Rat Brown Adipose Tissue Thermogenic Features are Altered During Mid-Pregnancy

Margalida Frontera, Esperanza Pujol, Sergio Rodríguez-Cuenca, Antoni Català-Niell, Pilar Roca, Francisco J. García-Palmer and Magdalena Gianotti

Grup de Metabolisme Energètic i Nutrició. Departament de Biologia Fonamental i Ciències de la Salut. Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Universitat de les Illes Balears, Palma de Mallorca, Spain

Key Words

Pregnancy • BAT • Uncoupling proteins • Adrenergic receptors • Mitochondria • Leptin

Abstract

Brown adipose tissue (BAT) thermogenesis is inhibited during late-pregnancy and lactation in the rat. However, scarce information concerning BAT functionality during mid-pregnancy is available. The aim of this work was to investigate uncoupling proteins and leptin expression during placentation in rat BAT as well as other key parameters in the thermogenic function of the tissue. BAT mitochondrial content was found to be reduced 50% in 11 and 13 day pregnant rats as compared to nonpregnant controls, although uncoupling protein 1 (UCP1) content was not modified. Furthermore, UCP3 mRNA levels were found to be highly increased during this period. β_3 -adrenergic receptor (β_3 -AR) decreased expression resulted in a higher α_2/β_3 ratio. Finally, leptin mRNA levels in BAT were found to be 3-fold up-regulated in pregnant animals. In conclusion, we show the existence of profound changes in thermogenic features in BAT during gestational days 11 and 13, pointing to the importance of this tissue during mid-pregnancy.

Introduction

Brown adipose tissue (BAT) plays an important role in the regulation of energy balance since it is a major site of facultative thermogenesis, mediated by uncoupling protein 1 (UCP1), a mitochondrial inner membrane protein whose function is to dissipate as heat the proton gradient generated by the respiratory chain. Besides UCP1, the so-called novel uncoupling proteins UCP2 and UCP3 mRNAs are also found in BAT [1, 2]. UCP2 is widely expressed throughout the organism, while UCP3 shows a more strict distribution, mainly expressed in skeletal muscle and BAT. Activity and expression of UCPs are under adrenergic control, mainly through the presence in brown adipocytes of adrenergic receptors (ARs) [3]. The balance between the cellular content of α_2 -AR (inhibitor) and β_3 -AR (activator) is central to the regulation of thermogenesis by modulating the net adrenergic signal response in the adipocyte [4].

During late pregnancy and lactation, diet-induced thermogenesis (DIT) is suppressed in rats [1, 5]. Inhibition of cold-induced thermogenesis in late-pregnant rats has also been reported [6]. This diminished thermogenic activity is related to lowered levels of UCP1 expression and GDP-binding activity [7, 8], as well as decreased lipoprotein

Copyright © 2005 S. Karger AG, Basel

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2005 S. Karger AG, Basel
1015-8987/05/0155-0203\$22.00/0

Accessible online at:
www.karger.com/journals/net

Dr. Magdalena Gianotti
Dept. Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears. Cra. Valldemossa km 7.5, E-07122-Palma de Mallorca (Spain)
Tel. +34 971 173173, Fax +34 971 173184
E-Mail magdalena.gianotti@uib.es

lipase and iodothyronine 5'-deiodinase activity levels [9]. In this way, the augmented energy intake of pregnant rats is efficiently deposited as fat in order to later support the energy costs of lactation. This physiological situation is similar to the suppression of DIT observed in certain diet-induced obesity models [10]. It has been suggested that the increased plasma levels of 17 β -estradiol and/or progesterone in pregnancy could be the main hormonal factors responsible for this inactivation of BAT thermogenesis [11, 12]. On the other hand, the role of decreased plasma leptin levels on the inhibition of uncoupling protein expression in BAT during lactation has also been recently reported [13]. The leptin gene codifies for a 16 kDa protein [14] that plays a central role in the control of energy homeostasis. Among its functions are inhibiting food intake and enhancing energy expenditure. During rat gestation, plasma leptin levels are increased, suggesting the existence of a leptin-resistant state at the hypothalamic level as high leptin levels coexist with the presence of hyperphagia [15].

Although the role of BAT thermogenesis in energy balance in late-pregnant rats is quite well established, its role during earlier energetic adaptations to pregnancy remains obscure. In rodents, placentation in mid-pregnancy is accompanied by a rise of placental steroid hormones synthesis: total rat placental progesterone production is low on day 10 of pregnancy and rises to high levels from day 12 to term [16]. This enhanced steroidogenic function of the placenta coincides with a peak in the plasma levels of progesterone from day 12 to day 16 of gestation [17].

Taking this into account, the aim of this work was to investigate uncoupling proteins and leptin expressions during mid-pregnancy, in the period around which the steroidogenic function of the placenta is established (gestational days 11 and 13), in rat BAT. Additionally, we studied important parameters in BAT thermogenesis, i.e. tissue composition and the expression of adrenergic receptors, in order to elucidate the role of this tissue in early energetic adaptations during pregnancy.

