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Effect of high fat diet on skeletal muscle uncoupling protein 3 and hypothalamic neuropeptide Y expression in rats

Nasser M Rizk
Physiology Department, Al-Mansoura Faculty of Medicine, Al-Mansoura University, Egypt

Abstract
Obesity results when energy intake is greater than energy expenditure. Skeletal muscles (SK.M) UCP3 and central NPY play an important role in energy balance. The aim is to study the effects of high-fat diet for 16 weeks on (SK.M) UCP3 expression [mRNA and protein], and hypothalamic NPYmRNA expression in rats. Obese-prone Sprague Dawley rats were fed beef tallow (46%) of energy as fat "HFD" and control group fed libitum diets containing 4.5% of energy as fat (control), for 16 weeks. Body weight and food intake were measured every 3 days throughout the experimental period. After the feeding period is completed, hindlimb skeletal muscle was isolated for subsequent determinations of triglyceride. Skeletal UCP3 mRNA& protein expression and hypothalamic NPY and long isoform of leptin receptor (Ob-Rb) mRNA were assessed by (RT-PCR) and Immunoblotting respectively. Plasma glucose, triglyceride, insulin, leptin, and free fatty acids levels were significantly higher in rats fed the HFD compared to control group, while circulating adiponectin level was significantly reduced in HFD rats. HFD resulted in a significant increase in (SK.M) triglyceride, and mRNA levels of skeletal UCP3 mRNA and protein by (3.84 folds) and (2.1 folds) respectively, but without significant change in hypothalamic NPY mRNA and Ob-Rb mRNA expressions compared to control group. The results show that high fat diet induces obesity with marked induction of UCP3 expression. The development of obesity in spite of the upregulation of UCP3 may indicate that obesity may be caused by mechanisms independent of thermogensis and the central mechanisms could be involved.

Introduction

Regulation of body weight involves coordination of dietary intake and energy expenditure. Obesity is the most common nutritional disorder in western populations and in developing countries. It is characterized by a chronic imbalance between energy intake and energy expenditure.[1] The discovery of several uncoupling proteins (UCPs) provides new molecular targets for increasing energy expenditure. The UCPs are integral membrane proteins of the mitochondrial inner membrane, where they function as a proton channel or shuttle. The action of these proteins creates a futile cycle that decreases the metabolic efficiency of the organism. Thus, UCPs are potentially important in disorders of energy balance such as obesity and diabetes.[2,3] Uncoupling protein 3 (UCP3) gene encodes a protein with 57% of amino acid sequence homology with UCP1,[4] suggesting that UCP3 protein might behave as proton translocator as well. Unlike UCP1, which is mainly expressed in BAT, UCP3 is highly expressed in skeletal muscle.[5] In view of the wide distribution of UCP3 genes and their action in uncoupling oxidative phosphorylation, their expressed proteins have been hypothesized to alter thermogensis, and as a result, may influence whole body energy balance, particularly energy expenditure.[6]

The hypothalamus is a major site of leptin action.[7] It can affect numerous neurotransmitter peptides that are involved in the control of feeding behavior and energy balance. Several data suggest that increased levels of hypothalamic neuropeptide Y (NPY) are manifestations of the obese phenotype and may therefore contribute to bring about their hyperphagia and increased body weight.[8] Exposure to high fat diets for a prolonged period results in positive energy balance and obesity in animals and humans.[9] The model of diet-induced obesity (DIO) in rodents is particularly suited to this task as DIO rats share a number of traits with human obesity. These include polygenic inheritance, insulin resistance, and hyperleptinemia.[10] Moreover, the recent and rapid increase in obesity in developed countries points to the important interaction between genes that predispose to obesity and an environment that facilitates expression of the obese phenotype, another trait shared with DIO rodent models.[11]

The aim of the present work is to investigate the effects of high- fat feeding on the expression of two genes implicated in energy balance, UCP3 and NPY. Sprague Dawley rats are well known to gain weight and to develop diet-induced obesity by overfeeding over long time.[12] We studied the hypothalamic expression of NPY implicated in control of food intake, and the hindlimb skeletal muscle of UCP3 expression implicated in energy expenditure as well as metabolites and hormones in the obesity-susceptible Sprague Dawley rats fed high fat after a total period of 16 weeks.

