Positive correlation of skeletal muscle UCP3 mRNA levels
with overweight in male, but not in female, rats

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Running head: sex-dependent muscle UCP3 induction by overweight

Keywords: skeletal-muscle UCP3, cafeteria diet, sex-associated differences, obesity.

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Abstract

The objective of this study was to investigate the sex-dependent regulation of skeletal muscle UCP3 mRNA expression in response to overweight and its relationship with serum levels of free fatty acids, leptin and insulin. Two obesity models were used: rats made obese by feeding them with a cafeteria diet for 14 weeks, and post-cafeteria overweight rats fed a chow diet for 10 weeks after consuming the cafeteria diet for 14 weeks. The effects of 24-hour fasting were studied in post-cafeteria rats and their age-matched controls. The cafeteria rats ate a high fat diet and attained an excess body weight that was higher in females (+59%) than in males (+39%). A trend to higher induction of abdominal muscle UCP3 mRNA in male rats than in females after cafeteria diet was apparent (+116% increase versus +26% increase). Post-cafeteria male, but not female rats, still showed the tendency to have increased UCP3 mRNA levels relative to their age-matched controls. A linear regression analysis showed a significant positive correlation of the UCP3 mRNA levels with overweight and with serum levels of leptin and insulin in males, but not in females, and no correlation with serum free fatty acid levels. A subsequent correlation analysis and a multiple linear regression analysis showed that overweight was the only parameter actually related to UCP3 mRNA levels in males. Fasting-induced up-regulation of muscle UCP3 mRNA levels was higher in males (5-7 fold) than in females (3-4 fold). Our results point to the existence of sex-associated differences in the control of muscle UCP3 expression in response to overweight and fasting, with an impaired induction in female rats under both conditions. The correlation of abdominal muscle UCP3 mRNA expression with overweight in males could be related to their relative resistance to gain weight after chronic overeating of a cafeteria diet, by the purported role of UCP3 in the regulation of lipid utilization.

Keywords: skeletal-muscle UCP3, cafeteria diet, sex-associated differences, obesity.
Introduction

Uncoupling protein 3 (UCP3) and 2 (UCP2) genes were cloned in 1997 (4, 12, 13, 42) as genes encoding proteins with high sequence homology to UCP1, a brown adipose tissue (BAT)-specific inner mitochondrial membrane protein which is the main effector of adaptive thermogenesis in BAT. When active, UCP1 can dissipate energy as heat by uncoupling oxidative phosphorylation (29, 33). Considering that BAT plays an important role in energy homeostasis in rodents, but it is scarce in adult humans, the discovery of novel putative uncoupling proteins that are expressed in different mammalian tissues apart from BAT, such as UCP2 and UCP3, led to a renewed interest on the potential involvement of uncoupling proteins in human energy metabolism. Different reports (17, 18) (reviewed in (33)) showed that UCP2 and UCP3 have uncoupling activity when ectopically expressed in heterologous yeast expression systems and in mammalian cells, although this activity is uncontrolled and may not represent their physiological function. More recently, analysis of UCP3 knockout mice provided evidence of uncoupling activity by UCP3 in skeletal muscle in vivo (7, 43), although results are conflicting (5).

By virtue of its specific tissue pattern of expression, in BAT and skeletal muscle (4, 14, 42), UCP3 is a candidate gene that may underlie regulatory thermogenesis in muscle, with a potential impact on body weight regulation. In favor of such a role is up-regulation of muscle UCP3 by thyroid hormone (14), a well known stimulator of basal metabolic rate, and the fact that transgenic mice overexpressing human UCP3 in muscle are lean even though they are hyperphagic (6). However, increased energy expenditure in the muscles of these animals was later attributed to a transgenesis artifact (5), and a number of results argue against a role of UCP3 in regulated energy dissipation. For instance, in both rats (14) and humans (27), muscle UCP3 mRNA expression is induced in fasting, a situation where whole body energy must be conserved, and knockout mice for UCP3 are not obese (16, 43), suggesting that UCP3 is not required for normal body weight regulation (although the existence of compensatory mechanisms in the knockout animals cannot be excluded). In addition, results from Cannon, Nedergaard and co-workers with UCP1 knockout mice
strongly suggest that UCP1 is probably the only physiologically relevant uncoupling protein for thermogenesis (reviewed in (28)).