Materials and Methods

Materials

All enzymes, substrates, and coenzymes were obtained from Sigma-Aldrich (Madrid, Spain). Antibodies for UCP1 were obtained from Alpha Diagnostic International (San Antonio, USA). Antibodies for β_3 -AR and α_{2A} -AR were from Santa Cruz Biotechnology Inc. (California, USA).

Routine chemicals used were supplied by Amersham Pharmacia Biotech (Freiburg, Germany), Panreac (Barcelona, Spain), Sigma-Aldrich (Saint Louis, USA) and Roche (Lewes, UK).

Animals

Female Wistar rats, three-months old, purchased from Charles River (Barcelona, Spain), were individually housed at 22°C, with a 12-h light/dark cycle, with free access to tap water and standard chow diet (Panlab, Spain). Female rats were mated with adult males, and the day 0 of pregnancy was determined by the presence of spermatozoa in the vaginal smears. The pregnant rats were studied on days 11 (P11) or 13 (P13) of gestation. Age-matched unmated female rats were used as non-pregnant controls (NP). Body weight and food intake was daily registered in a group of 6 non-pregnant and 6 pregnant animals. The experimental protocol was approved by the bioethical committee of the Universitat de les Illes Balears.

Sacrifice and tissue collection

All animals were killed by decapitation at the start of the light cycle. The interscapular BAT (IBAT) depot was dissected and weighed. For total protein content, cytochrome-c oxidase (COX; EC 1.9.3.1) activity determination, mitochondrial DNA quantification and Western blot analysis, tissue was immediately homogenized in TSE buffer (10 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, pH 7.5 for the mitochondrial DNA (mtDNA) quantification; 300 mM sucrose, 1 mM EDTA, 5 mM MOPS, 5 mM KH₂PO₄, 0.01% BSA, pH 7.4 for the rest of the analyses) with a teflon/glass homogenizer and then filtered through a 250 μ m silk filter. For DNA and triglycerides quantification and Northern analyses, tissue was rapidly dissected, weighed and frozen in N₂ liquid and stored at -70°C. DNA content was measured using diaminobenzoic acid method [18], and the triglycerides content was measured using the Folch extract [19] and determined with a Sigma chemical kit (Triglycerides 320A). Protein concentration was measured by the method of Lowry *et al.* [20]. COX activity was measured spectrophotometrically [21].

Determination of glucose, insulin, progesterone and 17- β -estradiol plasma levels

Plasma glucose was measured with an enzymatic colorimetric kit supplied by Roche Diagnostics. Insulin was determined in plasma aliquots by immunoassay using a double-antibody rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Progesterone and 17 β -estradiol were measured using a competitive immunoenzymatic colorimetric kit from Diametra (Milano, Italy). All assays were performed according to the manufacturers' protocols.

Extraction and quantification of mtDNA

mtDNA was extracted by digestion with Proteinase K (100 μ g/ μ l) in a buffer containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂ and 0.5% Tween 20. Samples were incubated overnight at 37°C and then boiled for 5 min. mtDNA was linearised by digestion with BclI restriction enzyme for 3 h and again boiled for 5 min in order to inactivate the enzyme. Samples

were centrifuged at 10000 rpm for 5 min and the resulting supernatant was used for amplification. A quantitative PCR assay was adapted to the LightCycler technology from Koekemoer *et al* [22], relating the initial number of template copies to the number of cycles at which exponential amplification of the PCR product first occurs. The standard curve was obtained as follows. PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-TACACGAT-GAGGCAACCAAA-3' and 5'-GGTAGGGGGTGTGTTGTGAG-3'. The PCR product was purified with the High Pure PCR template preparation kit (Roche) and the concentration of the purified template was determined spectrophotometrically. Increasing amounts of template were amplified in parallel reactions to obtain a standard curve. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche Diagnostics) using a total volume of 20 μ l containing 0.375 μ M of each primer, 3 mM MgCl₂, 2 μ l LightCycler-FastStart DNA Master SYBR® Green I (Roche Diagnostics) and 5 μ l of sample prepared as described above. The PCR reactions were cycled 35 times after initial denaturation (95°C, 10 min), with the following parameters: denaturation at 95°C for 10 s, annealing at 60°C for 12 s, extension at 72°C for 12 s with temperature transition rates of 20°C/s. Melting curve analysis was performed at the end of each PCR reaction by cooling the samples to 65°C and then increasing the temperature to 99°C at 0.1°C/s. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of the PCR products obtained was characterized by melting curve analysis followed by agarose gel electrophoresis, visualised by ethidium bromide staining. The absence of inhibitory factors in the unknown sample was assessed as described in Koekemoer *et al*. [23].

Northern blot analysis of UCP1-mRNA, UCP3-mRNA, leptin-mRNA, and 18S-rRNA

Total RNA was isolated from a sample of approximately 50 mg of IBAT using TriPure reagent (Roche), following the manufacturer's instructions.

Total IBAT RNA (30 μ g) was denatured with formamide/formaldehyde and fractionated by agarose gel electrophoresis [23]. The RNA was then transferred onto a nylon membrane in 20xSSC (saline sodium citrate, 1xSSC in 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) by capillary blotting for 16 h [24] and fixed with UV light.