Materials and Methods

In all experiments, animals were studied in the fed state, because study in the fasted state would result in altered UCP3 and NPY levels due to fasting.

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Correspondence to: Dr. Nasser M Rizk Deparment of Physiology, Al-Mansoura Faculty of Medicine, Al-Mansoura University-Egypt. E-mail: Nassrizk@gmail.com.

Chemicals

All chemicals were obtained from Sigma (International Egyptian Center, Cairo), unless mentioned elsewhere. TRI reagent (Sigma #T 9424), Enhanced avian RT first strand synthesis kit (Sigma #MB-555), Taq-polymerase (Sigma #MB-350), Deoxynucleotide mix (Sigma #D7295) and RNase inhibitor (Sigma #R1274) were purchased from Sigma. The oligonucleotide primer set for beta-actin was a product of Clontech, CA, USA (International Egyptian Center, Cairo). The primers for the long form of the rat leptin receptor (Ob-Rb) and UCP3 were designed using Oligo programme software and synthesized by MWG-biotech (Germany). The forward for Ob-Rb primer was 5-ACACTGTTAATTTCACACCAGAG-3 and the reverse primer 5-GGATAAACCCTTGCTCTTCA-3. The forward primer for UCP3 was: 5'-ATGCATGCCTACAGAACCAT and the reverse primer was 5'-CTGGGCCACCATCCTCAGCA. For amplification of NPY we used a published primer (13) which was synthesized by MWG-biotech (Germany). The forward primer for NPY was 5-GGGGCTGTGTGGACTGACCCTGG-3 and the reverse primer 5-GATGTAGTGTCGCAGAGCGGAG-3.

Animals and experimental design

A hundred male Sprague–Dawley rats were, housed to the physiology laboratory animal care in cages (5 in each) in a thermo neutral room on a 12-hour light/dark cycle. The mean initial weight for rats with standard deviation was similar (90 ± 5g). All animals had free access to water and supplied with standard chow food (Test Diet # 58Y2) containing 4.5% fat, and 21% protein, wt/wt, 3.5 kcal/g, for the acclimatization period. Obesity-prone and obesity-resistant rats were identified by a dietary screening process that predicts future weight gain (14). In short, the rats [3 weeks old] were standardized to facility and to the consumption of a low-fat diet (12% kcal fat, test diet #58G7) for 2 weeks. They were then switched to a high-fat diet (46% kcal fat, Test Diet# 85G8) for 1 week, while weight gain was monitored. The rats were then switched back to the low-fat diet for another week before entering into the study. The rats were ranked by their rate of weight gain during the high-fat dietary challenge. The top tertile was classified as obesity prone and the lower tertile was classified as obesity resistant. Rats from the middle and lower tertile were not used for this study. The animal protocol was approved by Institutional Animal Care and Use Committee at Mansoura Faculty of Medicine, Egypt.

Experimental procedures

Thereafter, thirty animals were weighed and divided into 2 groups (n=15) of approximately equal mean body weight for the ongoing experiments. The first group fed with standard chow food "4.5% Kcal fat" (control group) and the second group fed with high-fat diet "46% kcal fat", (HFD) for further 16 weeks.

Body weight and food intake were measured every 3 days throughout the experimental period. Food intake was measured by subtracting the weight of the chow pellets left over from the initial weight of the pellet divided by the number of the rats in a cage. Careful inspection of the cages revealed no detectable spillage of food. After 12 weeks of feeding, intraperitoneal (ip) glucose tolerance test (IGTT) in a dose of 1g/kg body weight was performed (n=5 rats/group). Blood was collected from tail vein and immediately analyzed for glucose by a refolux2 glucometer (Boreniger Mannheim). After the feeding period is completed, the rats were decapitated and blood samples were collected for plasma glucose, triglyceride, insulin, leptin, adiponectin and free fatty acid assays. Skeletal muscle tissue from hindlimb [mixed fibers] was isolated for subsequent determination of tissue triglycerides content. Skeletal UCP3 mRNA and hypothalamic NPY, and Ob-Rb mRNA were assessed by reverse-transcriptase polymerase chain reaction (RT-PCR). Skeletal UCP3 protein expression was assessed by Immunoblotting. Fat pads from mesenteric, retroperitoneal and epididymal fat were collected and weighted immediately.

