Mitochondrial Biogenesis and Thyroid Status Maturation in Brown Fat Require CCAAT/Enhancer-binding Protein α*

Received for publication, February 20, 2002, and in revised form, April 5, 2002
Published, JBC Papers in Press, April 8, 2002, DOI 10.1074/jbc.M201710200

M. Carmen Carmona‡, Roser Iglesias‡, Maria-Jesús Obregón§, Gretchen J. Darlington¶, Francesc Villarroya‡, and Marta Giralt‡‡

From the ‡Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona E-08028, Spain, the §Instituto de Investigaciones Biomédicas “Alberto Sols,” Centro mixto CSIC-UAM, Madrid E-28029, Spain, and the ¶Huffington Center on Aging, Baylor College of Medicine, Houston, Texas 77030

Brown adipose tissue (BAT)1 is a major site for nonshivering thermogenesis in mammals in response to either cold or overfeeding. Its thermogenic capacity is due to the presence of the uncoupling protein-1 (UCP1), a mitochondrial protein that uncouples oxidative phosphorylation from the respiratory chain, causing energy dissipation as heat (1). UCP1 is uniquely expressed in BAT and thus constitutes the unequivocal molecular marker of differentiated brown adipocytes. The development of BAT in rodents occurs during the perinatal period (2) and mainly relies on three differentiation programs: (i) the thermogenic program related to the specific induction of UCP1; (ii) the adipogenic program related to lipid synthesis, uptake, and multilocular fat droplet accumulation but also to lipid catabolism to provide the main fuel for thermogenic activity; and (iii) the mitochondrial biogenesis program related to the acquisition of a large content of highly respiratory active mitochondria. All of these processes take place before birth, thus providing fully functional tissue able to respond to the thermal stress associated with birth (3). The thermogenic activity of BAT and UCP1 gene expression are mainly regulated by the sympathetic nervous system innervating the tissue (4, 5). However, since innervation is not fully developed in the fetus (2, 3), other biological factors are expected to be involved in determining brown fat differentiation, including the onset of UCP1 gene transcription. Potential candidates as regulatory factors are thyroid hormones, since complete maturation of BAT thyroid status is achieved during late fetal development (6), due to a high activity of type 2 iodothyronine 5'-deiodinase (D2) necessary for local generation of active thyroid hormone T3 (7, 8). Moreover, there is general agreement on the involvement of T3 in controlling mitochondrial biogenesis, although the molecular mechanisms responsible are not well characterized (9). It has also been reported that endogenous T3 is required for BAT optimal thermogenic function (10), including T3 action through nuclear thyroid receptors (T3R) to up-regulate UCP1 gene expression (11). Other nuclear receptor-mediated pathways have also been implicated in the regulation of UCP1 gene transcription, such as retinoic acid receptors (12) and peroxisome proliferator-activated receptors α and γ (PPARα and -γ) (13), and also in promoting brown fat adipogenesis, such as PPARγ (14). The PPARγ coactivator-1 (PGC-1), which is highly expressed in brown but not in white fat, is involved in the regulation of mitochondrial biogenesis, and it activates UCP1 gene transcription probably by co-activating nuclear receptors (13, 15). The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors has also been described to induce transcription of the UCP1 gene (16) and is suggested to play an important role in the development of BAT (17).

C/EBP are transcription factors of the basic leucine zipper transmission electron microscopy; mtTFA, mitochondrial transcription factor A; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein.

This paper is available on line at http://www.jbc.org

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
family. Several members of the C/EBP family (such as C/EBPα, C/EBPβ, and C/EBPδ) have been described to have tissue-specific expression patterns and to display similar dimerization specificities and to recognize a common DNA-binding element (18, 19). C/EBPα is most abundantly expressed in brown and white adipose tissue, placenta, and liver (20). Several lines of evidence in cell culture systems led to the consideration that C/EBPα is a master regulator of white adipose tissue (WAT) development (19). Thus, C/EBPα overexpression in cultured cells caused adipose differentiation (21, 22), and the expression of C/EBPα antisense RNA blocked this process (23). However, since cell lines differentiate almost exclusively to WAT, little is known about brown adipocyte differentiation. Moreover, it is not known to what extent the observations on adiogenic transcription factor activation in cultured cells are relevant to adipocyte differentiation in vivo. Mice with targeted disruption of transcription factor genes are unique tools to dissect their role on adipose tissue ontogenesis.

Mice with a deletion in the gene for C/EBPα die shortly after birth due to severe hypoglycemia and defective hepatic glycogen storage and gluconeogenesis (24). Knockout mice lacking C/EBPα do not have discernible WAT (24, 25). The first histological analysis of BAT in the C/EBPα-deficient neonates showed a great reduction of fat depots, together with impairment of UCP1 mRNA expression (24). Late fetal development of BAT constitutes a unique model to study brown adipocyte differentiation. For each developmental age, at least two mice were taken from the same litter in each experiment. When possible, two mice were analyzed for each developmental age and genotype.

EXPERIMENTAL PROCEDURES

Animals—The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC and approved by the Comité Étique d’Expérimentation Animal of the University of Barcelona. Heterozygous female mice with a targeted deletion in the gene for C/EBPα (24) were mated with heterozygous males, and the day of pregnancy was determined by the presence of a vaginal plug (day 0). For studies on interscapular region sections, embryonic mice were perfused on day 16, 17, or 18 of gestation. For studies in neonates, pups were studied at 2–4 h after birth. Mice were killed by decapitation and genotyped by Southern blot (24). BAT was collected from the interscapular region. BAT, liver, and heart were harvested, immediately frozen with liquid nitrogen, and stored at −80 °C until RNA or proteins were isolated for analysis. Wild-type, heterozygous, and homozygous mice were taken from the same litter in each experiment. When possible, samples from two or three pups were pooled for each experimental situation. At least three different litters were analyzed independently for each developmental age.