Leptin mRNA, UCP3 mRNA, UCP1 mRNA, and 18S rRNA were analyzed sequentially on the same membrane, in the aforementioned order, by a chemiluminescence procedure based on the use of antisense oligonucleotide probes end-labelled with digoxigenin. We used the following probes against rat sequences, labeled at both ends and obtained from Roche: for UCP3 mRNA, 5'-GACTCCTTCTTCCTGGCGATGGTTC-TGTAGG-3'; for leptin mRNA, 5'-GGTCTGAGGCAGGG-

AGCAGCTCTTGGAGAAGGC-3'; for UCP1 mRNA, 5'-GTTGGTTTATTCGTGGTCTCCAGCATAG-3'; and, for 18S rRNA, 5'-CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3'.

Fixed membranes were pre-hybridized at 42°C for 1 h in DIG-Easy Hyb (Boehringer Mannheim) and then hybridized with the corresponding oligonucleotide probe (used at 35 ng/ml, except the corresponding 18S-rRNA probe, which was used at 70 pg/ml) in DIG-Easy Hyb at 42°C overnight. Hybridized membranes were washed twice in a solution of 2xSSC/0.1% sodium dodecylsulphate (SDS) at room temperature, followed by washes in 0.1xSSC/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 (Roche), essentially as in the protocols provided by the manufacturer. Finally, membranes were exposed to Hyperfilm ECL (Amersham). Bands in films were analyzed by scanner photodensitometry and quantified using the KODAK 1D Image Analysis Software 3.5 (Kodak). Stripping between analyses was performed by exposing the membranes to boiling 0.1% SDS.

Western-blot for UCP1 and adrenergic receptors (α_{2A} -AR and β_3 -AR)

Varying amounts of IBAT total protein of homogenate (15 μ g for UCP1 and 25 μ g for adrenergic receptors) were fractioned by SDS-PAGE (12% polyacrylamid for UCP1 and 9% polyacrylamid for adrenergic receptors) according to Laemmli [23] and electrotransferred onto a nitrocellulose filter as described elsewhere [25]. Staining with Ponceau S was used to provide visual evidence for correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Blocking and development of the immunoblots were performed using an enhanced chemiluminescence western blotting analysis system (Amersham). Rabbit polyclonal antibodies against UCP1 and goat polyclonal antibodies against α_{2A} -adrenergic receptor and β_3 -adrenergic receptor were used as primary antibodies. Bands in films were analyzed by scanner photodensitometry and quantified using the Kodak 1D Image Analysis Software.

Autoradiograms of membrane proteins revealed a protein exhibiting an apparent molecular mass of 32, 58, and 55 kDa, for UCP1, β_3 -AR and α_{2A} -AR, respectively. The difference between the values obtained for β_3 -AR and α_{2A} -AR and the corresponding calculated molecular mass based on the deduced amino acids of rat genes is attributed to the presence of carbohydrate moiety [26].

Statistical Analysis

All data are presented as group mean values $\pm$ SEM. Differences between groups were assessed by Student's t-test or one-way analysis of variance (ANOVA) and LSD as post-hoc comparison when an effect of pregnancy was found. Correlations of IBAT leptin expression with IBAT depot weight and triglyceride content were analyzed in non-pregnant rats and pregnant rats in gestational days 11 and 13 by simple linear regression and ANOVA analysis. The analysis was performed with SPSS 12.0 for Windows. The level of probability was set at $p < 0.05$ as statistically significant.

Results

Food intake was found to be increased in both P11 (+55%) and P13 (+58%) rats compared to non-pregnant rats (table 1). This enhanced food intake was accompanied with a progressive increase in body weight throughout the studied gestational period.

Plasma glucose and insulin levels showed an opposite trend during mid-pregnancy (table 2); in spite of increased food intake, plasma glucose was significantly reduced in gestational days 11 and 13, while 13-day pregnant rats showed mild hyperinsulinemia, in agreement with previous reports [27]. As a consequence, the glucose:insulin ratio decreased from 1.87 in the non-pregnant rats to 0.64 in P13 rats. 17 β -estradiol plasma levels tended to increase as pregnancy advanced, although they did not reach statistical significance. However, plasma progesterone levels sharply increased between gestational days 11 and 13 (2.5-fold P13 vs. P11), reaching a 6-fold increase on day 13 over non-pregnant values.

Table 3 displays the variation in IBAT weight during mid-pregnancy. IBAT weight significantly rose from day 11 to 13, reaching a higher value in 13-day pregnant rats (+20%) when compared to non-pregnant rats. Nevertheless, IBAT weight per body weight was significantly lower in each pregnant group (-27%, -17% respectively). The aforementioned increased tissue weight on day 13 was related to unchanged protein content as well as lower DNA content (per gram of tissue). Triglyceride content reached higher levels on day 11 (+60%) returning to non-pregnant levels on day 13. However, no differences were found when this value was corrected by the tissue DNA content, suggesting that there were no differences in the cellular lipid content during these days.