Apart from regulatory thermogenesis, other physiological roles have been proposed for the novel UCPs, such as the regulation of ATP production by mitochondria, the control of the NADH/NAD⁺ ratio, the limitation of reactive oxygen species (ROS) production, and the enhancement of lipid utilization (7, 39, 43) (reviewed in (33)). A role of UCP3 in lipid metabolism is consistent with the fact that gene expression of UCP3 (and UCP2) in muscle shifts from a state of up-regulation during fasting, when lipid fuel utilization in muscle is increased, to a state of down-regulation below control levels during refeeding, when body fat deposition is increased (39). Moreover, changes of UCP3 (and UCP2) expression during fasting and refeeding have been found to be more pronounced in a mixed fiber type muscle (gastrocnemius) with a high capacity to shift between glucose and lipids as fuel substrate, than in a muscle rich in slow-twitch oxidative fibers (soleus) (39). Also supporting a role of UCP3 in lipid utilization is the finding in humans of an association between certain polymorphisms of the UCP3 gene and a marked reduction of basal lipid oxidation (2). The mechanism(s) by which UCPs may enhance lipid catabolism is still unknown, but could rely on their ability to transport fatty acid anions outside of the mitochondrial matrix. One hypothesis is that excess acyl CoA in the mitochondria is enzymatically hydrolysed into fatty acid anion, which is exported through UCP3, and CoASH, needed for fatty acid oxidation in the beta-oxidation cycle and the tricarboxylic cycle. In this scenario, the activity of the UCPs would favor CoASH delivery to the mitochondria, thus permitting continued rapid fatty acid oxidation in the face of an oversupply (19), and, at the same time, it would protect against toxic accumulation of non-esterified fatty acid anions in the mitochondrial matrix (40).

Different molecules have been shown to up-regulate muscle UCP3 expression, such as thyroid hormone (14), leptin — which also favors the expression of other UCPs (8, 14, 38) — and insulin (30). A stimulatory role of free fatty acids, the circulating levels of which are elevated after prolonged fasting, has also been reported (45) and can be related to the possible role of UCP3 in the handling of lipids as a fuel substrate (19).
In most rodent studies on muscle UCP3, only male animals have been used. However, previous results of our group demonstrated the existence of sex-associated differences in cold-induced (32), diet-induced (34, 37) and overweight-induced (36) stimulation of the expression of the main uncoupling protein in BAT, the UCP1, which was also shown to be regulated by the sex steroids (35), raising the possibility that sex-dependent response could also hold for the other UCPs. Thus, the aim of the present study was to study the changes of skeletal muscle UCP3 mRNA expression in both male and female rats in two different models of obesity and during fasting, as well as their relationship with serum levels of free fatty acids, leptin and insulin. The obesity models used were: rats made obese by chronic (14 weeks) cafeteria diet feeding and overweight rats fed a chow diet for 10 weeks after they had consumed the cafeteria diet for 14 weeks. The latter model, known as post-cafeteria obesity (36), allows distinguishing the effect of overweight itself from the effect of dietary energy content.
Materials and Methods

Chemicals. RNA isolation reagent (TriPure), Hybond nylon membranes and most of the reagents for Northern blotting (digoxigenin-labelled probes, Dig-easy Hyb, blocking reagent, anti-digoxigenin antibodies and CDP-Star) were from Roche (Barcelona, Spain). Other reagents were from Sigma (Madrid, Spain) and routine chemicals were from Merck (Barcelona, Spain) and Panreac (Barcelona, Spain).

