5 |
| Cornstarch | 397 | 480 |
| Dextrinized cornstarch | 130.5 | 159 |
| Sucrose | 100 | 121 |
| Soybean oil | 70 | 70 |
| Fiber (cellulose) | 50 | 50 |
| Mineral mix (AIN 93 G)[a] | 35 | 35 |
| Vitamin mix (AIN 93 G)[a] | 10 | 10 |
| L-Cystine | 3 | 1 |
| Choline bitartrate | 2.5 | 2.5 |

[a] For detailed composition, see Reeves *et al*. [23]

(85 and 10 mg kg b $w^{-1}$, respectively) for the insertion of the transponder below the IBAT. To check the temperature of the IBAT, the Bio Medic Data System DAS-6002 system was used through radio frequencies emitted by the sensors (IPTT-3000, Co. BMDS). The animals were anesthetized with isoflurane at the end of the 15th day of treatment. A cannula (Silastic Medical Grade tubing, no. 602-135, Dow Corning, Midland, MI) was implanted into the right jugular vein for the infusion of noradrenaline (Arterenol DL-HCl, Sigma) at a dose of 20 µg 100 g b $w^{-1}$ $min^{-1}$. Immediately after the cannulation, the temperature of IBAT was read using the mobile receiving probe (SP-6005) before and 3 min after an infusion of noradrenaline.

### Immunohistochemistry

For the histology and immunohistochemistry experiments, all tissues were fixed with formaldehyde, embedded in paraffin and cut into 3-µm sections. The sections were dewaxed, hydrated and then subjected to endogenous peroxidase blocking with 3 % hydrogen peroxide in methanol and glacial acetic acid 20 % to reduce nonspecific background. The antigen retrieval was performed by heat mediation in citrate buffer, pH 6. The samples were incubated with the primary antibody Anti-UCP1 (1:250 in PBS) for 18 h at 4 °C. An HRP system was used as the secondary antibody, and DAB (3-3′-diaminobenzidine) was the substrate chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated and stabilized with mounting medium.

### Tissue Examination and Analysis

All specimens were viewed with an Axioscope A2 with a 100× objective, and the immunohistochemical staining for UCP1 in the BAT was assessed using computer-generated analysis [33]. Tissue images were analyzed using the Zeiss-Axio vision A2 software. Special care was taken to ensure that all images were captured using the same alignment, exposure and illumination intensity settings. The relative density of the antibody staining for the UCP1 protein was established using the following protocol. The original color images were first changed to grayscale with a pixel intensity ($Z$ value) range from 0 (white) to 256 (black). The intensity range defining the immunoreactivity signal in each treatment group was established using the threshold feature in the Axion Vision software. Each histological sample was divided into 10 random fields. Each area was measured 2 times, giving a total of 20 measurements for a sample.

### Western Blot for Protein Analysis

To detect the protein expression levels of UCP1, PKA, p38 MAPK, PKC, α1, DIO-2 and α-tubulin in the total extract, samples from IBAT were collected, and the tissue samples were immediately homogenized in 100 mmol $L^{-1}$ Tris–HCl buffer, pH 7.4, containing 1 % Triton X-100, 100 mmol $L^{-1}$ sodium pyrophosphate, 100 mmol $L^{-1}$ sodium fluoride, 10 mmol $L^{-1}$ EDTA, 10 mmol $L^{-1}$ sodium orthovanadate, 2.0 mmol $L^{-1}$ phenylmethylsulfonyl fluoride and 0.1 mg $mL^{-1}$ aprotinin at 4 °C. The nuclear extract of the IBAT was obtained as previously described by Siegrist-Kaiser *et al.* [34] and was used to evaluate the expression of CREB, ATF-2, PGC1α, TRβ1 and histones. The total protein concentration was determined using the Bradford method [35]. Samples containing 100 µL of total extract or nuclear extract from each experimental group were incubated for 5 min at 80 °C with 4x concentrated Laemmli sample buffer (0.250 mmol $L^{-1}$ Tris–HCl buffer at pH 6.8, 0.5 % bromophenol blue, 50 % glycerol, 10 % sodium dodecyl sulfate and 500 mmol $L^{-1}$ dithiothreitol) (4:1, v/v). The IBAT proteins (100 µg for the detection of the total proteins and 60 µg for the detection of nuclear proteins) were separated by 10 % SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies. Specific bands were detected using the super signal west pico chemiluminescent substrate, and the band intensity was quantified using the Scion Image Program (version 4.03, Frederick, Maryland, USA). The band intensities for UCP1, PKA, p38 MAPK, PKC, α1 and DIO-2 were normalized using the α-tubulin band intensity, and the band intensities of CREB, ATF-2, PGC1α, and TRβ1 were normalized using the histone band intensity. There was no difference between the α-tubulin band intensity and the histone band intensity among the groups. The results are expressed as a relative ratio.

