Sex-related differences in energy balance in response to caloric restriction

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Valle, A., A. Català-Niell, B. Colom, F. J. García-Palmer, J. Oliver, and P. Roca. Sex-related differences in energy balance in response to caloric restriction. Am J Physiol Endocrinol Metab 289: E15–E22, 2005. First published February 8, 2005; doi:10.1152/ajpendo.00553.2004.—Sex-related differences in energy balance were studied in young Wistar rats fed standard chow pellets either ad libitum or in restricted amounts (60% of ad libitum intake) for 100 days. Caloric intake, indirect calorimetry, organ and adipose tissue weights, energy efficiency, liver mitochondrial respiration rate, and brown adipose tissue (BAT) uncoupling protein-1 (UCP1) content were measured. Ad libitum-fed females showed greater oxygen consumption (VO_2) and carbon dioxide production (VCO_2) and lower energy efficiency than males. Caloric restriction induced a chronic drop of VO_2 and VCO_2 in females but not in males over the period studied. Restricted females showed a better conservation of metabolic active organ mass and a greater decrease in adipose depots than restricted males. Moreover, changes of BAT size and UCP1 content suggest that BAT may be more important for energy conservation seen when female rats are subjected to hypercaloric diets. Female rats subjected to hypercaloric diets have shown a greater propensity to obesity and lower induction of BAT thermogenesis than males (51, 52). It has been suggested that the mechanisms involved in CR adaptation could be the same as those that conserve energy in obese animals (30, 58, 59). In this way, to study the opposed energy situation could allow for a better understanding of sex differences in the mechanisms that regulate energy balance and the propensity to obesity. Thus the aim of this paper was to study energy balance and BAT thermogenic capacity in female and male rats subjected to 100 days of 40% CR to examine adaptive changes related to sex differences.

Another possibility to explain changes in energy efficiency related to sex implies differences in heat production. In rodents, nonshivering thermogenesis takes place mainly in brown adipose tissue (BAT), where thermogenesis activity depends on uncoupling protein-1 (UCP1), an inner-membrane mitochondrial protein expressed characteristically in this tissue, the function of which is to dissipate, as heat, the proton gradient energy generated by the respiratory chain (7, 48). These particular energy features mean that BAT has a high metabolic rate, contributing to a large extent to the energy expenditure under diet- (51, 52) or cold-induced conditions (57). Several researchers have shown in rodents that CR causes a decline in energy expenditure beyond what would be expected by change in metabolic mass (14, 37). This elevated metabolic efficiency may be related to the decrease in BAT thermogenic capacity reported by Rothwell and Stock (56). To date, little effort has been made to look for sex differences in heat production in response to CR. In previous reports, we have demonstrated important sex differences in diet- (51, 52) and cold-induced (46) thermogenesis. Female rats subjected to hypercaloric diets have shown a greater propensity to obesity and lower induction of BAT thermogenesis than males (51, 52). It has been suggested that the mechanisms involved in CR adaptation could be the same as those that conserve energy in obese animals (30, 58, 59). In this way, to study the opposed energy situation could allow for a better understanding of sex differences in the mechanisms that regulate energy balance and the propensity to obesity. Thus the aim of this paper was to study energy balance and BAT thermogenic capacity in female and male rats subjected to 100 days of 40% CR to examine adaptive changes related to sex differences.

MATERIALS AND METHODS

Materials. Routine chemicals used were supplied by Sigma-Aldrich (St. Louis, MO), Panreac (Barcelona, Spain), and Amersham Pharmacia Biotech (Little Chalfont, UK). Antibody for UCP1 was obtained from Alpha Diagnostics International (San Antonio, TX) and peroxidase-conjugated secondary antibody was purchased from Sigma-Aldrich.

Animals and feeding. All animals were treated in accordance with accepted standards of humane animal care. Six-week-old Wistar rats, 12 males and 12 females (supplied by Charles River, Barcelona, Spain) were housed individually in wire-bottom cages and acclimated in our animal facility (22°C; 12:12-h light-dark cycle, lights on from 10 AM to 10 PM). After 2 wk of acclimatization, animals were randomized into four experimental groups of six animals each: ad libitum male, ad libitum female, restriction male and restriction female. Ad libitum animals were fed standard chow pellets (A04; Panlab, Barcelona, Spain), and their caloric intake was calculated...