Transgenic mice that express C/EBPα under the control of the albumin enhancer/promoter were generated as described (26). This line was bred into the C/EBPα knockout strain to generate mice that express C/EBPα in the liver but in no other tissue. Pups were studied on day 2 or 7 after birth and genotyped as described previously (26). Transgenic wild-type (TG+, C/EBPα+/+) and homozygous (TG+, C/EBPα/−−) mice were taken from the same litter in each experiment, and at least three different litters were analyzed independently for each postnatal age.

Transmission Electron Microscopy—BAT, heart, and liver samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide and 0.8% FeCNK in phosphate buffer. After dehydration in a graded acetone series, tissue samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H600AB transmission electron microscopy at 75 kV. Two mice were analyzed for each developmental age and genotype.

Stereological Analysis—BAT was sliced to obtain a reference horizontal plane. Subsamples from first slicing were systematically rotated before postfixation and inclusion in Spurr resin as above. For each experimental condition, at least three sections from each of three different blocks from each tissue sample were systematically assessed. The percentage of mitochondrial volume in relation to cell volume (Volmit/Vollum) was estimated following the volume method (27), and surface density (Ss) was estimated following the vertical sections method (28). After estimation of Ss of both inner and external mitochondrial membrane in relation to mitochondrial volume, the surface ratio between membranes were calculated as follows: Smem/Sinten = Sinten × (Volinten/Volintmem) −1. The surface density of inner mitochondrial membrane per cell was calculated using Sinten/Vollum and Volint/Vollum parameters.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted using the Rneasy Mini Kit (Qiagen). For Northern blot analysis, 10–15 μg of total RNA was denatured, electrophoresed on 1.5% formaldehyde agarose gels, and transferred to positively charged nylon membranes (N+; Roche Molecular Biochemicals). Equal loading of gels was checked by ethidium bromide staining and hybridization with an 18 S rRNA probe. Prehybridization and hybridization were carried out as described (29). Blots were stripped and rehybridized sequentially as required in each case. Autoradiographs were quantified by densitometric analysis (Phoretics; Millipore Corp.).

Preparation of Protein Extracts and Western Blot Analysis—BAT was homogenized in buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) containing 0.2 M sucrose (17). The homogenates were centrifuged for 10 min at 1500g. The pellet was resuspended in buffer A (mitochondrial extract) and supernatant was then centrifuged for 10 min at 8500g. The pellet was resuspended in buffer A (mitochondrial extract). All steps were performed at 4 °C. Protein concentration was determined by the micromethod of Bio-Rad using bovine serum albumin as a standard.

For Western blot analysis, samples containing 40 μg of nuclear or mitochondrial protein were mixed with equal volumes of 2× SDS loading buffer, incubated at 90 °C for 5 min, and electrophoresed on SDS–12% (nuclear) or 15% (mitochondrial) polyacrylamide gels. Coomassie Blue staining of gels was performed systematically and showed similar patterns of major nuclear or mitochondrial proteins in the different extracts, thus indicating similar overall quality. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp.), and immunoreactive bands were detected using the following antisera: rabbit antiserum for C/EBPα, kindly provided by Dr. S. L. McKnight; antiserum against C/EBPβ (C-19) or C/EBPβ (C-22) from Santa Cruz Biotechnology, Inc.; monoclonal antibodies specific for cytochrome oxidase subunit IV (A-6409) and subunit I (A-6403) from Molecular Probes, Inc. (Eugene, OR); and rabbit antiserum specific for UCP1, kindly provided by Dr. E. Rial. Immunoreactive material was detected using the micromethod of Bio-Rad. Radiographic images were quantified by densitometric analysis (Phoretics; Millipore Corp.).

Determination of Mitochondrial DNA Abundance—According to previously established procedures, total DNA from interscapular BAT was prepared, and, after digestion with EcoRI endonuclease, 20 μg of DNA was subjected to Southern blot analysis, using the DNA for the 16 S rRNA as labeled probe (30). For nuclear DNA, the blot was hybridized with the murine C/EBPα genomic probe (51).

Thyroid Hormone Content and Iodothyronine 5’-Deiodinase Activity—Determination of tissue T3 content and iodothyronine 5’-deiodinase activity were carried out as previously described (8). Because of the small amount of interscapular BAT during fetal life, determination of T3 content required pools of 2–4 tissues/sample. Thyroid hormone concentration was determined in chloroform/methanol-purified tissue extracts using a specific and highly sensitive radioimmunoassay for T3 (8). Activity of iodothyronine 5’-deiodinase was assayed in BAT homogenates by quantifying the 125I− released from [125I]T3 (8).

Statistical Analysis—Where appropriate, statistical analysis was performed by Student’s t test, and significance is indicated.