Animals. Female and male Wistar rats (from CRIFFA, Barcelona, Spain) were used. They were acclimated to 22°C, with a 12-h day (light)/12-h night (dark) cycle, and initially distributed in four experimental groups of 18 animals each, two of males and two of females. Two groups, one of males and one of females, were offered ad libitum standard chow diet plus cafeteria diet from day 10 of age until day 110. The other two groups, one of males and one of females, were fed ad libitum standard chow only. At 110 days of age, 6 animals of each of the four groups were killed, making up the Cafeteria male and female groups and their respective age-matched controls. The remaining animals (12 per group) were fed ad libitum only with chow diet until the age of 180 days, when they were killed, making up the Post-cafeteria male and female groups and their respective age-matched controls. 6 animals of each group of 180-day-old rats were starved for 24 hours before they were killed. All the experiments were performed in accordance with the national ethical guidelines and the animals were humanely killed.

The cafeteria diet began to be offered while the animals were still in the stage of suckling, so as to accustom them to this diet. Animals were separated from their mother at week 4 of age, and their body weight and food intake were weekly measured from week 7 of age until the end of the experiment.

Diets. The cafeteria diet used included the following foodstuffs: cookies with liver pâté and sobrassada (a typical Majorcan cured sausage), candies, fresh bacon, biscuits, chocolate, salted peanuts, cheese, ensaï mada (a typical Majorcan pastry) and milk containing 20% (w/v) sucrose. The quantitative composition of this cafeteria diet was (by mass): 9.0%
protein, 21.0% carbohydrate, 13.6% lipid, 51.3% water and 5.1% others (41). The standard chow used (Panlab, Barcelona, Spain) had the following percentage composition: 23.5% protein, 48.9% carbohydrate, 5.0% lipid, 12% water, 5.7% ash and 4.0% cellulose. 24-hour energy intake was determined once a week, from the actual amount of standard chow and each individual component of the cafeteria diet consumed by the animals during 24 h and their composition and caloric equivalence (24).

**Tissue collection and muscle UCP3 mRNA Northern blotting analysis.** Animals were killed by decapitation at the start of the day (light) cycle. Samples of abdominal muscle from all experimental groups were obtained, frozen in liquid N₂ and stored at -70°C. All the samples were dissected taking the same part of the abdominal wall, a mixture of the rectus abdominis, transversal abdominis, internal oblique and external oblique muscles. Total RNA was isolated using TriPure reagent, following the instructions of the manufacturer. 30 µg total RNA, denatured with formamide/formaldehyde, were fractionated by agarose gel electrophoresis as described elsewhere (20). The RNA was then transferred onto a Hybond nylon membrane in 20 x SSC (saline sodium citrate buffer: 1 x SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) by capillary blotting for 16 h according to (20), and fixed at 120°C for 30 minutes.

Total UCP3 mRNA and 18S rRNA were analyzed sequentially on the same membrane by a chemiluminiscence procedure based on the use of antisense oligonucleotide probes end-labeled at both ends with digoxigenine. The probes used (obtained from Roche) were: 5'-GACTCCTTCTTCCCTGCGATGGTTCTGTAGG-3' for UCP3 mRNA, and 5'-CGCCTGCTGCCCTTCCCTGGATGGTGGTAGCCG-3' for 18S rRNA.

Fixed membranes were pre-hybridized at 42°C for 1 hour in DIG-Easy Hyb (Boehringer Mannheim) and then hybridized with the corresponding oligonucleotide probe (UCP3 mRNA probe at 35 ng/ml and 18 S rRNA probe at 70 pg/ml) in DIG-Easy Hyb at 42°C overnight. Hybridized membranes were submitted to 2 x 15 min washes in a solution of 2 x SSC/0.1% SDS at room temperature, followed by 2 x 15 min washes in 0.1 x SSC/0.1% SDS at 48°C. After blocking, the membranes were incubated first with an anti-digoxigenin-alkaline
phosphatase conjugate, and then with the chemiluminescent substrate CDP-Star, essentially as in the protocols provided by Boehringer Mannheim. Finally, membranes were exposed to hyperfilm ECL (Amersham). Bands in films were analyzed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, Mass., USA). For each sample analyzed, the integrated optical density (IOD) of the UCP3 mRNA band was divided by the IOD of the corresponding 18S rRNA band, to correct for loading and for transfer of RNA during blotting. The mean values of the male 110-day and 180-day old control groups were set as 100%. Stripping between analysis was performed by exposing the membranes to boiling 0.1% SDS.