### Statistical Analysis

Levene's test for homogeneity of variance was used initially to determine whether the data complied with the assumptions for parametric analysis of variance. Statistical significance was analyzed using Student's $t$ test for independent samples. Statistical analyses were performed using the Statsoft software (Tulsa, OK, USA). All data were expressed as the means ± standard errors (SE) for the number of animals indicated. For the experimental procedure that assessed the IBAT temperature, the results are presented as the means ± SE of the basal temperature. The stimulated (after norepinephrine infusion) temperature and the delta ($\Delta T$) measure (the difference of the temperature obtained after stimulation from the basal temperature) are presented similarly. $P < 0.05$ was used as the criterion of significance.

### Chemicals

Nitrocellulose membranes were purchased from Amersham Bioscience (NJ, USA). The antibodies against p38 MAPK

and ATF-2 were purchased from Cell Signaling Technology (MA, USA). The antibodies against UCP1, PKA, PKC, TRβ1, DIO-2, histones and α-tubulin were purchased from Santa Cruz Biotechnology (CA, USA). The antibody against the α1- adrenergic receptor was purchased from Abcam (MA, USA), and the anti-goat IgG and anti-rabbit IgG were purchased from Invitrogen (CA, USA). All other chemicals were analytical grade and were purchased from Sigma-Aldrich (MO, USA).

## Results

Despite the relative food intake being 24 % higher in LPHC-fed rats compared to control rats, their final body weight was 25 % lower (Table 2). The IBAT weight was 32 % higher when compared to the control group. The LPHC diet also promoted an approximately 60 % increase in the content of uncoupling protein 1 (UCP1) in IBAT (Fig. 1).

The measure of the temperature in the IBAT of the two groups demonstrated that the LPHC diet caused an increase of 1.1 °C in basal temperature. The infusion of noradrenaline increased the temperature of the IBAT by 0.3 °C in the C group and 0.5 °C in the LPHC groups when compared to basal temperature (Table 3).

Figure 2 shows the macroscopic images, the hematoxylin-eosin (HE) microscopic images and the immunohistochemistry staining of the IBAT from the animals treated with the control or LPHC diet. In addition to the difference in the color and size of the IBAT of the LPHC rats when compared with the C rats (Fig. 2a, b, respectively), hypertrophy was also observed in the HE images of LPHC rats (Fig. 2d). The immunohistochemistry staining showed greater staining (12 %) with the anti-UCP1 antibody in the IBAT of the LPHC group than the IBAT of the C group (Fig. 2e–g).

**Table 2** The initial and final body weights, relative daily food intakes and the IBAT weights of rats fed a C diet or an LPHC diet for 15 days

| | C | LPHC |
|---|---|---|
| Initial body weight (g) | 97.8 ± 3.4 | 97.1 ± 2.3 |
| Final body weight (g) | 205.5 ± 8.2 | 154.8 ± 6.6‡ |
| Food intake (g 100 g b $w^{-1}$ $day^{-1}$) | 10.7 ± 0.2 | 13.4 ± 0.4‡ |
| IBAT weight (g) | 0.397 ± 0.018 | 0.525 ± 0.030† |

The values are expressed as the means ± standard errors of 5–7 animals per diet group