Metabolic and Tissue Measurements

After death and decapitation, truncal blood was collected for measurement of metabolites. Plasma was immediately isolated and stored at −80°C until assayed for insulin, leptin and adiponectin. Plasma glucose and triglyceride were determined by an in vitro enzymatic colorimetric method (Biomerieux, France). Free fatty acids "FFA" were determined by calorimetric method using a "NFFA kit C" obtained from (Wako, Germany) according to the method described by manufacturer protocol. Insulin, leptin and adiponectin concentrations were assessed by rat ELISA kits supplied by (Linco Research, St. Louis, MO) according to the manufacturer protocol. Triglycerides concentrations was determined in SK.M after extraction of 50-60 mg of tissue by brief sonication in 0.1M PBS/0.1% BSA (sigma) followed by centrifugation at 10.000g for 5 minutes at room temperature. The resulting supernatant was then analyzed with the kits described above.[15]

Molecular Biology Procedures

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The yield was determined spectrophotometrically at 260 nm. First-strand cDNA synthesis from 5 μg of total RNA was performed according to the manufacturer instructions using oligo (dT) primer.

The PCR reaction mixture was added directly to 5 μl of template cDNA, to yield final concentrations of: 1xPCR buffer (10 mM TrisHCl, 50mM KCl, 1.5mM MgCl2, pH8.3), 200 mM of dNTP, 0.2 mM of each primer (sense and antisense) and 1.25U of Taq polymerase, in a final volume of 50 μl. Co-amplification of NPY and beta-actin was undertaken in the same tube using 94^0C C for 1 min, 58^0C C for 45 s and 72^0C for 1 min with 30 cycles. Amplification of the UCP3, Ob-Rb, and beta-actin was undertaken in separate tubes. UCP3 was amplified using the following conditions; 94^0C for 45 s, 61^0C for 60 s and 72^0C for 60 s with 27 cycles. Ob-Rb was amplified using 94^0C for 1 min, 55^0C for 1 min and 72^0C for 1 min with 30 cycles. Beta-actin was amplified using 94^0C for 1 min, 55^0C

Figure 1

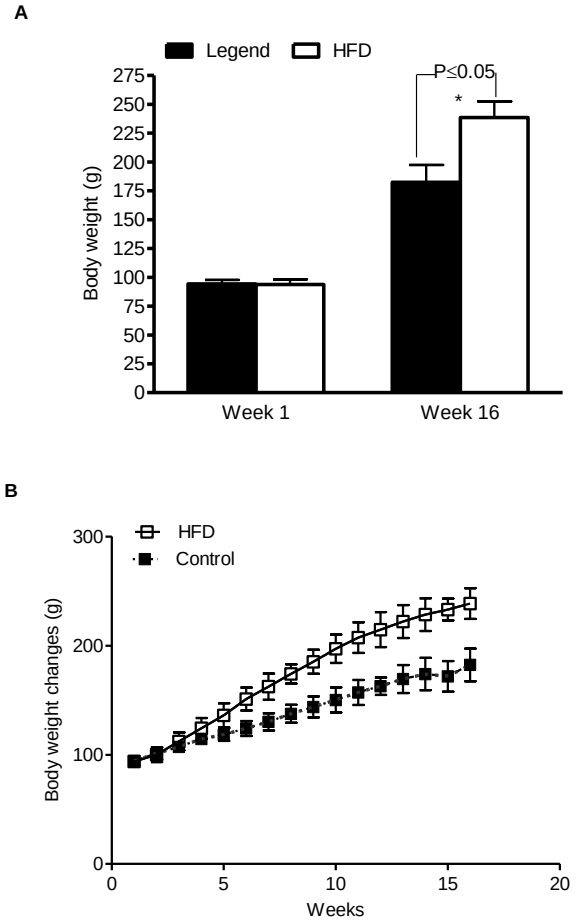


Figure 1: Body weights and the rate of weight regain. (A) Obesity-prone rats were subjected to normal chow (control) and high fat diets (HFD) for 16 weeks. HFD rats had significantly (*) greater BW than the control ($p \leq 0.05$). (B) The rate of weight regain for control and HFD rats over 16 weeks period is represented. Data are expressed as means ± SE (n=15 for each group).