**Determination of leptin, NEFA and insulin serum levels.** Serum levels of leptin, non-esterified free fatty acids (NEFA) and insulin were determined, respectively, using a RIA method (Rat Leptin RIA Kit, Linco Research, INC.), an enzymatic colorimetric method (NEFA C, Wako), and an EIA method (Rat Insulin ELISA, DRG Instruments GmbH).

**Statistical analysis.** Body weight, energy intake, serum leptin and insulin levels and UCP3-mRNA data are presented as mean value ± SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA), three-way ANOVA and Student’s t-test for *post-hoc* comparisons. In the two-way ANOVA analysis, D indicates effect of cafeteria-diet treatment, S effect of sex, and DxS interaction between diet treatment and sex. In the three-way ANOVA analysis, P indicates effect of post-cafeteria state, S effect of sex, F effect of fasting and PxS,PxF, SxF and PxSxF interactive effects. Results were considered statistically significant at the P<0.05 level.

Correlations of muscle UCP3 mRNA levels with overweight and serum leptin, insulin and NEFA levels, and of the serum parameters with overweight, were analyzed separately in male and female rats by simple linear regression analysis. Overweight was estimated as the percent difference between the body weight of each animal and the mean body weight of sex- and age-matched control animals (fed standard chow only throughout their entire life-span). Cafeteria and post-cafeteria fed animals and their corresponding controls, but not fasted animals, were pooled for these analysis. A multiple linear regression analysis and a
correlation analysis of UCP3 mRNA levels respect to overweight and serum leptin, insulin and NEFA levels were also carried out.

All the statistic analysis were performed using the program SPSS® for Windows (SPSS, Chicago, USA).
Results

Body mass, energy intake and fat intake in cafeteria and post-cafeteria male and female rats

Cafeteria diet feeding from day 10 to day 110 of age resulted in an excess body mass that was higher in females (+59%) than in males (+39%) (table 1). In both sexes, the energy intake of cafeteria rats practically tripled that of control rats throughout the whole experimental period (34); hyperphagia of the cafeteria animals is illustrated by the energy intake data in table 1, which correspond to the last week on cafeteria diet. Female cafeteria rats displayed a slight but sustained (34) higher energy intake per gram of animal than male rats, which can account in part for the higher degree of obesity they reached. The composition of the diet voluntarily eaten by the cafeteria rats was the same for both sexes and highly imbalanced in comparison to that consumed by the control animals: lipid-derived calories represented 44% of the total energy intake in the cafeteria animals and only 12% in the control animals, entailing a strong reduction of the percentages of carbohydrate-derived and protein-derived energy in the cafeteria rats (carbohydrates, 44% vs. 60%; protein, 12% vs. 28%). The obese rats left to recover from obesity by feeding them a chow diet from day 110 on (post-cafeteria rats) showed a lower percentage of overweight by day 180, when the experiment was finished. However, they still maintained a significant excess of body mass relative to their age-matched controls (12% for males and 21% for females), despite their measured energy intake was the same as their controls during most of the post-cafeteria period (36) (in table 1, the energy intake data of the last week of the post-cafeteria experiment are shown). The effects of 24-hour fasting were analyzed in part of the post-cafeteria animals and their controls: the percentage of body weight lost upon fasting was slightly but significantly higher in females than in males, and was not affected by the post-cafeteria state (table 1).

Skeletal muscle UCP3 mRNA levels in cafeteria and post-cafeteria male and female rats
Abdominal muscle UCP3 mRNA levels were induced after chronic overeating a cafeteria diet, with the induction being more evident in males (116% increase) than in females (26.4% increase) (figures 1A and 2). Post-cafeteria male, but not female, rats still showed the tendency to have increased muscle UCP3 mRNA levels relative to their age-matched controls (by 83%) (figures 1B and 2). 24 hours of fasting brought about an up-regulation of muscle UCP3 mRNA in post-cafeteria rats and their controls, as expected. Fasting-induced up-regulation was significantly more pronounced in male (5-7 fold) than in female (3-4 fold) rats, independently of overweight.