BAT thermogenic function relies on its characteristic mitochondria expressing UCP1. Table 4 compiles the evolution of some mitochondrial parameters (mtDNA content, COX activity, UCP1 content and COX/UCP1 ratio) in IBAT during the gestational period studied. The mitochondrial content of the tissue was decreased more than 60% in pregnant rats when compared to non-pregnant rats, as revealed by the mtDNA content analysis. COX activity, a marker for oxidative capacity of the tissue, was also diminished at day 11. Nonetheless, in spite of showing a lower number of mitochondria, the oxidative capacity of the tissue completely recovered on day 13. Moreover, no changes were observed in UCP1 content (table 4) or UCP1 mRNA levels (table 5) throughout the gestational period studied. This would imply that, as the

	NP	P11	P13
Body weight (g)	259 $\pm$ 9	292 $\pm$ 4 ^a	300 $\pm$ 3 ^a
% body weight increase	6.28 $\pm$ 0.53	14.8 $\pm$ 0.3 ^a	19.1 $\pm$ 0.2 ^{a,b}
Food intake (g)	15.4 $\pm$ 1.2	23.9 $\pm$ 1.3 ^a	24.4 $\pm$ 1.2 ^a

Table 1. Body weight and food intake in non-pregnant and pregnant rats on gestational days 11 and 13. Body weight and food intake were measured in the day of sacrifice. % body weight increase was calculated in relation to initial weight in gestational day 0 in P11 and P13 rats or 13 days before the day of sacrifice for NP rats. Data are the means $\pm$ S.E.M. of 5-6 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

	NP	P11	P13
Glucose (mM)	8.76 $\pm$ 0.35	7.60 $\pm$ 0.31 ^a	6.44 $\pm$ 0.20 ^{a,b}
Insulin (μ g/L)	4.68 $\pm$ 0.48	7.44 $\pm$ 2.52	10.1 $\pm$ 2.2 ^a
17- β -estradiol (pg/mL)	5.21 $\pm$ 1.48	7.77 $\pm$ 1.03	10.5 $\pm$ 2.1
Progesterone (ng/mL)	52.2 $\pm$ 6.7	123 $\pm$ 10 ^a	299 $\pm$ 28 ^{a,b}

Table 2. Effect of pregnancy on fed-state plasma glucose, insulin and sex hormones concentrations. Data are the means $\pm$ S.E.M. of 5-6 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

	NP	P11	P13
IBAT weight (g)	0.30 $\pm$ 0.01	0.29 $\pm$ 0.01	0.36 $\pm$ 0.02 ^{a,b}
g IBAT/kg body weight	1.42 $\pm$ 0.07	1.04 $\pm$ 0.01 ^a	1.18 $\pm$ 0.01 ^a
DNA content			
mg per g of tissue	6.32 $\pm$ 1.37	8.77 $\pm$ 1.03	4.55 $\pm$ 0.66 ^b
mg per tissue	1.84 $\pm$ 0.26	1.77 $\pm$ 0.19	1.35 $\pm$ 0.16
Protein content			
mg per g of tissue	106 $\pm$ 10	110 $\pm$ 8	100 $\pm$ 8
mg per tissue	38.2 $\pm$ 4.9	32.2 $\pm$ 2.6	35.4 $\pm$ 4.6
Triglyceride content			
mg per g of tissue	344 $\pm$ 48	549 $\pm$ 67 ^a	309 $\pm$ 64 ^b
mg per tissue	101 $\pm$ 14	118 $\pm$ 22	102 $\pm$ 24
mg per mg of DNA	71.1 $\pm$ 12.8	64.5 $\pm$ 8.2	68.3 $\pm$ 19.5

Table 3. IBAT weight and composition in non-pregnant and pregnant rats on gestational days 11 and 13. Data are the means $\pm$ S.E.M. of 6 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

number of mitochondria in IBAT is greatly decreased during this period, the content of UCP1 and respiratory chain machinery (as shown by COX activity) per

	NP	P11	P13
mtDNA content			
µg per g of tissue	32.2 ± 7.0	10.7 ± 5.5 ^a	11.1 ± 4.8 ^a
µg per tissue	8.99 ± 1.75	3.36 ± 1.81 ^a	3.80 ± 1.71 ^a
COX activity			
IU per g of tissue	107 ± 9	76.0 ± 7.2 ^a	101 ± 5 ^b
IU per tissue	38.1 ± 3.2	22.3 ± 2.1 ^a	35.5 ± 4.9 ^b
UCP1 content			
AU per g of tissue	100 ± 12	149 ± 18	105 ± 22
AU per tissue	100 ± 16	118 ± 17	92.3 ± 15.1
COX/UCP1 ratio	1.27 ± 0.28	0.59 ± 0.07 ^a	1.28 ± 0.28 ^b

Table 4. IBAT mitochondrial content, cytochrome c oxidase activity and UCP1 protein content in non-pregnant and pregnant rats on gestational days 11 and 13. UCP1 content mean values of the non-pregnant rats were set as 100%. Data represent the means ± S.E.M. of 6-8 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

Fig. 1. Relationship between IBAT leptin gene expression and IBAT weight in non-pregnant and pregnant rats in gestational days 11 and 13.