for 1 min and 72^0C for 1 min with 30 cycles. The length of the amplified fragments was: 312 bp for UCP3, 146 bp for NPY, 445 bp for Ob-Rb, and 540 bp for B- actin. 10 µl was withdrawn from the amplified PCR products and separated in a 2% agarose gel containing ethidium bromide. Amplified DNA bands were visualized with a UV transilluminator and analyzed by densitometry, (Pharmacia, Sweden).

Western blot analysis of muscle UCP3 protein levels

An amount of 30 µg (UCP3) of muscle protein were fractionated on 15% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies against rat UCP3 was used as primary antibodies (#ab3477, ABcam, USA). Anti-rabbit IgG-alkaline phosphatase antibodies were used as secondary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). Autoradiograms revealed an apparent molecular mass of 34 kDa for UCP3.

Statistics

Significance of reported differences was evaluated using the null hypothesis and Student's t-test statistics for unpaired data. Results were considered statistically significant at the $P \leq 0.05$ level. All the statistical analyses were performed using the program SPSS (version 14) for Windows (SPSS, Chicago, IL).

Results

Effect of HFD on body weight, body weight gain food consumption, relative food consumption and fat pads

Body weight at the end of the 16-week dietary study was significantly higher in HFD group (238.6± 14.0 g) compared to control group (182.4 ± 15.0 g) respectively. As shown in (Fig 1), the body weight gain of the HFD group started to differentiate from the control group at the beginning of the fourth week of diet protocol (124.3 vs. 114.4 g) respectively and continued thereafter. A body weight differential of 37 to 56 g between HFD and control group was maintained throughout the 8^{th} week up to the 16^{th} week of the study. The mean value of food consumption all over the dietary protocol in HFD group (27.5±1.34 g/day) was significantly higher than that of control group (22.75± 1.0 g/day), p= 0.001 as shown in (Fig 2). Fat pads from mesenteric, retroperitoneal and epididymal, and its percent to total body weight were significantly higher in HFD rats after 16 weeks than control rats fed standard chow (15.13g vs. 3.49g and 6.3% vs. 2.06%),p= 0.001 respectively (table1).

Table 1: Changes in body weight, food consumption, fat pad mass, blood analytes and muscle triglycerides in animals fed high fat diet for 16 weeks vs. chow-fed controls.

	Controls (n=15)	HFD (n=15)
Body weight (g)		
Initial	94.30± 3.50	93.80±4.50
Final	182.40±7.04	238.60±12.32*
Food consumption (g/day)	22.88±1.00	27.50±1.29
Fat pads (g)	3.489±0.08	15.13±0.09*
Plasma glucose (mM)	6.883±0.637	11.618±1.484*
Plasma triglycerides (mM)	0.4290±0.039	0.7270±0.0429*
Plasma free fatty acids (mM)	0.2120±0.032	0.4330±0.0437*
Plasma insulin (ng/ml)	1.420±0.329	4.500±0.531*
Plasma leptin (ng/ml)	3.358±1.2	15.33±1.229*
Plasma adiponectin (µg/ml)	5.82±0.55	3.13±0.66*
Tissue triglyceride (µmol/g)	47.92±2.916	166.5±6.663*

Data shown as means ± SE. * P < 0.05, HFD vs. controls.