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Mitochondrial viability was checked by the respiratory control ratio (RCR) and carbon dioxide production (V\(^\text{\text{CO}_2}\)) were measured every 2 wk (4 animals in each group) by open-circuit respirometry (flow rate 1 liter/min). The measurements were obtained during the last 2 h of the dark phase with the aid of a red light. Room temperature was maintained at 22°C. The animals were individually placed in metabolic cages 30 min before data collection initiation, which was continued for 120 min. The first 30 min of data recordings were discarded; so altogether, animals were allowed to become acclimated for 1 h. Data recordings were made sequentially in each cage during 90-s periods. V\(^\text{O}_2\) and V\(^\text{CO}_2\) were adjusted for surface area (ml•min\(^{-1}\)•kg\(^{-0.67}\)) and averaged over the collection period (62).

Tissue and organ weights. One hundred days after the beginning of restriction, all animals were killed by decapitation at the start of the light cycle. Heart, interscapular BAT, liver, kidneys, lungs, brain, and white adipose tissue depots (WAD; retroperitoneal, mesenteric,inguinal, and gonadal) were dissected and weighed.

Measurement of mitochondrial respiration rate. Liver samples (5 g) were homogenized in 35 ml of ice-cold isolation buffer (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4) with a Teflon/glass homogenizer. The nuclei and cell debris were removed by centrifugation at 500 g for 10 min. The supernatant was centrifuged at 8,000 g to yield the mitochondrial pellet. The pellet was washed once by resuspension and centrifuged (8,000 g), and the final pellet was resuspended in the same buffer. Mitochondrial protein was measured by the Bradford method (4).

Liver mitochondrial V\(^\text{O}_2\) was measured polarographically, with minor modifications as described previously (35). Mitochondria were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O\(_2\) electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 1.8 mg mitochondrial protein/ml in buffer containing 145 mM KCl, 30 mM HEPES, 5 mM KH\(_2\)PO\(_4\), 3 mM MgCl\(_2\), 0.1 mM EGTA, and 0.1% BSA (pH 7.4 at 37°C). Pyruvate-malate (2.5 mM/2.5 mM) was used as the substrate in the presence (state 1) and in the absence (state 4) of ADP. Mitochondrial viability was checked by the respiratory control ratio (RCR = state 3/state 4 V\(^\text{O}_2\)).

Western blot for UCP1. Interscapular BAT samples were washed in isolation buffer at 4°C. White adipose tissue and muscle fibers were removed, and samples (100–200 mg) were minced and homogenized in 15 ml of buffer with a Teflon/glass homogenizer. Total protein content was determined by the Bradford method (4). Fifteen micrograms of BAT total protein were fractionated by SDS-PAGE (10% polyacrylamide) according to Laemmli (33) and electrotransferred onto a nitrocellulose filter, as described elsewhere (45). Staining with Ponceau S was used to provide visual evidence for correct loading and immunoblotting analysis system (Amersham, Little Chalfont, UK). Rabbit polyclonal antibody against UCP1 was used as primary antibody (Alpha Diagnostics International). Bands in films were analyzed by scanner photodensitometry and quantified using the Kodak 1D Image Analysis Software.

Statistics. The Statistical Program for the Social Sciences software for Windows (SPSS, version 12.0) was employed for all statistical analyses. Data are presented as means ± SE. Differences between groups were assessed by two-way analysis of variance (ANOVA) and Student’s t-test post hoc analysis. Bivariate linear regression and ANOVA were used to assess the ability of body composition parameters to explain the variance in whole animal oxygen consumption (w\(\text{VO}_2\); see Table 3). Statistical significance was set at P < 0.05.