**Serum parameters in cafeteria and post-cafeteria male and female rats**

Serum leptin and insulin levels were increased in the cafeteria animals as compared to their age-matched controls, and were reduced in the post-cafeteria groups, although leptin levels in the latter were still significantly higher than in the corresponding controls (table 1). Serum leptin levels tended to be reduced by 24-hour fasting in all groups, a feature that reached statistical significance in post-cafeteria males. Serum leptin levels were in general higher in male than in female rats, in accordance with previous reports (22). Circulating NEFA levels were not significantly affected by any of the conditions studied (not shown).

**Correlations between muscle UCP3 mRNA levels, overweight and serum leptin, insulin and NEFA levels**

Pooling cafeteria and post-cafeteria fed animals and their corresponding controls, a simple linear regression analysis revealed a highly significant positive correlation between abdominal muscle UCP3 mRNA levels and overweight in males, but not in females (figure 3A). Positive correlation of muscle UCP3 mRNA levels with serum leptin (figure 3B) and insulin (not shown) levels was also found in males only. A highly significant positive correlation between serum leptin levels (figure 3C) and overweight, and between serum insulin levels and overweight (not shown) was evident in both sexes. We did not find any correlation between muscle UCP3 mRNA levels and circulating NEFA levels, neither in males nor in females (not shown). A correlation analysis of muscle UCP3 mRNA levels respect to overweight and serum leptin, insulin and NEFA levels revealed that, in males, the
parameter actually related to UCP3 mRNA levels in abdominal muscle was overweight (table 2). This was further confirmed by the results of a multiple linear regression analysis (not shown).
Discussion

In this work, we provide evidence of sex-dependent differences in the regulation of skeletal muscle UCP3 expression, at the mRNA level, in response to overweight, overfeeding, and fasting. The results of both simple and multiple regression analysis revealed a positive correlation between muscle UCP3 mRNA levels and overweight in male, but not female, animals. Induction of muscle UCP3 mRNA levels after cafeteria diet feeding showed a trend to be higher in males than in females. Sexual dimorphism was also present, in this case reaching statistical significance, in the response of abdominal muscle UCP3 mRNA expression to a 24-hour fasting, up-regulation being more pronounced in male (5-7 fold) than in female (3-4 fold) rats. Changes of mRNA levels do not always correlate with similar changes in protein levels or activity, but they are usually good predictors of gene expression regulation.

Changes in muscle UCP3 mRNA expression levels in cafeteria animals may be in part a consequence of overfeeding; in particular, the high amount of fat consumed by the cafeteria animals may be an important factor. Induction of muscle UCP3 mRNA expression after high-fat diet feeding has been reported in both humans (40) and male rodents (11, 15, 26, 44). Remarkably, in the human study (40), in which the two sexes were studied, induction of muscle UCP3 after high fat diet was only of statistical significance for male subjects, which agrees with the present report. Nevertheless, we detected an effect of overweight itself on muscle UCP3 mRNA levels, which was also sex-dependent, as indicated by the fact that post-cafeteria overweight males (but not females) still had the tendency to have higher abdominal muscle UCP3 mRNA levels than their age-matched controls despite no differences between the two groups in energy and fat intake, and confirmed by the results of the linear and multiple regression analyses performed.

UCP3 mRNA levels in the abdominal muscle of male rats positively correlated in linear regression analysis not only with overweight (figure 3A), but also with serum leptin (figure 3B) and insulin levels (not shown), which may suggest a regulatory role of both hormones on muscle UCP3 expression. However, a correlation analysis (table 2) and a
multiple linear regression analysis (not shown) revealed that expression of muscle UCP3 mRNA in male rats was only related to overweight; the positive correlation in linear regression analysis with serum leptin and insulin could be understood as a consequence of the significant positive correlation between the latter parameters and overweight that we found in both sexes (table 2 and figure 3). In fact, even though there are reports that both leptin and insulin can stimulate UCP3 gene expression (8, 23, 30, 38), changes in leptin or insulin cannot explain (27, 45) the well known fasting-induced up-regulation of this gene (14, 27), suggesting that additional regulatory signals are involved.