RESULTS

Defective Morphological Differentiation of Brown Adipocytes in Homozygous C/EBPα-null Mice—To study whether lack of C/EBPα affects differentiation of brown adipocytes in vivo, transmission electron microscopy (TEM) analysis of developing BAT from C/EBPα-null mice was performed and compared with their wild-type and heterozygous littermates (see Fig. 1). In heterozygous mice, BAT morphology, as well as further results analyzed in the present work, were similar to the wild-type ones (data not shown). BAT section of wild-type mice at birth showed the characteristic ultrastructural morphology of mature brown adipocytes, which is multilocular lipid droplets;
Impaired Brown Fat Mitochondriogenesis in C/EBPα-null Mice

To gain insight into the molecular mechanism underlying the impaired morphological differentiation of C/EBPα-null BAT, we first examined the expression of genes whose functions are related to thermogenesis and adipose metabolism. As depicted in Fig. 2A, the pattern of mRNA expression for UCP1 increased abruptly from day 17 of fetal life, just when it started as we previously described (32). In C/EBPα-null mice, expression of UCP1 mRNA was absent in fetal BAT and only minimal in neonatal BAT. Concordant data were obtained by immunoblot analysis of UCP1 protein levels in mitochondria isolated from developing BAT (data not shown). The expression of the phosphoenolpyruvate carboxykinase gene, a key enzyme in glyceroenogenesis and also target gene for C/EBPα transactivation (33), was impaired in fetal C/EBPα-null BAT but recovered by nearly 50% of wild type in neonates. The expression of other adipogenic markers such as adipocyte-fatty acid-binding protein, lipoprotein lipase, and glucose transporter-4 genes was delayed in C/EBPα-null fetuses but recovered in neonates. In contrast, β-actin mRNA expression was unaltered in the C/EBPα-null mice. Thus, differentiation-dependent expression of genes involved in thermogenic and adipogenic BAT function was either impaired or delayed in the absence of C/EBPα.

C/EBPα Regulates Gene Expression of Transcription Factors Involved in Controlling Brown Adipocyte Differentiation—Next, we examined whether the expression of other transcription regulators involved in adipocyte differentiation was affected by lack of C/EBPα. As shown in Fig. 2B, mRNA levels of PPARγ and of adipocyte determination and differentiation-dependent factor-1 (also called sterol regulatory binding protein-1) were reduced in fetal C/EBPα-null BAT but recovered at birth. A similar profile of expression was observed for the PPARα gene, high expression of which is a differential feature of BAT with respect to WAT and related to its high capacity of lipid oxidation (29). In contrast, ubiquitously expressed PPARδ/β mRNA was unchanged in C/EBPα-null BAT. Taken together, the present findings indicate that besides a direct role of C/EBPα in regulating brown adipocyte gene expression, an indirect effect through its regulation upon expression of other adipogenic transcription factors can also be involved.

Defective Mitochondrial Biogenesis in Brown Fat, but Not in Heart or Liver, of Homozygous C/EBPα-null Mice—As indicated above, TEM analyses of developing BAT sections showed defective mitochondrial maturation in C/EBPα-null mice. As shown in Fig. 3A, mitochondrial maturation during perinatal development was observed in wild-type BAT: mitochondrial size increased, and internal membranes (cristae) were more developed and acquired their characteristic parallel orientation. The stereological quantitation of the surface density of inner mitochondrial membrane per cell, an index of oxidative capacity, showed a significant increase from late fetal to neonatal stage (see Table I). In contrast, not only the percentage of cytoplasm occupied by mitochondria was significantly lower in C/EBPα-null BAT (Table I), but further, mitochondrial mor-

![Fig. 1. Morphology of brown adipocytes in developing brown fat from wild-type and C/EBPα-null mice.](image)

**TABLE I**

Stereological analysis of BAT during perinatal development of wild-type (+/+) and C/EBPα-null (−/−) mice

| Developmental stage | Genotype | Vol_{cell}/Vol_{cell} | Surface ratio S_{IMM}/S_{MEM} | Surface density IMM/Vol_{cell} |
|---------------------|----------|----------------------|-----------------------------|-------------------------------|
| Fetus day 17        | +/+      | 54 ± 2               | 7.88 ± 0.38                 | 4.14 ± 0.24                  |
| Fetus day 17        | −/−      | 15 ± 1***            | 2.73 ± 0.13***              | 0.38 ± 0.02***               |
| At birth            | +/+      | 63 ± 1***            | 9.05 ± 0.65                 | 5.52 ± 0.39*                 |
| At birth            | −/−      | 43 ± 2***##**        | 2.98 ± 0.27***              | 1.48 ± 0.20***##**          |

*Vol_{cell}/Vol_{cell}*, percentage of cell volume occupied by mitochondria.

*IMM*/MEM*, surface of inner mitochondrial membrane with respect to surface of external mitochondrial membrane.

*IMM*/Vol_{cell}, surface density of inner mitochondrial membrane per cell.
phology was defective, with few and randomly oriented cristae (Fig. 3A). C/EBPα-null mitochondria had significantly lower surface density of inner membrane both per external membrane (index of mitochondrial maturation) and per cell volume (index of cell oxidative capacity) when compared with wild-type values at any developmental stage (Table I).

To determine whether defective mitochondrial biogenesis was a more general defect in C/EBPα-null mice, we performed TEM analyses of developing heart, a tissue in which mitochondrial gene expression in C/EBPα-null mice at day 17 (d17) or 18 (d18) of intrauterine life or newborn (NB, 2–4 h after birth) pups. Data are expressed as percentage relative to the point of maximum expression in wild type, which was set to 100. Results are shown as means of three or four independent analyses ± S.E., each one performed by comparing littermates. At least three different litters were analyzed independently for each developmental age. Statistical significance of comparison between genotypes is shown (*, p ≤ 0.05). A representative Northern blot analysis is depicted at the bottom.