RESULTS

Body weight and caloric intake. The body weight and caloric intake curves after correction for differences in surface area are shown in Fig. 1, A and B, respectively. As illustrated by Fig. 1A, the growth rate of the ad libitum rats slowly decreased throughout the experiment, with the females tending to plateau earlier. CR produced a deceleration of growth rate, which was more marked in the first 2 wk of restriction. Restricted female rats showed a significantly lower weight than ad libitum females from the first week onward (P < 0.05), whereas for restricted males this occurred from the second week onward. At the end of the period studied, restricted males and females both showed the same weight difference related to ad libitum groups (~26%). No statistically significant differences were found in caloric intake between sexes in either group.

Whole animal respirometry. Energy expenditure can be indirectly measured as V\(^\text{O}_2\) under a set of standard conditions...
To study the changes induced by CR, we measured \( V\dot{O}_2 \) and \( V\dot{C}O_2 \) in whole organism by indirect calorimetry. Respirometry results are compiled in Fig. 2. Ad libitum females showed a higher \( V\dot{O}_2 \) and \( V\dot{C}O_2 \) than the rest of experimental groups over the period studied. CR produced a significant decrease in the \( V\dot{O}_2 \) and \( V\dot{C}O_2 \) in both sexes in the third week of restriction, but, from the fifth week, significant differences between ad libitum and restriction males disappeared. No significant differences were found in respiratory quotient.

**Effect of sex and CR on energy efficiency.** Growth is an important factor in the whole body energy balance in young animals. Hence, an important part of calories from the diet are converted into new structures or consumed during the needed work to carry out the de novo synthesis. To know the course of this energy flow, we decided to calculate the energy efficiency (gain in body weight/total food eaten) over the period studied. Figure 3 shows the evolution of energy efficiency calculated each month. In ad libitum-fed animals, as growth rate declined (Fig. 1), the energy efficiency also gradually decreased. In the first month, restricted animals showed a marked decrease of energy efficiency, even reaching, in the female rats, negative values. Nevertheless, during the second month, restricted animals recovered their efficiency quite a lot, coming close to ad libitum animals and following a similar profile from then on.

Ad libitum females showed a lower energy efficiency than males, which is consistent with the greater energy expenditure observed in this sex (see above). These differences in energy efficiency were diminished by CR, and more so the longer the restriction. This trend in energy efficiency is in accordance with the greater ability, seen by other authors, of female rats to conserve energy during food restriction (26, 39, 63).

**Effect of CR on tissue weights.** Table 1 shows organ and tissue weights grouped by similar metabolic characteristics. The more metabolically active organs [organ weight (OW)] includes the sum of brain, liver, kidneys, and heart weight (21). Although these organs comprise <10% of total body weight, they account for the majority of whole body basal energy usage (6, 15, 17). Compared with OW, white adipose tissue has a lower metabolic rate, as it is a tissue specializing mainly in energy storage. The sum of the mesenteric, gonadal, inguinal, and lumbar WAD was used as an adiposity index. BAT was kept out of these groups due to its particular energy characteristics. Weight data are presented as absolute values, percentage relative to body weight (RBW), and percentage of loss in restricted animals relative to control group (RCG).
Weight loss induced by CR was observed in all organs except brain (data not shown). CR caused a significantly more pronounced loss of OW in male than in female rats (−24.3 vs. −16.1%, respectively). In this way, female rats maintained a higher percentage of body weight as metabolically active mass; nevertheless, this difference between sexes did not reach significance. Significant differences in WAD weight were observed between the sexes in ad libitum and restricted animals. These data were in accord with several studies that have reported in males a greater percentage of the body as fat than females (31, 34, 44). CR induced a more pronounced loss of BAT mass in females (31, 34, 44). CRG induced a more pronounced loss of OW in males than in females (−72.8 vs. −68.6% RCG respectively), although this did not reach statistical significance. This decrease in body fat also contributes to the greater percentage of lean organ mass observed in females.

With regard to BAT, relative BAT weight is higher in ad libitum females, which is in agreement with previous studies (53). BAT showed differences related to sex and diet, but, furthermore, its RBW showed a statistically interactive effect that was not observed in any of the other parameters measured, due to the greater loss of BAT mass in females (−53.5 in females vs. −43.1% in males).