*IBAT* interscapular brown adipose tissue, *C* control diet, *LPHC* low-protein, high-carbohydrate diet

† $P < 0.05$ *versus* control, as determined by Student's *t* test

‡ $P < 0.01$ *versus* control, as determined by Student's *t* test

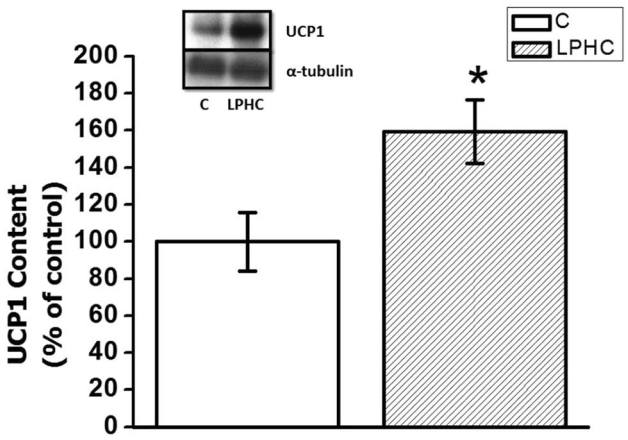


**Fig. 1** The effect of the low-protein, high-carbohydrate diet on the content of uncoupling protein 1 (UCP1) in interscapular brown adipose tissue. The results are expressed as the means ± standard errors; C ($n = 5$) and LPHC ($n = 5$). *$P < 0.05$ *versus* C group (Student's *t* test). C (control diet), LPHC (low-protein, high-carbohydrate diet)

**Table 3** The baseline temperature, the noradrenaline-stimulated temperature and the difference of temperature ($\Delta T$) in the IBAT of rats fed a C diet or an LPHC diet for 15 days

| | C | LPHC |
|---|---|---|
| Baseline (°C) | 36.3 ± 0.4 | 37.4 ± 0.1† |
| Noradrenaline stimulated (°C) | 36.5 ± 0.7 | 38.0 ± 0.1† |
| $\Delta T$ (°C) | 0.3 ± 0.05 | 0.5 ± 0.04† |

The values are expressed as the means ± standard errors of 4–7 animals per diet group

*IBAT* interscapular brown adipose tissue, *C* control diet, *LPHC* low-protein, high-carbohydrate diet

† $P < 0.05$ *versus* control, as determined by Student's *t* test

Figure 3 shows the Western blot analysis of the cytosolic proteins involved in the regulation of the UCP1 expression. The protein expression levels of PKA and p38 MAPK represent the activation of the β3 adrenergic receptor (β3-AR). Although the PKA content in IBAT was not altered by the LPHC diet, the protein level of p38 MAPK, which is activated by its phosphorylation by PKA, was 40 % increased. The expression levels of α1-AR and PKC (Fig. 3), proteins related to the activation of the expression of UCP1 by noradrenaline through this receptor, were similar between the groups. Similarly, the levels of the enzyme DIO-2 (Fig. 3) and the receptor of T3 (TRβ1) (Fig. 4) were not altered by the LPHC diet.

Among the transcription factors, the expression level of CREB was 30 % reduced, and the expression level of ATF-2 increased approximately 60 % in the IBAT of the LPHC group (Fig. 4). No difference in the level of PGC1α was observed between the two groups (Fig. 4).