Figure 2

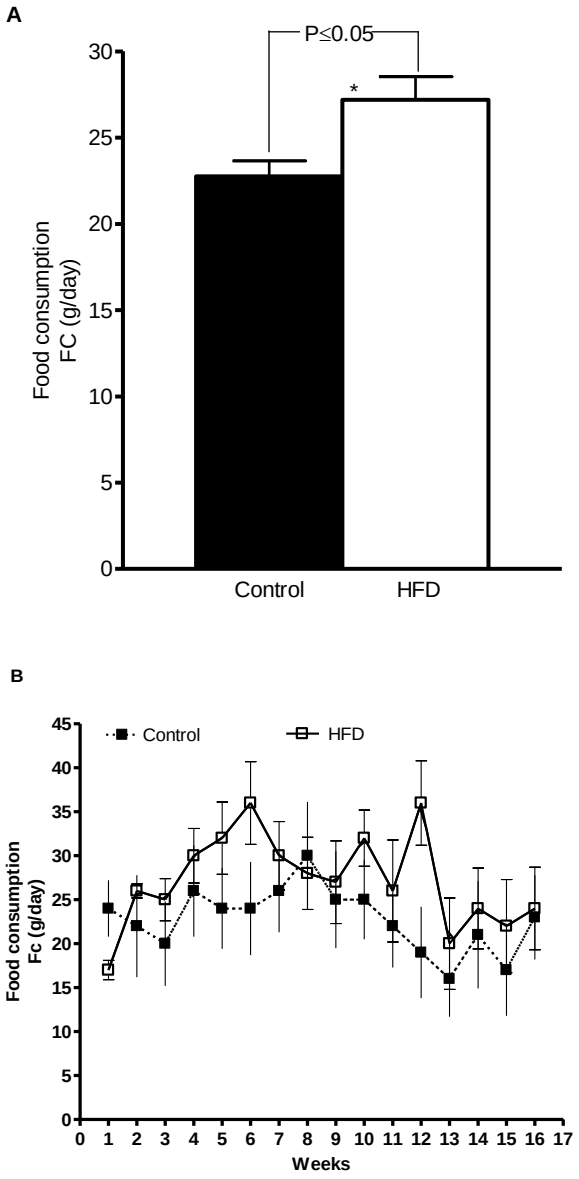


Figure 2: Food consumption "FC" grams /day in rats (A) and FC during the study periods (B). Obesity-prone rats were subjected to normal chow (control) and high fat diets (HFD) for 16 weeks. HFD rats had significantly (*) greater FC than the control ($p \leq 0.05$). Numbers in parentheses denote the number of rats. Data are expressed as means ± SE (n=15 for each group).

Effect of HFD on circulating metabolites, hormones and glucose tolerance

As shown in table 1, plasma leptin levels and triglycerides concentrations, determined at the end of the dietary protocol, showed a significant difference between groups: (3.358±1.2 ng/mL vs. 15.33±2.18 ng/mL for leptin) and (0.43±0.04 vs. 0.727±0.043 mM for triglycerides) in the control group, and in the HFD group, respectively. In addition, HFD resulted in a 2-fold elevation of the plasma free fatty acid "FFA" levels compared with the samples from the control group (0.443 ±0.04 mM vs. 0.223 ± 0.03 mM; respectively) with a p value ≤ 0.05. HFD group exhibited a significant higher plasma glucose levels and plasma insulin levels compared with control group, (13.99±0.66 and 7.25±0.25 mM for glucose) and (6.5±0.59, and 1.42 ± 0.33 ng/ml for insulin) respectively. HFD rats showed a significant lower circulating adiponectin level than control group with (p value= 0.022) (table 1). The muscle content of stored metabolites of triglycerides was significantly higher in HFD rats compared to control rats (166.52 ± 6.66, and 47.92 ± 2.92 μmol/g) respectively. At 12^{th} week of feeding program, glucose solution (50%) was injected at dose of 1g/kg via intraperitoneal route and samples of the blood were collected from the tail veins at different time points 0, 15, 30, 60 and 90 minutes Fig 3. The rats fed HFD demonstrated impaired tolerance to glucose compared to control group.