Respiratory rate in liver mitochondria. Liver plays a major role in energy metabolism, contributing to a great extent (10–20%) to energy expenditure in relation to the percentage of body weight that it represents (3.4–5.5%) (6, 15, 47). Hence, we decided to measure the mitochondrial respiratory rate in liver to study variations in energy expenditure in specific organs. Table 2 shows the mitochondrial respiration rate in the four groups studied. The mitochondrial respiratory capacity in liver was higher in female than in male rats, with females showing significantly greater respiratory rate in both states. A similar sex effect was obtained using succinate (5 mM) and glutamate-malate (2.5 mM/2.5 mM) as substrates (unpublished data). However, significant differences were not observed in mitochondrial respiratory capacity in relation to CR.

Bivariate analysis regression. In view of the observed changes in body composition and energy expenditure between sexes and diet groups, we decided to employ bivariate linear regression analysis to estimate the ability of the different organs to explain the variance in whole body energy expenditure. Table 3 compiles the results of the regression analysis between whole animal VO2 at the end of the experiment (wVO2) as a dependent variable and body weight (BW), OW, and BAT weight as independent variables. These regressions were carried out separately for sexes (Fig. 4), diets, and the whole experimental group with the aim of finding which were the best regressions in each case.

Females showed a better correlation than males with the different expressions of metabolic mass, especially with BW and BAT. It should be noted that BW is strongly affected by CR in both sexes (Fig. 1A), whereas energy expenditure (VO2) seems to be more affected by CR in females (Fig. 2), suggesting that weight loss is not the determining factor in explaining changes in wVO2.

Table 2. Effect of CR on liver mitochondrial respiration rates in male and female rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>482±14</td>
<td>261±8*</td>
</tr>
<tr>
<td>OW</td>
<td>18.8±0.8</td>
<td>10.7±0.4*</td>
</tr>
<tr>
<td>RBW, %</td>
<td>3.91±0.17</td>
<td>4.12±0.16</td>
</tr>
<tr>
<td>WAD</td>
<td>30.8±2.8</td>
<td>12.5±1.5*</td>
</tr>
<tr>
<td>BAT</td>
<td>6.38±0.57</td>
<td>4.8±0.6</td>
</tr>
</tbody>
</table>

Table 3. Linear bivariate regression analysis

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Dependent Variable</th>
<th>wVO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>n</td>
<td>BW</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>0.476</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>0.913‡</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>8</td>
<td>0.516*</td>
</tr>
<tr>
<td>Restricted</td>
<td>8</td>
<td>0.939‡</td>
</tr>
<tr>
<td>Whole group</td>
<td>16</td>
<td>0.716‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals per group. CR, caloric restriction; OW, metabolically more active organ weight; sum of heart, brain, liver, and kidney weights; WAD, white adipose depots: sum of inguinal, gonadal, mesenteric, and retroperitoneal depots; BAT, brown adipose tissue; RBW, tissue weight as percent relative to body weight; RCG, percent weight loss relative to control group. ANOVA (P < 0.05): S, effect of sex; D, effect of diet; S × D, interaction of sex and diet; NS, no significant difference. Post hoc analysis Student’s t-test (P < 0.05): † restricted vs. ad libitum; * females vs. males.
With regard to the analysis between diet groups, the results point out that restricted animals had a greater correlation between \( wV\dot{O}_2 \) and body mass expressions than ad libitum animals. Considering the whole experimental group, the parameter that was best able to explain the variance in \( wV\dot{O}_2 \) and, therefore, in the energy expenditure, was BAT weight. In fact, it is well known that facultative thermogenesis is an important component of energy expenditure in rodents (57), with overall \( \dot{V}O_2 \) being tightly correlated to BAT \( \dot{V}O_2 \) (19).