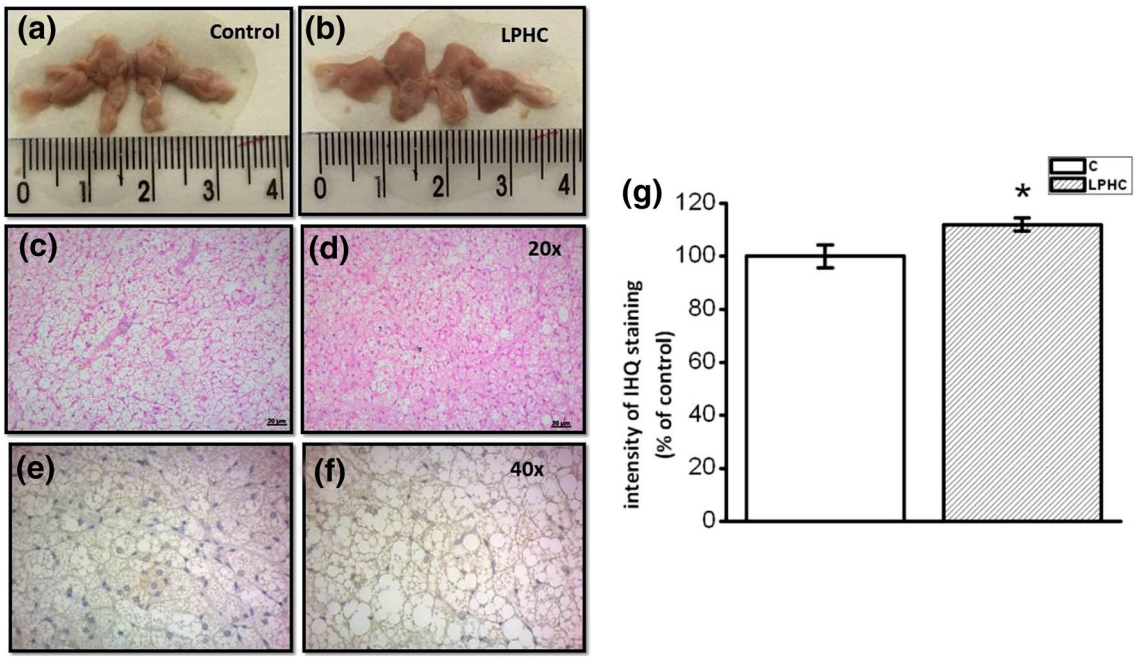


**Fig. 2** The macroscopic images (**a**, **b**); the microscopic hematoxylin-eosin (HE) images (**c**, **d**), and the immunohistochemistry (IHQ) staining images (**e**, **f**) of the interscapular brown adipose tissue from animals treated with the control (**a**, **c**, **e**) or LPHC (**b**, **d**, **f**) diet for 15 days. The results of the quantitative analysis of the protein expression of UCP1 using sections of interscapular brown adipose tissue from animals treated with the control or LPHC diet for 15 days (**g**) ($n = 6$). *$P < 0.05$ *versus* C group (Student's *t* test). C (control diet), LPHC (low-protein, high-carbohydrate diet)

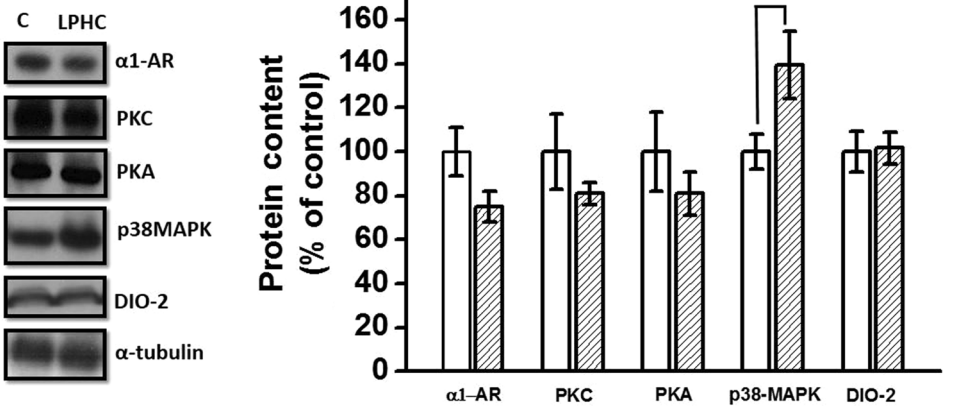


**Fig. 3** The Western blot analysis of the interscapular brown adipose tissue from animals fed a control (C) or low-protein, high-carbohydrate (LPHC) diet for 15 days. The representative blots for the expression levels of α1-AR, PKC, PKA, p38 MAPK and DIO-2 from the rats fed C and LPHC diets. The *bars* of the mean ± SE of the pooled data from rats fed the control ($n = 5$–8) and LPHC ($n = 5$–6) diets. *$P < 0.05$ *versus* C group (Student's *t* test)