Figure 3

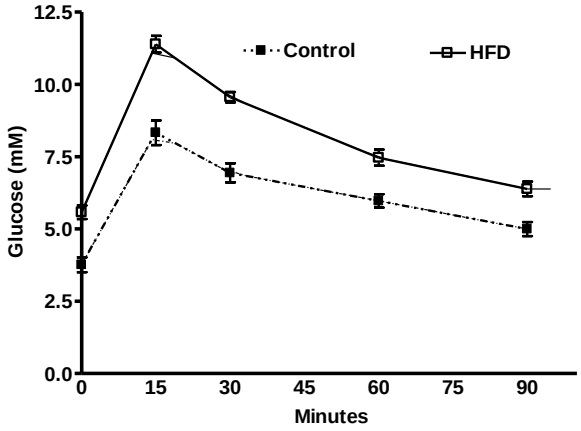


Figure 3: Intraperitoneal glucose tolerance test in rats. Obesity-prone rats were subjected to normal chow (control) and high fat diets (HFD) for 16 weeks. The open squares represent plasma glucose values from control group and the closed squares represent these values from HFD group. The glucose bolus was given as 50% glucose solution in saline in a volume corresponding to 1 g glucose/kg body weight at 12 weeks of feeding protocol as described in under methods. Data are expressed as means ± SE (n=4 for each group).

Effect of HFD on (SK) UCP3 mRNA & protein expression and hypothalamic NPY and leptin receptor mRNA expression

UCP3 mRNA and protein expression analyzed by RT-PCR and western blotting revealed that high fat diet induces the expression of UCP3 in skeletal muscle of the hindlimb significantly compared to chow-fed diet by (3.84 and 2.1 folds), respectively (Fig 4a and 4b). The hypothalamic expression of leptin receptor (Ob-Rb) and NPY (a marker of food intake) showed no significant difference to different dietary protocols in rats (Fig 5a and 5b).

Discussion

In the present investigation, we examined the effects of HFD on two important genes implicated in the regulation of

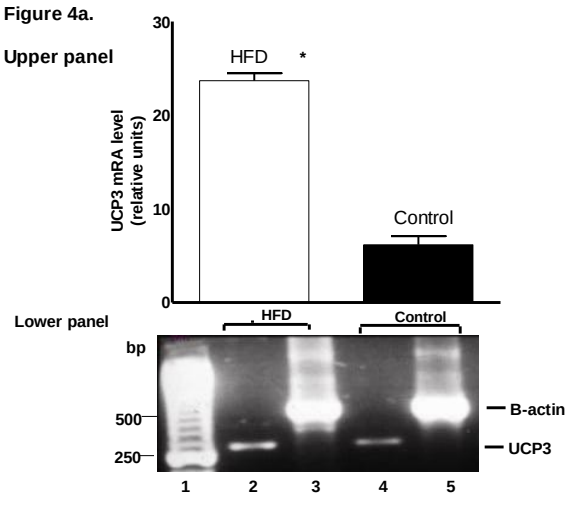


Figure 4b.

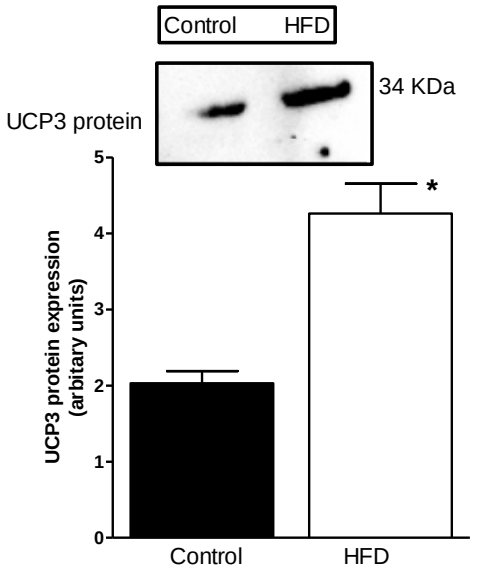
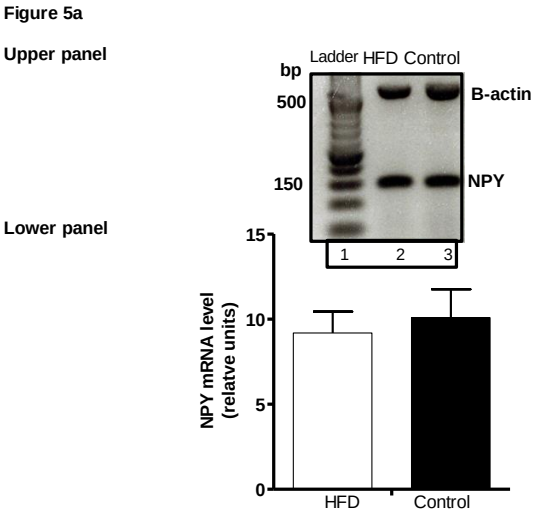