mitochondrial unit increases, suggesting that changes in replication but not in translation of mtDNA are taking place. Strikingly, UCP3 mRNA was found to be greatly up-regulated (table 5) during mid-pregnancy (+180% and +250% on days 11 and 13 respectively). The COX/UCP1 ratio represents the tendency to uncouple the mitochondrial proton gradient generated by the respiratory chain. Thus, a diminution of this relationship is expected to indicate an increase in mitochondrial uncoupling [28]. 11-day pregnant rats showed a 50% reduction in their COX/UCP1 ratio when compared to non-pregnant rats although this parameter recovered the control value on day 13. This diminution was due to decreased COX activity found in this period rather than to any changes in UCP1 content. In vivo studies have shown that leptin is expressed in BAT [29]. Leptin expression in this tissue

	NP	P11	P13
UCP1 mRNA (%AU)	100 ± 38	161 ± 28	115 ± 29
UCP3 mRNA (%AU)	100 ± 27	283 ± 69 ^a	351 ± 95 ^a
leptin mRNA (%AU)	100 ± 16	145 ± 24	298 ± 38 ^{a,b}

Table 5. Effect of pregnancy on UCP1, UCP3 and leptin relative mRNA levels. UCP1, UCP3 and leptin mRNA mean values of non-pregnant rats were set as 100%. AU, arbitrary units. Data represent the means ± S.E.M. of 5-6 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

	NP	P11	P13
α_2 -AR content			
AU per g of tissue	100 ± 13	114 ± 21	91.3 ± 12.6
AU per tissue	100 ± 14	92.3 ± 17.6	86.5 ± 12.8
β_3 -AR content			
AU per g of tissue	100 ± 12	119 ± 16	49.8 ± 6.6 ^{a,b}
AU per tissue	100 ± 16	93.6 ± 10.6	49.6 ± 10.1 ^{a,b}
α_2/β_3 -AR ratio	1.00 ± 0.11	1.07 ± 0.15	1.94 ± 0.34 ^{a,b}

Table 6. Effect of pregnancy on the relative levels of α_2 - and β_3 -AR protein in IBAT. Adrenergic receptor mean values of the non-pregnant rats were set as 100%; the ratio of α_2/β_3 -AR of non-pregnant rats was set as 1. Data represent the means ± S.E.M. of 8 animals per group. Analysis of variance significance was $p < 0.05$; *a* significant differences versus NP; *b* significant differences versus P11.

seems to be closely related to the energy status of the tissue, with its expression being strongly down-regulated in situations of activated thermogenesis and elevated sympathetic activity [29]. Given this, we considered it interesting to measure leptin mRNA levels in BAT during mid-pregnancy. Leptin mRNA levels were increased 3-fold over non-pregnant values on day 13 (table 5). This sudden increase correlated well in a positive manner with the tissue weight ($r = 0.703$, $p = 0.002$) (fig. 1). Interestingly, the correlation between leptin expression and tissue weight was mainly due to day 13, as shown by the individual correlation values (NP, $r = 0.491$, $p = 0.323$; P11, $r = 0.291$, $p = 0.575$; P13, $r = 0.994$, $p = 0.001$). Surprisingly, despite the lipid content of the tissue being considered the main factor regulating leptin expression in BAT [29], leptin mRNA levels did not show any significant correlation with triglyceride content ($r = 0.093$, $p = 0.733$).

In order to assess the possible existence of changes in the adrenergic signaling pathway, we studied the α_2 - and β_3 -AR levels in IBAT during mid-pregnancy (table 6). α_2 -AR content showed no significant changes between the studied days, while β_3 -AR content was decreased by 50% on day 13. This diminished β_3 -AR content resulted in a 2-fold higher α_2/β_3 ratio. It has been proposed that

an increased α_2/β_3 ratio could be involved in a lower thermogenic capacity as well as a greater tendency to lipid accumulation in the tissue [28].

Discussion

During rat pregnancy the energy requirements of maternal organism are augmented in order to maintain both developing *conceptus* growth and lipid storage to sustain lactation later on. So as to meet these higher energy needs, BAT thermogenesis is inhibited during late-pregnancy and lactation in rats [5].

Gestational body weight increase in the rat accounts not only for the growing *conceptus* and reproductive organs, but for enhanced fat deposition as well [27, 30]. In this study, we found a substantial increase in maternal body weight in the presence of hyperphagia during mid-pregnancy, together with the existence of profound changes in rat BAT characteristics during mid-pregnancy, pointing to the importance of this tissue at this stage. It is worth noticing that some of the changes here described were rapid, as they took place between gestational days 11 and 13. These days are characterized in the rat by the establishment of the steroidogenic function of the placenta [16]. In accordance with this, we show that plasma levels of 17β -estradiol and especially progesterone increase between gestational days 11 and 13 in the rat. Several studies have put under evidence that steroid sex hormones may exert direct or indirect effects on mitochondrial function [31, 32]. Furthermore, direct effects of sex hormones in brown adipocytes thermogenic features have also been reported [33, 34] and they have been claimed to underlie the sex-dependent differences found in BAT [28]. On the other hand, glucose metabolism is also modified during mid-pregnancy [27]: insulin secretion is enhanced from early-pregnancy, when insulin responsiveness is improved, while as pregnancy proceeds insulin-mediated glucose disposal decreases, thus developing an insulin-resistant state in the mother. Therefore, the significant changes in the maternal plasma levels of these hormones during the placentation stage might be central to explain the mechanisms driving to BAT inactivation in the late-pregnant stage.