## Discussion

Our objective in this work was to evaluate the IBAT thermogenic capacity and the involvement of adrenergic stimulation in this activation in rats treated with an LPHC diet because previous experiments showed that the diet promoted an increase in the sympathetic flux to the tissue [22] but a reduction in β3-AR [27]. In addition to the evaluation of the protein expression levels of UCP1, the expression levels of the proteins involved in its expression were also evaluated after adrenergic stimulation using NA. The levels of DIO-2 and the T3 receptor (TRβ1) were also determined because the thyroid hormone is related not only to its permissive action on UCP1 activity but also its expression.

The higher basal and noradrenaline-stimulated thermogenesis in the IBAT of LPHC-fed rats confirmed our hypothesis that the thermogenesis in these animals is

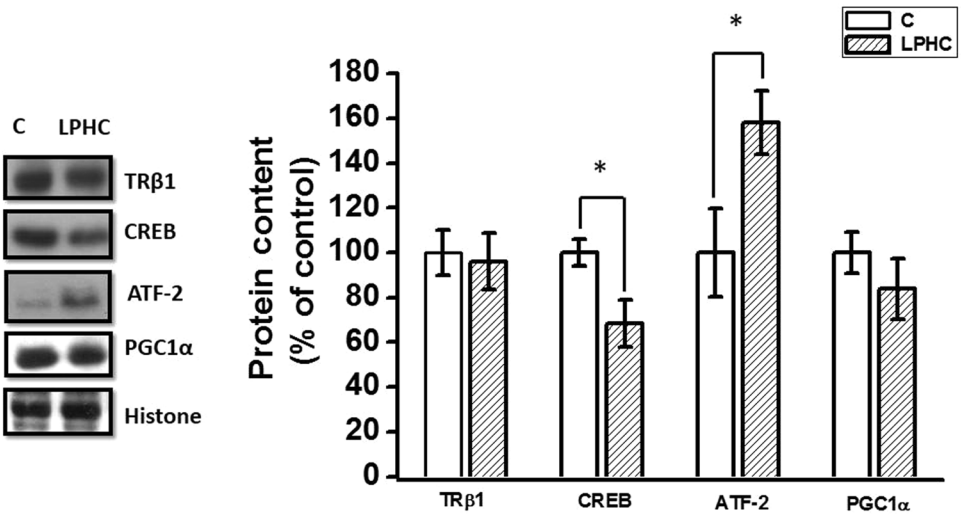


**Fig. 4** The Western blot analysis of the interscapular brown adipose tissue from animals fed a control (C) or low-protein, high-carbohydrate (LPHC) diet for 15 days. The representative blots for the protein expression levels of TRβ1, CREB, ATF-2 and PGC1α from rats fed C and LPHC diets. The *bars* of the mean ± SE of the pooled data from rats fed the control ($n = 6$) and LPHC ($n = 5$) diets.*$P < 0.05$ *versus* C group (Student's *t* test)