![Fig. 2. Developmental regulation of thermogenic and adipogenic gene expression in brown fat from wild-type and C/EBPα-null mice.](http://www.jbc.org/)

While the transcriptional activity of PGC-1α and its downstream targets in C/EBPα-null mice was delayed. In contrast, COI and COIV were not reduced in liver or in heart (data not shown).

We next analyzed whether, besides reduced mitochondrial mRNA expression in C/EBPα-null BAT, other processes involved in mitochondrial genome expression were altered. Results indicated that neither mitochondrial ribosomal 16 S RNA expression nor mitochondrial DNA abundance were altered (Fig. 4, B and C). Taken together, these findings indicate that lack of C/EBPα specifically impairs transcription/translation of nuclear and mitochondrial encoded genes for mitochondrial proteins, but it does not alter mitochondrial DNA replication.

**C/EBPα Regulates Gene Expression of Transcription Factors Involved in Controlling Mitochondrial Oxidative Capacity**—Since gene promoters for OXPHOS proteins are not direct targets of C/EBPα (34), the expression of the main transcription factors involved in regulation of OXPHOS genes was assessed (Fig. 5). Results showed that whereas mRNA expression of nuclear respiratory factor (NRF)-1 and mitochondrial transcription factor A (mtTFA) was not impaired in C/EBPα-null BAT, the expression of the main transcription factor A (mtTFA) was not impaired in C/EBPα-null BAT, the expression of the main transcription factor A (mtTFA) was not impaired in C/EBPα-null BAT, other processes involved in mitochondrial genome expression were altered. A significant decrease in mRNA expression was also observed for T.R genes α and β at day 17 of fetal life. PGC-1 mRNA levels were 50% reduced at day 17 of fetal life in C/EBPα-null BAT. Thus, altered expression of NRF-2, T.R, and/or their coregulator PGC-1 can be involved in mediating defective mitochondrial gene expression in C/EBPα-null BAT.

**Thyroid Hormone Content and Iodothyronine 5′-Deiodinase Activity Are Decreased in Brown Fat of Homozygous C/EBPα-null Mice**—To further investigate whether thyroid hormone, a
BAT showed maximum values on day 18 of fetal life, as previously assessed it (Fig. 6). The ontogenic profile of D2 in wild-type were observed between genotypes.

BAT (IBAT) was also diminished in developing C/EBP development stage (Table II). As the weight of interscapular tissue T3 content was markedly lower in C/EBP mice, the amount of active thyroid hormone expressed as total concentration was found in the C/EBP/H9251 mice. As depicted in Fig. 7A, immunoblot analysis of nuclear protein from wild-type developing BAT showed that transcriptionally active C/EBP (LAP isoforms) and C/EBPβ were overexpressed in BAT probably to compensate for the absence of C/EBPα.

C/EBPα-null mice transgenically expressing C/EBPα in liver show major recovery of the brown fat differentiated phenotype by day 7 after birth.—To determine whether mitochondrial biogenesis recovered during postnatal development, survival of C/EBPα-null mice was achieved by generating a transgenic line with liver-specific expression of C/EBPα (26). TEM analyses of BAT from fetuses at term indicated similar disturbances in transgenic C/EBPα-null with respect to C/EBPα-null mice. Two-day-old transgenic C/EBPα-null pups showed increased lipid accumulation and number of mitochondria when compared with C/EBPα-null neonates but still fewer mitochondria, with smaller size and less developed cristae, than BAT from 2-day-old transgenic wild-type animals (data not shown). When BAT was analyzed on postnatal day 7, an almost total recovery of mitochondrial number and morphology was observed in transgenic C/EBPα-null BAT, although cells contained larger lipid droplets. As shown in Fig. 8, Western blot analysis showed a decrease in the content of COI, COIV, and UCP1 in BAT mitochondria from 2-day-old transgenic C/EBPα-null mice, whereas the levels of these proteins were essentially recovered by day 7. When expression of other C/EBP proteins was analyzed, C/EBPβ was always overexpressed. C/EBPβ abundance was similar but with a slightly higher LAP/LIP ratio, in transgenic C/EBPα-null BAT (data not shown). Thus, the differentiated brown fat phenotype can be recovered postnatally in transgenic C/EBPα-null BAT, probably due to compensatory mechanisms mediated by other C/EBPs.

**DISCUSSION**

BAT differentiation in mice is fully achieved at birth, and here we have established that C/EBPα is essential for this process. Defects on BAT lipid storage observed in the absence of C/EBPα are probably related to impaired lipid metabolism in the tissue. This is reinforced by present results on delayed expression of genes for lipid and glucose metabolism in developing C/EBPα-null mice. This involves not only genes with C/EBPα-regulated promoters, such as phosphoenolpyruvate carboxykinase (33), adipocyte-fatty acid-binding protein (36), and Glut-4 (37), but also other adipogenic-specific marker genes such as the lipoprotein lipase gene. In fact, lack of C/EBPα also alters the expression of other transcription factors involved in adipogenesis in BAT. A parallel alteration in the profile of expression is found for both PPARγ and adipocyte determination and differentiation-dependent factor-1/sterol regulatory binding protein-1: reduced fetal expression but recovery at birth. Different studies have demonstrated that PPARγ is necessary for WAT cell differentiation (for a review, see Ref. 38) but also that PPARγ ligands promote BAT adipo-
Several adipogenic genes are regulated directly by PPARγ activation, including those encoding lipoprotein lipase (39), adipocyte-fatty acid-binding protein (40) and phosphoenolpyruvate carboxykinase (41). The PPARγ gene itself has been described to be directly regulated by C/EBPα (42), but at present, nothing is known about a role for C/EBPα upon adipocyte determination and differentiation-dependent factor. However, expression of the PPARα gene is also impaired in BAT from developing C/EBPα-null mice. PPARα, which is highly expressed in BAT and in terminally differentiated brown adipocytes, is likely to regulate mitochondrial and peroxisomal fatty acid oxidation rates in the tissue (29). Furthermore, we have recently identified PPARα, as well as PPARγ, as
Thyroid hormone content in liver and BAT from wild-type (+/+) and C/EBPα-null (−/−) mice during perinatal development