BAT is hypertrophied in late-pregnancy, mainly due to lipid accumulation [5, 8, 27]. Here we report an already greater tissue weight on day 13 that was not related to increased triglyceride or protein content. It could be therefore proposed that hypertrophy taking place on day 13 would be related to greater tissue water content rather

than changes in other components as other authors have previously shown higher water retention in pregnant rats resulting in increased tissue weights [35, 36].

To our knowledge, this is the first report for an early diminution in the mitochondrial content in BAT during pregnancy. Martin et al. [7] reported diminished mitochondrial content in late-pregnant rats (gestational day 20), but they did not find any differences in rats on day 10. The observed decreased mitochondrial content could be indicative of early changes in mitochondrial biogenesis responsible for the subsequent inactivation of the thermogenic response. Strikingly, this 60% decreased mitochondrial content was not related to lower UCP1 expression, as shown by the protein and mRNA levels. In this picture, unmodified UCP1 content in the tissue together with a decreased number of mitochondria would imply a higher UCP1 content per mitochondrion. An increased UCP1 content in BAT mitochondria during mid-pregnancy was previously reported [37]. This result might be a consequence of the higher plasma sex steroid levels during this period, as progesterone has been shown to increase UCP1 expression in brown adipocytes [33].

UCP3 mRNA levels were found to be progressively increased from gestational day 11 to 13, thus showing a differential pattern from UCP1 mRNA that would support the hypothesis that UCP1 and UCP3 have different functions in BAT. Although a functional role for this protein has not yet been established, some authors suggest that it could be involved in the regulation of lipids as fuel substrate [38, 39] rather than thermogenesis. Fatty acids are considered to be the main substrate for oxidative activity in BAT. However, glucose is also an important fuel for thermogenesis [40]. Glucose uptake by brown adipocytes is diminished during mid-gestation [41], as it is mainly diverted towards the feto-placental unit. In this situation, UCP3 up-regulated mRNA levels could be related to this enhanced utilization of fatty acids in BAT as a result of the impossibility of achieving glucose as substrate. The observed diminution in triglycerides per mg of BAT between day 11 and 13 would be in agreement with this hypothesis.

As a consequence of decreased β_3 -AR content in BAT on gestational day 13, the α_2/β_3 ratio was 2-fold higher than that from non-pregnant animals. This altered ratio could underlie the posterior decrease in UCP1 expression [13] as well as triglyceride deposition [30] in the tissue during late-pregnancy. Progesterone and 17β -estradiol, already showing elevated plasma levels during this period, have been shown *in vivo* to reduce the density of β_3 -ARs in BAT [12] hence being appealing candidates

to be involved in the decreased β_3 -AR levels we report. However, the role of other hormones affecting β -ARs expression such as insulin [42] cannot be ruled out.

Surprisingly, in coincidence with the diminished expression of β_3 -AR, we found 3-fold increased leptin mRNA levels in IBAT. Plasma leptin levels are increased in pregnant rats, with a peak of concentration from gestational days 12 to 19 [43]; this fact has been explained as a result of increased leptin expression in the maternal white fat depots [43] or/and expression and secretion of leptin by the feto-placental unit [44]. Our results indicate that maternal brown fat depots might also be significantly involved in gestational hyperleptinemia. Modulation of leptin expression in BAT has been suggested to be closely associated with alterations in cell lipid content rather than with changes in sympathetic activity in the tissue [29]. Unexpectedly, enhanced leptin mRNA levels in IBAT during mid-pregnancy did not correlate with the lipid content of the tissue. An alternative factor regulating leptin expression in BAT during pregnancy could be insulin, as a stimulatory effect of insulin in leptin expression in brown adipocytes has been reported [45]. Although a precise role for leptin in BAT of 13 days pregnant rats remains to be elucidated, a growing body of evidence indicates that it could have autocrine actions in this tissue as leptin receptors are expressed in brown adipocytes [46]. Leptin has been reported to negatively affect the insulin- signaling pathway in brown adipocytes, resulting in acute and transient induction of insulin resistance [47] thus inhibiting insulin-induced glucose uptake. Therefore, insulin and leptin would interact in a negative feed-back

system in brown adipocytes during mid-pregnancy, in which insulin would activate leptin synthesis in BAT while leptin would exert its effects in an autocrine way producing insulin resistance in the tissue, thus reducing glucose uptake. Furthermore, increased plasma leptin in women suffering gestational diabetes [48] also supports a role for leptin in insulin resistance during pregnancy.

Taking evidences together, we propose that the particular hormonal milieu found during mid-pregnancy, with elevated insulin and progesterone plasma levels, would impair BAT function by the inhibition of the sympathetic system signaling pathway (as shown by the increased α_2/β_3 -AR ratio), that together with the diminution in the mitochondrial content of the tissue would be involved in the reported inactivation of BAT during late-pregnancy and lactation. Nevertheless, further investigation will have to be carried out in order to clarify this point.