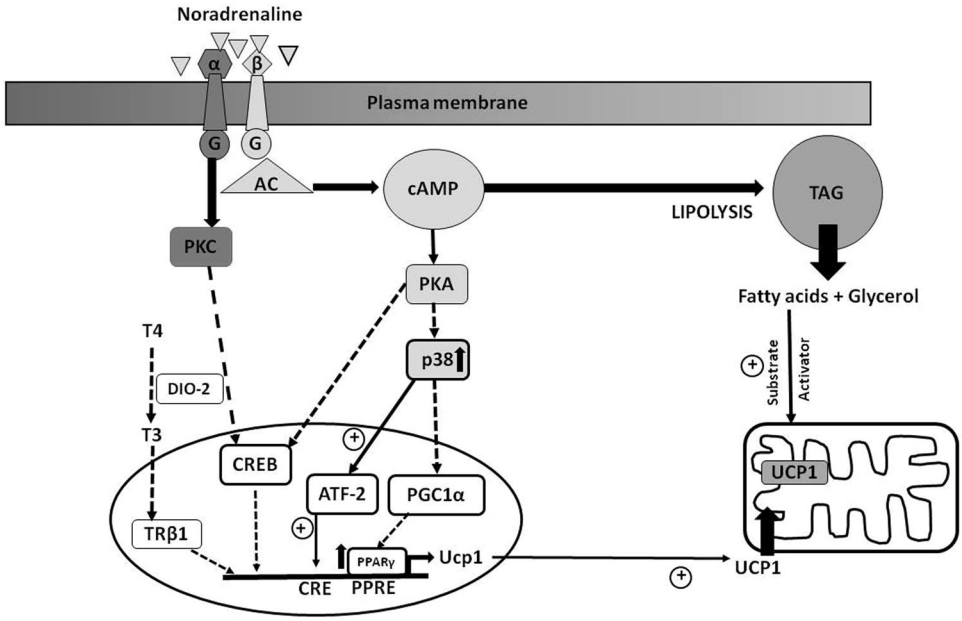


**Fig. 5** A schematic diagram of the adrenergic regulation of the transcription of UCP1 in the brown adipose tissue of LPHC-fed rats. *Solid lines* represent the proposed activation for the expression of UCP1 in LPHC-fed rats. *Dashed lines* represent the likely routes that do not participate in the activation of UCP1 expression in LPHC-fed rats

more active and more responsive to noradrenaline stimulation than that of the control rats, despite the reduction in the level of β3-AR, which has been observed previously [27]. The increase in the protein expression level of UCP1 explains the increase in the thermogenic capacity of the tissue.

As the level of T3 in the serum [24] and the levels of DIO-2 and the TRβ1 isoform in the tissue are similar between the two groups, it is probable that the effect of this hormone on the increase in the expression of UCP1 in the IBAT of LPHC rats is not significant.

As the increase in the expression of noradrenaline-stimulated UCP1 occurs mainly through the β3 and α1 receptors, we evaluated the expression level of α1-AR, and we observed that the content of this receptor was not altered by the LPHC diet. The activation of the expression of UCP1 through α1-AR is mediated by CREB. The reduction in the level of CREB suggests that the increase in the expression level of UCP1 is not related to this receptor. Thus, we concluded that the effect of noradrenaline on the expression of UCP1 must be mediated mainly by β3-AR.

In addition to CREB, ATF-2 and PGC1α can mediate the increase in the expression of UCP1 through the binding of noradrenaline to β3-AR. In these three cases, the protein p38 MAPK participates upstream. The increase in the content of p38 MAPK, the reduction of CREB and the fact that the level of PGC1α was not altered led us to believe that this activation can occur through ATF-2 because it is increased in the IBAT of LPHC rats. Other studies have also identified p38 MAPK as a central obligatory component of

the signaling cascade mediating the β-adrenergic regulation of UCP1 expression in BAT during thermogenesis activation [19, 36]. By phosphorylating ATF-2 and PGC-1α, p38 MAPK controls the expression of the UCP1 gene through their respective interactions with CRE and the PPAR response element (PPRE), considering that both reside within a critical enhancer motif of the UCP1 gene [19]. UCP1 expression may also be regulated through the stimulation of PPARγ signaling pathways, which synergistically and directly stimulates the transcription of the Ucp1 promoter because of cross talk between cAMP and the PPARγ pathways [37, 38]. We had previously demonstrated that PPARγ is increased in IBAT of LPHC rats [27], which could also be contributing to increased UCP1 expression.

Therefore, we concluded that the higher sympathetic flux to IBAT, which is the consequence of the administration of an LPHC diet to rats soon after weaning, activates thermogenesis in the BAT and contributes to an increase in the expenditure of energy. The stimulation of β3-AR and the increase in the expression level of the p38 MAPK and ATF-2 proteins seem to be associated with an increase in the expression level of UCP1 (see Fig. 5).

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