Figure 4: 4 A Upper panel: Uncoupling protein3 (UCP3) mRNA levels in hindlimb skeletal muscles of rats fed HFD and chow-diet for 16 weeks. Quantification of PCR products was performed and UCP3 mRNA levels are expressed relative to the beta-actin signals. Data represent mean values_± SE of four separate experiments. *Significantly different from control with P <0.05.**Lower panel:** Skeletal muscle expression of UCP3. Total RNA was isolated from the hindlimb skeletal muscle and reverse transcribed, as outlined in Methods. The resulting cDNA was run in a polymerase chain reaction (PCR) with sense and antisense primers for UCP3 and beta-actin. PCR products were loaded on a 2% agarose gel and visualized by ethidium bromide staining. Upper panel: Lane 1: DNA ladder; lane 2 UCP3 of HFD (312 bp), 3: Beta-actin of HFD (540 bp), lane 4: UCP3 of control (312 bp), and lane 5: Beta-actin of control (540 bp). Fig 4b._Skeletal muscle UCP3 protein level detected by Immunoblotting. Data represent mean values± SE of four separate experiments. *Significantly different from control with P <0.05.



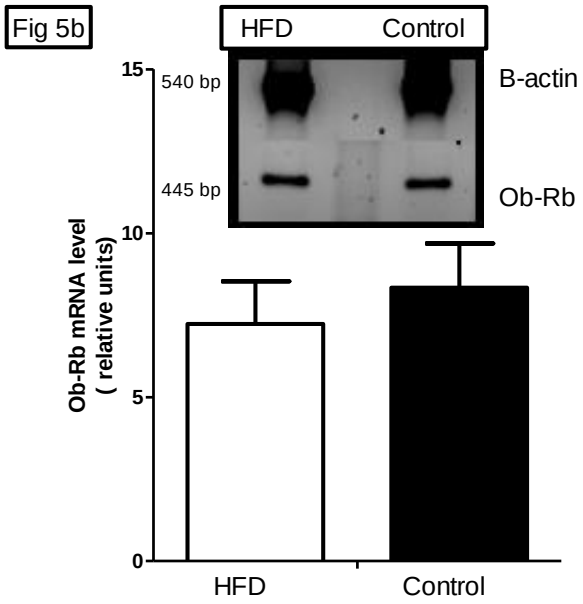


Figure 5: 5a Upper panel*: A* representative PCR for hypothalamic neuropeptide-Y (NPY). 10 µl of PCR products for NPY and B-actin were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Upper panel: Lane 1: DNA ladder; lane 2 NPY of HFD (146 bp) lower band, Beta-actin of HFD (540 bp) upper band, lane 3: NPY of control (146 bp) upper band, and Beta-actin of control (540 bp) lower band. Lower panel. Mean (±SE) of Neuropeotide-Y (NPY) messenger RNA (mRNA) expression in hypothalamic tissue (*n* = 10). Results are expressed as optical density units, adjusted proportionally to housekeeping "B-actin ". 5b: Upper panel: *A* representative PCR for hypothalamic long isoform of leptin receptor (Ob-Rb). Procedures are described in methods section. Lower panel: Mean (±SE) of Ob-Rb messenger RNA (mRNA) expression in hypothalamic tissue (*n* = 10). Results are expressed as optical density units, adjusted proportionally to housekeeping "B-actin ".

energy balance; UCP3 and NPY. Diet induced obesity "DIO" models in rodents represent the crossroads of nature and nurture. The main finding of the present study was an upregulation of HFD on UCP3 mRNA and protein expression in skeletal muscle without significant changes in hypothalamic NPY mRNA expression.