Acknowledgements

We are grateful to Dr. Ana Proenza, Dr. Bel Lladó and Dr. Jordi Oliver for critical reading of the manuscript. This investigation was supported by Dirección General de Enseñanza Superior e Investigación Científica of the Spanish Government (BFI2000-0988-C06-06) and by Fondo de Investigaciones Sanitarias (PI021339). M. Frontera and E. Pujol were funded by grants of the Ministerio de Educación Cultura y Deporte of the Spanish Government. S. Rodríguez-Cuenca was funded by a grant of the University of Illes Balears.

References

- 1 Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D, Warden CH: Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* 1997;15:269-72.
- 2 Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB: UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Commun* 1997;235:79-82.
- 3 Lafontan M, Berlan M: Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res* 1993;34:1057-91.
- 4 Valet P, Grujic D, Wade J, Ito M, Zingaretti MC, Soloveva V, Ross SR, Graves RA, Cinti S, Lafontan M, Lowell BB: Expression of human alpha 2-adrenergic receptors in adipose tissue of beta 3-adrenergic receptor-deficient mice promotes diet-induced obesity. *J Biol Chem* 2000;275:34797-802.
- 5 Abelenda M, Puerta ML: Inhibition of diet-induced thermogenesis during pregnancy in the rat. *Pflugers Arch* 1987;409:314-7.
- 6 Imai-Matsumura K, Matsumura K, Morimoto A, Nakayama T: Suppression of cold-induced thermogenesis in full-term pregnant rats. *J Physiol* 1990;425:271-81.
- 7 Martin I, Giralt M, Vinas O, Iglesias R, Mampel T, Villarroya F: Adaptive decrease in expression of the mRNA for uncoupling protein and subunit II of cytochrome c oxidase in rat brown adipose tissue during pregnancy and lactation. *Biochem J* 1989;263:965-8.
- 8 Villarroya F, Felipe A, Mampel T: Sequential changes in brown adipose tissue composition, cytochrome oxidase activity and GDP binding throughout pregnancy and lactation in the rat. *Biochim Biophys Acta* 1986;882:187-91.
- 9 Giralt M, Villarroya F, Mampel T, Iglesias R: Impaired basal and noradrenaline-induced iodothyronine 5'-deiodinase activity in brown adipose tissue from pregnant and lactating rats. *Biochem Biophys Res Commun* 1986;138:1315-21.