Increased UCP3 expression under conditions of enhanced fat utilization by muscles, such as fasting and high-fat diets, has favored the idea that UCP3 may be involved in the handling of lipids as a fuel substrate in muscles (25, 39, 45). An increase in circulating free fatty acid levels in these conditions was suggested to mediate the increased muscle UCP3 expression (3, 45). However, the actual mediator may be an increase in free fatty acid flux to muscles, not necessarily associated with detectable changes in the level of circulating free fatty acids; in fact, increases of muscle fat oxidation and UCP3 expression in humans under high-fat diets are not accompanied by increases of circulating free fatty acid levels (40). This could be in agreement with our results, because the changes of abdominal muscle UCP3 mRNA levels that we detected did not parallel changes in the levels of circulating free fatty acids.

Supporting the idea of a role of UCP3 in lipid utilization is the finding that fasting-induced up-regulation of UCP3 expression is more marked in a mixed fiber type muscle (gastrocnemius) with a high capacity of switching between glucose and lipids as a fuel than in a predominantly slow-twitch oxidative muscle (soleus), which already has a high oxidative capacity in the fed state (39). The composition of muscles in slow-twitch (type I) and fast-twitch (type II) fibers is important to their function and to their oxidative/glycolytic capacities; the oxidative potential of rat muscle is greatest when composed primarily of type IIA fibers, in the rank order of type IIA>I>IID/X>IIB (being the latter the most glycolytic) (9). Nevertheless, it has been shown that muscle fibers are capable of transforming from one fiber type extreme
to another (e.g., from type IIB to type I) in response to altered functional demands, hormonal signals or changes in neural input (31). The abdominal muscles studied here are predominantly fast-twitch muscles with a mixed fiber composition of 21-32% type I, 2-9% type IIA, 17-24% type IID/X and 44-50% type IIB fibers (as studied in male rats) (9). Fast-twitch muscles constitute the major muscle type of the total skeletal muscle mass in rodents and humans, and especially predominant in rat muscles are the fast-twitch IIB fibers (9). Thus, the abdominal muscles studied here, and the observed changes of UCP3 in them, may be representative of a significant part of the muscles of the rat.

Induction of muscle UCP3 expression may allow an increase of the use of lipids as fuel substrate, which, in the face of a dietary oversupply (high-fat diet feeding), could contribute to a relatively reduced body weight gain. In this scenario, the trend to a more efficient induction of muscle UCP3 expression in response to the dietary challenge found in male rats may be part of the explanation why they reached a much lower percentage of overweight at the end of the cafeteria period (39%) than females (59%), a result that cannot be accounted for solely by the small differences found in energy intake (34). Data from knockout mice also support the idea that enhanced induction of muscle UCP3 in male rats may contribute to their relative resistance to a body weight gain when offered a high-fat diet, as an increased tendency to diet-induced obesity was observed in male, but not in female, UCP3 knockout mice, as compared to their sex-matched wild type controls (43). In addition, a defective dietary induction of UCP3 in skeletal muscle has been described in obesity-prone male rats (44). Besides the enhanced induction of muscle UCP3 expression and the slightly lower energy intake, another factor that may contribute to the reduced susceptibility of male rats to dietary obesity in our experiment is that males induced the BAT thermogenic machinery during the cafeteria period more efficiently than females (34). A fourth factor that may also contribute is the fact that normal development is slower in females (as shown previously for our rats in (36)), because the cafeteria diet was offered during a period of active growth.
Fasting-induced body weight loss was slightly but significantly higher in female (5.2-5.8 %) than in male rats (3.7-4.1 %) (table 1). This may be somehow contradictory with our result of higher fasting-induced up-regulation of muscle UCP3 expression in males than in females (note the interactive effect of sex and fasting in figure 1B). It is possible that the impact of muscle fat oxidation rate — which we assume related to muscle UCP3 expression levels — on body weight becomes apparent only in the face of a dietary oversupply. On the other hand, other factors and tissues, different to muscle UCP3, could be influencing body weight losses during periods of caloric restriction. For instance, we have previously shown (36) that, during the post-cafeteria period, BAT is more efficiently activated in the females (the opposite that happens in the cafeteria period), who experienced higher body weight losses than males after removal of the cafeteria diet.