| Developmental stage | Genotype | T3 | T3 | T3 | IBAT | Samples |
|---------------------|----------|----|----|----|------|---------|
|                     |          | ng/g liver | ng/g IBAT | pg/IBAT | mg   | (tissues/sample) |
| Fetus day 17         | +/+      | 0.19 ± 0.05 | 1.03 ± 0.39 | 5.36 ± 1.72 | 5.25 ± 0.27 | 4 (4) |
| Fetus day 17         | −/−      | 0.25 ± 0.02 | 0.61 ± 0.18 | 2.12 ± 0.04 | 3.55 ± 0.85 | 2 (4) |
| Fetus day 18         | +/+      | 0.36 ± 0.06 | 2.94 | 12.1 ± 0.06 | 6.8 | 1 (2) |
| Fetus day 18         | −/−      | 0.34 ± 0.05 | 0.80 | 5.62 | 6.8 | 1 (2) |
| Neatones             | +/+      | 0.69 ± 0.11 | 2.50 ± 0.39 | 32.2 ± 3.5 | 20.9 ± 0.8 | 3 (2) |
| Neatones             | −/−      | 0.45 ± 0.10 | 0.19 | 1.51 | 7.9 | 1 (2) |

FIG. 6. Developmental changes in iodothyronine 5’d-deiodinase activity in brown fat from wild-type and C/EBPα-null mice. Iodothyronine 5’d-deiodinase activity was determined in interscapular BAT of wild-type (□) or homozygous (○) C/EBPα-null mice at days 17 (d17) or 18 (d18) of intrauterine life or newborn (NB) pups. Data are expressed as final of 1’ released/μg of protein or total tissue and are means ± S.E. of 2–6 mice from independent litters. Statistical significance of comparison between genotypes is shown (*, p < 0.05).

A direct activator of UCP1 gene transcription (13).

The thermogenic capacity of BAT mainly relies on UCP1 gene expression, which is markedly impaired in C/EBPα-null mice. Then the present data demonstrate that C/EBPα is required for appropriate thermogenic differentiation of brown fat. A complex regulation of the gene encoding UCP1 allows for tissue-specific, thermogenic activation and differentiation-dependent expression of the gene, through a distal enhancer (4, 12, 11, 13) and the proximal promoter region (4, 5), in which two C/EBP-responsive elements have been located (16).

Present findings indicate that not all gene promoters trans-activated by C/EBPα in cell culture are similarly affected by lack of C/EBPα in vivo. Differences can be attributed to the temporal pattern of expression, which affects gene promoters such as UCP1 in which expression is switched on later in development (3, 32), or also to the action of other transcriptional regulators of these promoters. For instance, the UCP1 gene, expression of which is most affected by the absence of C/EBPα, is also regulated by PPAR, T₃R, and coactivator PGC-1, whose expression is also altered. A complex transcriptional regulation has also been reported for phosphoenolpyruvate carboxykinase gene expression, including C/EBPβ but also T₃R and PPARγ (33, 41, 43). Thus, besides a direct effect of lack of C/EBPα in the regulation of brown adipocyte gene expression, an indirect effect through its regulation upon expression of other transcription factors can also be involved in leading to defective brown adipocyte gene expression.

Present results suggest that C/EBPα is expressed earlier during brown adipocyte differentiation than C/EBPβ and C/EBPδ, in agreement with previous data (17, 44) and in vitro studies (12, 45). This is in contrast to the white adipocyte model of differentiation proposed from 3T3 cells, in which C/EBPβ and C/EBPδ are expressed earlier in the differentiation program to initially activate transcription of the C/EBPα gene (for a review, see Ref. 46). However, C/EBPα is normally expressed in WAT and BAT of double knockout of C/EBPβ and C/EBPδ mice, suggesting that it may be induced by other factors in vivo (47). In BAT of C/EBPα-null mice, a compensatory mechanism for loss of C/EBPα function is promoted by overexpressing C/EBPβ and C/EBPδ. Furthermore, a biphasic profile of expression is found for C/EBPβ; first it is underexpressed early in fetal development, but later on, just before birth, it is overexpressed. In addition, the ratio of C/EBPβ isoforms (LAP (active) and LIP (inhibitory)) is increased in C/EBPα-null BAT, further contributing to higher C/EBP-dependent transcriptional activation. C/EBPβ isoforms can be generated in liver by two mechanisms, alternative translation (35) and post-translational generation of LIP (48). This last mechanism is regulated by C/EBPα and involves specific proteolytic cleavage of C/EBPβ, which is completely abolished in liver of C/EBPα-null mice (48). Whether present data on change in LAP/LIP ratio in developing BAT are due to a similar mechanism remains to be determined.