**UCP1 content in BAT.** Previous studies in male rats have shown that the decrease in energy expenditure that occurs during CR may be due, in part, to a decrease in BAT thermogenic activity (56). Taking into account the decrease in \( \dot{V}O_2 \) induced by CR in females, and in view of the higher correlation between BAT weight and \( wV\dot{O}_2 \) in the same sex, we decided to assess the sex-dependent changes induced by CR in UCP1 content, the main indicator of BAT thermogenic capacity (7).

Figure 5 shows a representative Western blot of UCP1 in BAT. In ad libitum rats, females showed significantly greater levels (175%) of UCP1 compared with their male counterparts \((P < 0.05)\). When the UCP1 levels were adjusted per gram of body weight, which better reflects the physiological relevance, females reached a fourfold higher UCP1 content than males, in accord with previous reports (51, 53).

CR induced a statistically significant drop in UCP1 content in female but not in male rats. In this way, CR seemed to diminish the sex differences found previously in ad libitum animals.

**DISCUSSION**

On the whole, our results showed that CR produced a deactivation of thermogenesis in females, decreasing their energy expenditure. These changes were accompanied by a tendency in restricted females to lose fat and preserve metabolically active organ mass to a greater extent than restricted males.

**Sex differences in ad libitum animals.** In ad libitum feeding conditions, females showed greater energy expenditure and lower energy efficiency than males, suggesting the existence of sex differences in the maintenance of energy balance. Several studies have reported this sexual dimorphism and have pointed to the effect exerted by sex hormones in energy balance (49, 61). In female rats, ovariectomy leads to an increase in energy gain, which is prevented or reversed by estrogens (49, 61), whereas in male rats removal of testosterone causes a reduction in energy gain (50). Although the implication of sex hormones...
in energy balance seems to be clear, the mechanisms involved are still unclear.

One of the possible reasons for energy inefficiency in female rats is the higher proportion of metabolically active organs per unit of body mass. However, this lean phenotype in female rats may be the result rather than the cause of the higher energy consumption. In addition, changes in metabolic rate of specific organs or tissues could also be involved in the higher energy expenditure observed in ad libitum females. In accord with this, liver mitochondria of ad libitum females showed a greater oxidative capacity. These potential differences in hepatic metabolic rate may be due to thyroid hormone action, as female rats have been reported to have a greater hepatic efficiency in the conversion of thyroxine to triiodothyronine (11). A greater activity of thyroid hormone could be responsible for a higher expression of mitochondrial structural and functional related genes, resulting in a greater respiratory capacity (12, 36).

Moreover, ad libitum female rats showed a higher degree of thermogenic capacity than males under usual rodent housing temperature, as indicated by the higher BAT weight as well as the greater UCP1 content. In a previous report (53), we described this sexual dimorphism and observed sex-dependent differences in both morphological mitochondrial features and adrenergic regulation. It should be noted that the housing temperature used in this study (22°C) is far from thermoneutrality (28°C). Sex-related differences in thermogenesis may be due to differences in the threshold temperature at which BAT is stimulated. In agreement with this, a previous work demonstrated that the threshold is set at higher values for females (~22°C) than that of males (~18°C) (46), supporting the thermogenic activation seen in ad libitum female rats.

Taken together, our results support the idea that females may have greater energy expenditure than males due to a higher basal metabolic rate and thermogenic activation.

**Sex differences in the response to CR.** One of the important adaptive responses to CR is the decrease in growth rate (38). In this study, rats showed a similar weight decrease in both sexes; however, there were some qualitative differences in the weight loss of specific tissues. CR induced a loss of weight in metabolically active organs in both sexes, which was more marked in males. This relative resistance to loss of lean mass in females has been observed in several situations in which energy balance tends to be negative, as in the case of exercise (9, 40, 41), diabetes (8), or starvation (63). Taking into account the higher proportion of metabolically active organ mass in restricted than in ad libitum females, greater energy expenditure should be expected when adjusted per unit of body mass. Nevertheless, restricted females showed an important decrease in energy expenditure with respect to ad libitum female rats. This fact indicates that, as other researchers have pointed out (26), the loss of lean body mass alone cannot explain the energy conservation seen when female rats are subjected to food restriction. Thus females seem to activate other mechanisms that allow them to economize energy expenditure to a greater extent than males. Several authors have argued that a reduction in basal metabolic rate of specific organs may be a reasonable mechanism to achieve this decrease in whole body energy expenditure (47). However, in this study, no differences were found in liver mitochondrial respiration rate after 100 days of CR in either sex, suggesting that there are probably no differences in mitochondrial machinery in relation to CR.