The sexual differences of muscle UCP3 induction observed here could be mediated, at least in part, by some sex steroids, either by a direct influence on UCP3 expression, or indirectly by influencing other muscle parameters. Gender differences in fiber composition may account, at least in part, for the difference (although non significant) in basal UCP3 gene expression (see figure 1) and for the different induction of UCP3 mRNA between sexes reported here, and differences in fiber composition could themselves be a consequence of the action of sex hormones. In fact, sex hormones are known to influence different features of skeletal muscle in rodents, such as fiber diameter (21) and myosin heavy chain expression (10). What is more, it has been suggested that female sex steroids may control the oxidative capacity of different tissues, including muscle, as the lack of female steroids in ovariectomized rats induces a generalized stimulation of COX activity that can be reversed by treatment with progesterone and estradiol (1). More direct effects of sex steroids on UCP3 gene expression, although not yet studied, cannot be discarded; remarkably, progesterone and testosterone have been recently demonstrated to regulate the expression of another member of the uncoupling protein family, UCP1, in brown adipocytes differentiated in culture (35).
Sex-dependent regulation of skeletal muscle UCP3 expression — and of BAT UCP1 expression (34) — may have a physiological significance. In particular, the reduced ability of female rats, compared to males, to induce muscle UCP3 and BAT UCP1 over the basal levels upon cafeteria diet feeding could contribute to the higher capacity of females to save fat stores when food is in excess, which could be an advantage to face biological functions only present in females, i.e. the reproductive lactation functions.

In conclusion, our results point to the existence of sex-associated differences in the control of muscle UCP3 expression in response to overweight and fasting, with an impaired induction in female rats under both conditions. Our results entail the possibility that sex-dependent strategies should be considered in molecular studies on obesity and management of the disease.
Acknowledgments

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References


45. **Weigle DS, Selfridge LE, Schwartz MW, Seeley RJ, Cummings DE, Havel PJ, Kuijper JL, and BeltrandelRio H.** Elevated free fatty acids induce uncoupling protein 3
Figure Legends

**Figure 1.** A: Changes of UCP3 mRNA levels in abdominal muscle of 110-day-old male and female Wistar rats in response to 14 weeks of cafeteria diet. **B:** Changes of UCP3 mRNA levels in abdominal muscle of 180-day-old male and female Wistar rats in response to post-cafeteria state and 24-hour fasting. Post-cafeteria animals were fed standard chow for 10 weeks after consuming the cafeteria diet for 14 weeks. UCP3 mRNA levels are expressed relative to the levels found in 110-day-old or 180-day-old male control rats (fed standard chow throughout life), the mean value of which was set at 100%. Each group was made up of 6 animals. C110: 110-day-old control rats; CAF: cafeteria rats; C180: 180-day-old control rats; POST: post-cafeteria rats. Significant differences, Student’s t-test (P < 0.05): ᵅ obese (cafeteria or post-cafeteria) versus control; ᵇ starved versus fed. Two-way ANOVA significance (P < 0.05): D effect of cafeteria-diet. Three-way ANOVA significance (P < 0.05): F effect of fasting; SxF interaction of sex and fasting.

**Figure 2.** Representative Northern images of the changes of UCP3 mRNA levels in abdominal muscle of 110-day-old and 180-day-old male and female Wistar rats in response to cafeteria diet, post-cafeteria state and 24-hour fasting. Each group was made up of 6 animals. Postcaf indicates post-cafeteria state. 30 μg of total muscle RNA were loaded per lane. The membranes were probed first for UCP3 mRNA, and thereafter stripped and re-probed for 18 S rRNA, to check loading and transfer. Developed Northern blot membranes were exposed to Hyperfilm ECL (Amersham).