The recovery of the BAT differentiated phenotype in 7-day-old transgenic C/EBPα-null mice (see also below) suggests compensatory mechanisms by C/EBPβ and/or C/EBPδ. Accordingly, while this study was in progress, C/EBPα expressed from the C/EBPα gene locus was reported to functionally replace C/EBPα in liver but not in WAT (49). Histological analysis of BAT showed increased lipid accumulation and normal expression of UCP1 mRNA, indicating that C/EBPβ can replace C/EBPα, at least when temporally expressed as is C/EBPα (49). In contrast, the A-ZIP/F1 transgenic mice, in which all C/EBP family members were eliminated by the expression of an artificial dominant negative protein, fail to develop WAT and BAT (50). These observations indicate that the development of BAT in vivo requires the action of any of the members of the C/EBP family. This is consistent, for instance, with the finding that C/EBPα, C/EBPβ (16), and C/EBPδ show a similar capacity to transactivate the UCP1 gene promoter in cultured brown adipocytes.

None of the previously reported studies have addressed BAT mitochondrial biogenesis in any knockout or transgenic mouse model. No OXPHOS gene promoter has ever been reported to be a target of C/EBPα transcriptional regulation (34). However, our present data demonstrate defective mitochondrial biogenesis in BAT due to the absence of C/EBPα. We report that both developmentally related proliferation of mitochondria and mitochondrial differentiation (i.e., acquisition of specific structural, molecular, and functional capabilities of mitochondria related to brown adipocyte function) are impaired in C/EBPα-null mice. Mitochondrial abnormalities in BAT are not a general defect in C/EBPα-null mice due to their severe metabolic derangement, since no obvious differences between genotypes

2 M. C. Carmona, F. Villarroya, and M. Giralt, unpublished observations.

3 P. Yubero, F. Villarroya, and M. Giralt, unpublished observations.
were observed either in heart or liver mitochondria. Furthermore, impaired mitochondrial biogenesis is also found in transgenic C/EBPα-null mice although almost recovered by day 7 of postnatal life. Taken together, present data raise the point that the control of mitochondrial biogenesis is itself a tissue-specific process and, as such, tightly linked to brown adipocyte differentiation.

Brown fat is one of the mammalian tissues with a high content of mitochondria as well as of expression of OXPHOS genes, and furthermore, it is probably the tissue in which these parameters are most modified in response to physiological and environmental stimuli (30, 51). Mitochondrial biogenesis requires the co-ordinate regulation of the expression of the mitochondrial genome and of nuclear genes for the OXPHOS system. Changes in transcriptional rates are considered a major mechanism for regulation of OXPHOS gene expression during development and in response to thyroid hormone (9, 34), although post-transcriptional mechanisms can also be involved (52). The nuclear respiratory factor NRF-2 has been recently described to play a major role in the regulation of OXPHOS gene expression in association with brown adipocyte differentiation (51). Another BAT-enriched factor is PGC-1, which coregulates nuclear receptors (15) and also stimulates mitochondrial biogenesis through regulation of NRF-1 and NRF-2 expression and transcriptional function (50). Since expression of PGC-1 is greatly induced by thermogenic stimulus in BAT (15), a role for this coactivator in transducing physiological stimuli to the coordinate regulation of thermogenesis and mitochondrial biogenesis and function in brown fat has been proposed (38). Present findings indicate that lack of C/EBPα specifically impairs transcription/translation of both nuclear and mitochondrial encoded genes for mitochondrial proteins in BAT. The delay in gene expression of both NRF-2 subunits as well as of PGC-1 may contribute to anomalous OXPHOS gene expression and transcriptional function (53). Since expression of PGC-1 is greatly induced by thermogenic stimulus in BAT (15), a role for this coactivator in transducing physiological stimuli to the coordinate regulation of thermogenesis and mitochondrial biogenesis and function in brown fat has been proposed (38). Present findings indicate that lack of C/EBPα specifically impairs transcription/translation of both nuclear and mitochondrial encoded genes for mitochondrial proteins in BAT. The delay in gene expression of both NRF-2 subunits as well as of PGC-1 may contribute to anomalous OXPHOS gene expression and transcriptional function (53).
expression. In contrast, neither mitochondrial DNA content nor mitochondrial rRNA transcription are altered in BAT from C/EBPα-null mice, which is in agreement with unaffected expression of its direct regulator mtTFA. However, normal expression of mtTFA as well as of NRF-1 is surprising, since expression of PGC-1, which has been described to induce gene expression of NRF-1 and, through co-activation of NRF-1, of mtTFA (53), is found to be delayed. This suggests that either PGC-1 target genes are differentially regulated by PGC-1 or, most probably, that the strength of PGC-1 effect depends on other transcription factors co-activated by it. In that sense, delayed gene expression in C/EBPα-null BAT of nuclear receptors co-activated by PGC-1, such as PPARs or TRs, may also be involved in the marked decrease in OXPHOS gene expression found, as it is likely to occur for impaired UCP1 gene expression.