Another possibility to explain the drop in energy expenditure seen in female rats may be a decrease in physical activity. In this study, we did not measure physical activity; however, the literature (16, 24, 38, 43) and our daily observation of animals indicate that restricted animals are as active as ad libitum animals.

Because the aforementioned components of energy balance were not able to explain energy conservation in females, nonshivering thermogenesis may be the main cause of the sex-dependent drop in energy expenditure. In this study, we have considered UCP1 in BAT to be the main system effector of thermogenesis; nevertheless, it should be taken into account that mitochondrial proton leak in other tissues, such as skeletal muscle, also contributes, to a large extent, to resting energy expenditure (55, 66). In this way, other uncoupling proteins, particularly UCP3 in skeletal muscle, may function like UCP1, dissipating the mitochondrial membrane potential and contributing to energy expenditure (3, 18, 20, 28, 65). However, the role of these UCPs is a subject of significant controversy, and their physiological functions are yet to be determined (2, 5, 22, 27). Further studies are necessary to establish their role and their involvement in sex-related differences in energy balance.

The evidence presented in this study, such as bivariate analysis regression results, BAT weight, and UCP1 content, suggests that the sexual dimorphism in energy expenditure may be due to changes in BAT heat production. BAT inactivation in response to CR was first described by Rothwell and Stock (56) in male rats subjected to 17 days of food restriction. Our results are in agreement with Rothwell and Stock, as both males and females showed a marked decrease in VO₂ during the initial weeks (Fig. 2). However, with longer-term CR (100 days), only females maintained VO₂ differences between ad libitum and restricted rats. These metabolic findings are not consistent with the reduction in metabolic rate reported in male rats by other authors in response to long-term CR (13). This inconsistency could be explained by the different recording periods used in this study.

Taken together, our results could indicate that CR reduces the sex differences in energy balance observed in ad libitum conditions at 22°C by means of a deactivation of BAT thermogenic capacity in the female rats. The precise molecular basis for the sex-dependent deactivation of BAT thermogenesis in response to CR may be related to sex hormone action, as estrogen-mediated changes in energy expenditure are dependent on BAT and the innervation of this tissue (1, 42, 60, 61). However, in our study, the estrous cycle continued in the restricted female rats, indicating that the decrease in BAT capacity was not due to a loss of the endogenous rhythm of sex steroids.

Evolutionary theories suggest that, during times of short food supply, mammalian females may have been subjected to more severe selection pressure than males. Hence, females evolved mechanisms to exploit their energy resources more efficiently to facilitate their own and their progeny’s survival (29). These mechanisms may involve a reduction in heat production as we have shown in this study. According to James and Trayhurn’s theory, the mechanisms involved in CR adaptation may be the same as those that conserve energy in obese animals (30, 58, 59). Previous results in our laboratory (51) showed that, with cafeteria diet feeding, female rats reach a greater degree of obesity (50% weight gain) than males (34%).
and that this is accompanied by a lower activation of BAT in females than males. Therefore, BAT may be part of the same mechanisms that allow females to achieve energy conservation during CR and lead to a higher propensity for obesity under hypercaloric conditions. Further studies are necessary to clarify the mechanisms involved in this sex-dependent deactivation of BAT and the importance of this component in the maintenance of energy balance when a subject is faced with changes in caloric intake.

In summary, female rats appear to decrease energy expenditure by protecting metabolically active organs to a greater extent than male rats during CR. Sex-dependent deactivation of BAT may be critical to achieving conservation of energy in females, promoting their own survival and that of the species. This aspect should be taken into account in metabolic rate studies and could explain the sex-related differences found in several conditions affecting energy balance.

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REFERENCES