- 10 Seydoux J, Benzi RH, Shibata M, Girardier L: Underlying mechanisms of atrophic state of brown adipose tissue in obese Zucker rats. *Am J Physiol* 1990;259:R61-9.
- 11 Abelenda M, Castro C, Venero C, Puerta M: Reduced oxygen consumption of brown adipocytes isolated from progesterone-treated rats. *Can J Physiol Pharmacol* 1994;72:1226-30.
- 12 Malo A, Puerta M: Oestradiol and progesterone change beta3-adrenergic receptor affinity and density in brown adipocytes. *Eur J Endocrinol* 2001;145:87-91.
- 13 Xiao XQ, Grove KL, Grayson BE, Smith MS: Inhibition of uncoupling protein expression during lactation: role of leptin. *Endocrinology* 2004;145:830-8.
- 14 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425-32.
- 15 Lopez-Soriano J, Carbo N, Lopez-Soriano FJ, Argiles JM: Leptin levels and gene expression during the perinatal phase in the rat. *Eur J Obstet Gynecol Reprod Biol* 1998;81:95-100.
- 16 Matt DW, MacDonald GJ: In vitro progesterone and testosterone production by the rat placenta during pregnancy. *Endocrinology* 1984;115:741-7.
- 17 Macdonald GJ, Matt DW: Adrenal and placental steroid secretion during pregnancy in the rat. *Endocrinology* 1984;114:2068-73.
- 18 Thomas PS, Farquhar MN: Specific measurement of DNA in nuclei and nucleic acids using diaminobenzoic acid. *Anal Biochem* 1978;89:35-44.
- 19 Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497-509.
- 20 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- 21 Wharton CD, Tzagoloff A: Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol* 1967;10:245-246.
- 22 Koekemoer TC, Downing TG, Oelofsen W: An alternative PCR assay for quantifying mitochondrial DNA in crude preparations. *Nucleic Acids Res* 1998;26:2829-30.
- 23 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- 24 Jacobsson A, Stadler U, Glotzer MA, Kozak LP: Mitochondrial uncoupling protein from mouse brown fat. Molecular cloning, genetic mapping, and mRNA expression. *J Biol Chem* 1985;260:16250-4.
- 25 Puigserver P, Llado I, Palou A, Gianotti M: Evidence for masking of brown adipose tissue mitochondrial GDP-binding sites in response to fasting in rats made obese by dietary manipulation. Effects of reversion to standard diet. *Biochem J* 1991;279:575-9.
- 26 Cervantes-Olivier P, Durieu-Trautmann O, Delavier-Klutchko C, Strosberg AD: The oligosaccharide moiety of the beta 1-adrenergic receptor from turkey erythrocytes has a biantennary, N-acetylglucosamine-containing structure. *Biochemistry* 1985;24:3765-70.
- 27 Ramos MP, Crespo-Solans MD, del Campo S, Cacho J, Herrera E: Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. *Am J Physiol Endocrinol Metab* 2003;285:E318-28.
- 28 Rodriguez-Cuenca S, Pujol E, Justo R, Frontera M, Oliver J, Gianotti M, Roca P: Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue. *J Biol Chem* 2002;277:42958-63.
- 29 Moinat M, Deng C, Muzzin P, Assimacopoulos-Jeannet F, Seydoux J, Dulloo AG, Giacobino JP: Modulation of obese gene expression in rat brown and white adipose tissues. *FEBS Lett* 1995;373:131-4.
- 30 Lopez-Luna P, Maier I, Herrera E: Carcass and tissue fat content in the pregnant rat. *Biol Neonate* 1991;60:29-38.
- 31 Campbell SE, Febbraio MA: Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am J Physiol Endocrinol Metab* 2001;281:E803-8.
- 32 Morkuniene R, Jekabsone A, Borutaite V: Estrogens prevent calcium-induced release of cytochrome c from heart mitochondria. *FEBS Lett* 2002;521:53-6.
- 33 Rodriguez AM, Monjo M, Roca P, Palou A: Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes. *Cell Mol Life Sci* 2002;59:1714-23.
- 34 Monjo M, Rodriguez AM, Palou A, Roca P: Direct effects of testosterone, 17 beta-oestradiol, and progesterone on adrenergic regulation in cultured brown adipocytes: potential mechanism for gender-dependent thermogenesis. *Endocrinology* 2003;144:4923-30.
- 35 Parsons DS, Van R: Perinatal Changes in the Fluid and Electrolyte Content of Rat Liver. *Nature* 1963;200:268-9.
- 36 Atherton JC, Dark JM, Garland HO, Morgan MR, Pidgeon J, Soni S: Changes in water and electrolyte balance, plasma volume and composition during pregnancy in the rat. *J Physiol* 1982;330:81-93.
- 37 Chan E, Swaminathan R: Role of prolactin in lactation-induced changes in brown adipose tissue. *Am J Physiol* 1990;258:R51-6.
- 38 Samec S, Seydoux J, Dulloo AG: Role of UCP homologues in skeletal muscles and brown adipose tissue: mediators of thermogenesis or regulators of lipids as fuel substrate? *FASEB J* 1998;12:715-24.
- 39 Dulloo AG, Samec S, Seydoux J: Uncoupling protein 3 and fatty acid metabolism. *Biochem Soc Trans* 2001;29(Pt 6):785-91.
- 40 Mampel T, Herrera E: Effects of insulin on the utilization of 14C-glycerol and 14C-glucose in hepatectomized nephrectomized rats. *Diabetes Metab* 1985;11:118-24.
- 41 Nieuwenhuizen AG, Schuiling GA, Bonen A, Paans AM, Vaalburg W, Koiter TR: Glucose consumption by various tissues in pregnant rats: effects of a 6-day euglycaemic hyperinsulinaemic clamp. *Acta Physiol Scand* 1998;164:325-34.
- 42 Hadri KE, Charon C, Pairault J, Hauguel-De Mouzon S, Quignard-Boulange A, Feve B: Down-regulation of beta3-adrenergic receptor expression in rat adipose tissue during the fasted/fed transition: evidence for a role of insulin. *Biochem J* 1997;323:359-64.
- 43 Kawai M, Yamaguchi M, Murakami T, Shima K, Murata Y, Kishi K: The placenta is not the main source of leptin production in pregnant rat: gestational profile of leptin in plasma and adipose tissues. *Biochem Biophys Res Commun* 1997;240:798-802.
- 44 Garcia MD, Casanueva FF, Dieguez C, Senaris RM: Gestational profile of leptin messenger ribonucleic acid (mRNA) content in the placenta and adipose tissue in the rat, and regulation of the mRNA levels of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation. *Biol Reprod* 2000;62:698-703.
- 45 Buyse M, Viengchareun S, Bado A, Lombes M: Insulin and glucocorticoids differentially regulate leptin transcription and secretion in brown adipocytes. *FASEB J* 2001;15:1357-66.
- 46 Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Permin A, Chin WW, Cusin I, Rohner-Jeanrenaud F, Burger AG, Zapf J, Meier CA: Direct effects of leptin on brown and white adipose tissue. *J Clin Invest* 1997;100:2858-64.
- 47 Kraus D, Fasshauer M, Ott V, Meier B, Jost M, Klein HH, Klein J: Leptin secretion and negative autocrine crosstalk with insulin in brown adipocytes. *J Endocrinol* 2002;175:185-91.
- 48 Kautzky-Willer A, Pacini G, Tura A, Biegelmayer C, Schneider B, Ludvik B, Prager R, Waldhausl W: Increased plasma leptin in gestational diabetes. *Diabetologia* 2001;44:164-72.