Many studies have pointed to thyroid hormone as a major regulator of mitochondrial biogenesis and respiratory function in vivo (9). Thyroid hormone up-regulates the expression of several nuclear encoded OXPHOS genes through TRβs (54). In addition to these effects on nuclear genes, it has been recently demonstrated that thyroid hormone directly regulates transcription of mitochondrial DNA and further increases the relative mitochondrial mRNA/rRNA ratio (55). The identification of a truncated form of the nuclear receptor TRα1 in the mitochondrial matrix able to activate transcription of mitochondrial DNA only in the presence of T3 provides further support for a direct thyroid hormone-dependent pathway within the mitochondrion (56). Present results on a delay in TRα1 and TRβ mRNA expression together with the dramatic decrease in T3 levels in BAT from C/EBPα-null mice indicate that thyroid hormone-dependent pathways are impaired. This is consistent with defective OXPHOS gene expression. Furthermore, it is also concordant with changes in mitochondrial transcripts despite unaltered abundance of mitochondrial DNA, indicating a specific effect upon transcriptional mechanisms not caused by changes in gene dosage, identical to that proposed for direct T3 action on mitochondrion (55).

This is the first report of involvement of C/EBPα in determining the thyroid status of a tissue. Maturation of intracellular thyroid status in BAT is unique among other mammalian thyroid-sensitive tissues, since BAT matures completely during late fetal development (6). In fact, maximum T3 binding capacity and T3R expression are attained in BAT before birth (6). Moreover, tissue T3 concentration is higher in fetal BAT than in any other fetal tissue, except thyroid gland itself (8, 57). Circulating T3 levels are low during the fetal period (57), and it is the high activity of type 2 iodothyronine 5′-deiodinase in fetal BAT that determines the high local generation of T3 from circulating T4 (7, 8). In fact, activation of T4 to T3 is catalyzed by two types of iodothyronine 5′-deiodinase, namely D1 and D2. D1 is highly expressed in the liver and thyroid gland among other tissues and is considered to produce the majority of circulating T3. In contrast, D2 is expressed predominantly in BAT, brain, and anterior pituitary, in which it plays a key role in producing T3 locally. The ontogenic profile of D2 in BAT, which peaks in late fetal life, is highly tissue-specific among other thyroid-sensitive tissues in which maximum activity is attained after birth (6). The present data on impaired D2 in BAT from C/EBPα-null mice identify this enzyme as a putative direct target of C/EBPα regulation. In agreement, several potential C/EBP binding sites have been reported in the proximal promoter region of the murine D2 gene (58). Although further research is necessary to determine whether D2 is a direct C/EBPα target gene, our results point to altered thyroid status as a major mechanism by which lack of C/EBPα impairs brown adipocyte differentiation and mitochondrial biogenesis in BAT.

In conclusion, C/EBPα is identified as a master gene for BAT differentiation in vivo. C/EBPα is found to be involved in the acquisition of the thermogenic, adipogenic and mitochondrial-tissue differentiated phenotype of the brown adipocyte. Besides a direct role of C/EBPα regulating transcription of several BAT genes, such as the UCP1 gene, an indirect effect of C/EBPα through thyroid-dependent pathways and other transcription factors is proposed (see Fig. 9). The critical role of C/EBPα in the early stages of BAT differentiation in vivo is potentially replaced by C/EBPβ and/or C/EBPδ in later stages during postnatal life.

Acknowledgments—We thank Dr. Josep Garcia-Valero for assistance in the stereological methods. Technical support by the staff of the Electron Microscopy Service and the Animal Facility of the Faculty of Biology of the University of Barcelona is also acknowledged. We thank Dr. S. L. McKnight, P. Johnson, D. Riquier, R. Hanson, B. Spiegelman, A. Zorzano, S. Enerback, S. Green, P. Grimaldi, C. Mascaro, C. Vallejo, H. Towle, V. Poli, and E. Rial for cDNA probes and antisera.