In the current study, consumption of high fat diet resulted in elevated circulating levels of free fatty acids, leptin and triglycerides in HFD group. The results of the current study are in agreement with prior studies showing upregulation of skeletal muscle UCP3 mRNA and protein expression in rodents and humans.[16-19]

Several factors can induce UCP3 expression in skeletal muscle. Weigle and associates[20] showed that the elevation of circulating fatty acids in rats caused by the infusion of intralipid plus heparin strongly and stimulates UCP3 expression in skeletal muscle. Several authors have proposed that fatty acids and triglycerides represent an important signal for the regulation of UCP3 activity.[21,22] The current study demonstrated that rats fed HFD were characterized by markers of insulin resistance such as whole body glucose intolerance, hyperinsulinemia, hyperleptinemia, hyperglycemia, hypertriglyceridemia, higher levels of free fatty acids and increased skeletal muscle triglycerides and fat pads content with decreased circulating total adiponectin. Our results are supported by prior studies demonstrated that HFD diet could induce insulin resistant with hypoadipo-nectinemia.[23,24]

The consumption of the HFD-through the increase of the fat supply to muscle provokes triglycerides accumulation in skeletal muscles, in spite of their increased mitochondrial oxidative capacity.[25] Moreover, the accumulation of fats in skeletal muscles by HFD correlates more strongly with insulin sensitivity in rats than abdominal fat.[24] Although an impairment of muscle fatty acid oxidation has been proposed to play a key role in the intramuscular accumulation of lipids and their deleterious effects on insulin action,[26] Turner and collaborators[19] have recently suggested that in C57BL/6J mice, fat-fed rats, obese Zucker rats, and db/db mice, all characterized by intramuscular lipid accumulation and insulin resistance state had an increase muscle UCP3 mRNA expression, such as we have also observed in the current study. These results can be understood as a homeostatic response to challenge to compensate for the elevated availability of lipids and to limit their accumulation in muscle.[27]

Elevated plasma leptin level observed in HFD group of this study could be another inducer of skeletal muscle UCP3. A previous study demonstrated that leptin could induce UCP3 expression.[6] The leptin induced upregulation of muscle UCP3 might also contribute to regulating fatty acid mobilization and use.[28] Despite reports showing associations between the UCP3 gene and markers for energy expenditure under a wide range of conditions,[6,29] there are no compelling data unequivocally linking UCP3 to energy expenditure.[30] Based on the design of our experiments and on the results of this study, skeletal muscle UCP3 in HFD animals may play a role in lipid metabolism.[31,32] The gradual increment in weight and development of obesity in rats fed HFD in our study may not support the role of UCP3 as a thermogenic agent. Moreover, it could not be discarded an additional role for this UCP3 induction as a mechanism of adiposity control in situations of high fuel availability, as it has been recently reported in humans.[18] Moreover, it was suggested that UCP3 contributes to the export of fatty acids from the mitochondrial matrix rather than the regulation of energy expenditure via thermogensis. [33] The export of fatty acid from the mitochondrial matrix by UCP3 may prevent the accumulation of fatty acids in mitochondria and help to maintain muscular fat oxidative capacity.

It was proposed that, among the variety of orexigenic peptides in the hypothalamus, NPY is important regulator of food intake and energy metabolism.[8] Leptin inhibits NPY expression in rats that have normal leptin levels,[34] and the circulating leptin appears to reflect levels of fat depots.[35] In the present study, despite the high leptin level observed in rats on HFD, they consumed more food and gained more body weight than rats on chow food during the dietary protocol. The results of the current study, taken in the context of prior studies,[36,37,38] suggests that HFD rats have a reduced central sensitivity to the inhibitory effect of leptin. These results are supported by the lack of significant changes in mRNA expression of Ob-Rb in hypothalamus of HFD rats in response to the elevated circulating leptin level, which could refer to a state of leptin resistance. As consequence, defects in leptin action developed with impaired response on hypothalamic neuropeptide Y as demonstrated by several studies.[39,40] This could explain the increment in food intake and weight gain observed in HFD group in the current study.

In conclusion, a 16-wk period of high fat feeding resulted in an upregulation of UCP3 mRNA and protein expression in skeletal muscle. In contrast, NPY and leptin receptor mRNA expression in the hypothalamus were not modified, indicating different in vivo gene regulation. The pathogenesis of diet induced obesity in rats on high fat intake is independent of thermogensis but reduced central leptin sensitivity could play a role. Further investigations are required to elucidate of the exact mechanisms responsible for the effects of high fat diet on obesity development at different time points and utilizing different hypothalamic neuropeptides involved in energy balance.

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