**Figure 3.** Correlation of muscle UCP3 mRNA levels with overweight (A) and leptin (B), and of leptin with overweight (C). A linear regression analysis was made. The figure shows the regression lines for male and female rats (17-19 animals per sex). For each gender, cafeteria
and post-cafeteria fed animals as well as their corresponding age-matched controls were pooled. Overweight was estimated as the percent difference between each individual weight and the mean body weight of sex- and age-matched control animals. Overweight negative values are not shown in the graphs, but they were included in the statistical analyses. n.s. indicates non-significant differences. The UCP3 mRNA data are the ratio of specific mRNA levels to the corresponding 18S rRNA (A.U., arbitrary units).
Table 1. Body mass, energy intake and serum leptin and insulin levels in cafeteria and post-cafeteria rats.

<table>
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<th>180-DAY-OLD</th>
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<tbody>
<tr>
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<td>Body mass (g)</td>
<td></td>
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<tr>
<td>M</td>
<td>441±3 (18)</td>
<td>611±28 § (18)</td>
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<tr>
<td>F</td>
<td>259±4 *</td>
<td>411±9 § * (18)</td>
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<td>% Loss</td>
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<td>F</td>
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</tr>
<tr>
<td>Insulin (µg L⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.15±0.03 (6)</td>
<td>2.46±1.18 § (6)</td>
</tr>
<tr>
<td>F</td>
<td>0.34±0.10 (6)</td>
<td>1.15±0.66 (6)</td>
</tr>
</tbody>
</table>

Data are the means ± SEM of the number of animals given in brackets. Energy intake data correspond to the last week of the cafeteria and the post-cafeteria experiment. Significant differences Student’s t-test (P<0.05): § obese versus control; * females (F) versus males (M), † starved versus fed. Two-way ANOVA significance (P<0.05): D effect of cafeteria-diet, S effect of sex, P effect of post-cafeteria state and DxS interaction of cafeteria-diet and sex. Three-way ANOVA significance (P<0.05): P effect of post-cafeteria state, S effect of sex, F effect of fasting, and SxF interaction of sex and fasting.
Table 2. Correlation matrix of the different parameters studied in male and female rats: abdominal muscle UCP3 mRNA levels, overweight and serum leptin, insulin and NEFA levels.

<table>
<thead>
<tr>
<th></th>
<th>UCP3</th>
<th>Overweight</th>
<th>Leptin</th>
<th>Insulin</th>
<th>NEFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UCP3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson’s correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Overweight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson’s correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.814**</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>0.329</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson’s correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>-0.059</td>
<td>0.856**</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>0.086</td>
<td>0.935**</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson’s correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.195</td>
<td>0.762**</td>
<td>0.665**</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>-0.136</td>
<td>0.535*</td>
<td>0.529**</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td><strong>NEFA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson’s correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>-0.215</td>
<td>-0.348</td>
<td>-0.188</td>
<td>-0.208</td>
<td>1.000</td>
</tr>
<tr>
<td>Females</td>
<td>-0.197</td>
<td>-0.019</td>
<td>0.211</td>
<td>0.002</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Cafeteria and post-cafeteria groups, as well as control animals, were pooled; each group contained 17-19 animals. The Pearson’s correlation index is given; ** indicates significant correlation at the P<0.01 level and * indicates significant correlation at the P<0.05 level (bilateral).
Figure 1.
Figure 2.
Figure 3.

A. Males $R^2 = 0.663 \ p = 0.000$

Females $R^2 = 0.108 \ n.s.$

B. Males $R^2 = 0.446 \ p = 0.002$

Females $R^2 = 0.073 \ n.s.$

C. Males $R^2 = 0.732 \ p = 0.000$

Females $R^2 = 0.874 \ p = 0.000$