REFERENCES

1. Nicholls, D. G., Cunningham, S. A., and Rial, E. (1986) in Brown Adipose Tissue (Trayhurn, P., and Nicholls, D. G., eds) pp. 52–65, Edward Arnold Publishers, London.
2. Nederhaard, J., Connolly, E., and Cannon, B. (1986) in Brown Adipose Tissue (Trayhurn, P., and Nicholls, D. G., eds) pp. 152–213, Edward Arnold Publishers, London.
3. Giralt, M., Martin, I., Iglesias, R., Viñas, O., Villarroya, F., and Mampel, T. (1990) J. Endocrinol. 193, 297–302.
4. Caudass-Doulier, A. M., Gelly, C., Fox, N., Schremeti, J., Rainmant, S., Krause, S., Forest, C., Bouillaud, F., and Riquier, D. (1993) Mol. Endocrinol. 7, 497–506.
5. Yubero, P., Barbera, M. J., Alvarez, R., Viñas, O., Mampel, T., Iglesias, R., Villarroya, F., and Giralt, M. (1998) Mol. Endocrinol. 12, 1023–1037.
6. Turca, A., Giralt, M., Villarroya, F., Viñas, O., Mampel, T., and Iglesias, R. (1993) Endocrinology 132, 1910–1920.
7. Giralt, M., Martin, I., Mampel, T., Villarroya, F., Iglesias, R., and Viñas, O. (1988) Biochem. Biophys. Res. Commun. 156, 481–489.
8. Oprea, M. J., Ruiu, D. O., Ké, B. K., Oliveira, R. B., Escobar del Rey, F., and Morreale de Escobar, G. (1989) Am. J. Physiol. 257, E625–E631.
9. Pillar, T. M., and Seitz, H. J. (1997) Eur. J. Endocrinol. 136, 231–239.
10. Bianco, A. C., and Silva, J. E. (1987) J. Clin. Invest. 79, 295–300.
11. Rabelo, R., Schifman, A., Rubio, A., Sheng, X., and Silva, J. E. (1995) Endocrinology 136, 1003–1013.
12. Alvarado, J. de, And, J., Yubero, P., Viñas, O., Mampel, T., Iglesias, R., Giralt, M., and Villarroya, F. (1995) J. Biol. Chem. 270, 5666–5673.
13. Barbera, M. J., Schlüter, A., Pedraza, N., Iglesias, R., Villarroya, F., and Giralt, M. (2001) J. Biol. Chem. 276, 14863–14893.
14. Tai, T. A. C., Jennermann, C., Brown, K. K., Oliver, B. B., MacGinnitie, M. A., Wilkison, W. O., Brown, H. R., Lehmann, J. M., Kliwer, S. A., Morris, D. C., and Graves, R. A. (1996) J. Biol. Chem. 271, 29909–29914.
15. Puigserver, P., Wu, Z. P., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998) Cell 92, 829–839.
16. Yubero, P., Manchado, C., Caudass-Doulier, A. M., Mampel, T., Viñas, O., Iglesias, R., Giralt, M., and Villarroya, F. (1994) Biochem. Biophys. Res. Commun. 195, 653–659.
17. Manchado, C., Yubero, P., Viñas, O., Riet, M., Villarroya, F., and Mampel, T. (1994) Biochem. Biophys. Res. Commun. 195, 1146–1155.
18. Yubero, P., Barbera, M. J., Alvarez, R., Viñas, O., Mampel, T., Iglesias, R., Villarroya, F., and Giralt, M. (1996) J. Biol. Chem. 271, 24753–24760.
19. Linhart, H. G., Ishimura-Oka, K., de Mayo, F., Ribe, T., Repka, D., Poindexter, B., Sick, R. J., and Darlington, G. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12532–12537.
20. CELL Press, Inc., New York.
21. Weibel, E. R. (1979) in Stereological Methods, Vol. 1, pp. 23–57, Academic Press, New York.
22. Baddeley, A. J., Gundersen, H. J. G., and Cruz-Orive, L. M. (1986) J. Microsc. 142, 259–276.
23. Valenzuela, A., Carmona, M. C., Barbera, M. J., Viñas, O., Mampel, T., Iglesias, R., Villarroya, F., and Giralt, M. (1999) Mol. Cell. Endocrinol. 154, 101–109.
24. Martin, I., Giralt, M., Viñas, O., Iglesias, R., Mampel, T., and Villarroya, F. (1995) Biochem. J. 308, 749–752.
25. Semponti, L., Romani, L., Musi, P., Reisi, K., Fattini, A., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, L., Lattanzio, G., Bistori, F., Prati, L., Cortese, R., Gulino, A., Ciliberto, G., Costantini, F., and Poli, V. (1995) EMBO J. 14, 1932–1941.
32. Carmona, M. C., A. Valmaseda, A., Brun, S., Viñas, O., Mampel, T., Iglesias, R., Giralt, M., and Villarroya, F. (1998) Biochem. Biophys. Res. Commun. 243, 224–228
33. Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990) Mol. Cell. Biol. 10, 6264–6272
34. Scarpulla, R. C. (1997) J. Bioenerg. Biomembr. 29, 109–119
35. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
36. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989) Genes Dev. 3, 1323–1335
37. Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 251–255
38. Rosen, E. D., and Spiegelman, B. M. (2000) Annu. Rev. Cell Dev. Biol. 16, 145–171
39. Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Stueler, B., and Auwerx, J. (1996) EMBO J. 15, 5336–5348
40. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) Genes Dev. 8, 1224–1234
41. Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) Mol. Cell. Biol. 15, 351–357
42. Elberg, G, Gimble, J. M., and Tsai, S. Y. (2000) J. Biol. Chem. 275, 27815–27822
43. Giralt, M., Park, E. A., Gurney, A. L., Liu, J., Hakimi, P., and Hanson, R. W. (1991) J. Biol. Chem. 266, 21991–21996
44. Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) Mol. Cell. Biol. 15, 351–357
45. Rehnmark, S., Antonson, P., Xanthopoulos, K. G., and Jacobsen, A. (1993) FEBS Lett. 318, 235–241
46. Lane, M. D., Tang, Q. Q., and Jiang, M. S. (1999) Biochem. Biophys. Res. Commun. 266, 677–683
47. Tanaka, T., Yoshioka, N., Kishimoto, T., and Akira, S. (1997) EMBO J. 16, 7432–7443
48. Weim, A. L., Timchenko, N. A., and Darlington, G. J. (1999) Mol. Cell. Biol. 19, 1685–1704
49. Chen, S. S., Chen, J. F., Johnson, P. F., Muppala, V., and Lee, Y. H. (2000) Mol. Cell. Biol. 20, 7292–7299
50. Moitra, J., Mason, M. M., Olive, M., Kylov, D., Gavrilova, O., Marcus-Samuels, B., Peigenbaum, L., Lee, E., Asyama, T., Eckhaus, M., Reitman, M. L., and Vinson, C. (1998) Genes Dev. 12, 3168–3181
51. Villena, J. A., Viñas, O., Mampel, T., Iglesias, R., Giralt, M., and Villarroya, F. (1998) Biochem. J. 331, 121–127
52. Izquierdo, J. M., Ricart, J., Ostronoff, L. K., Egea, G., and Cuezva, J. M. (1995) J. Biol. Chem. 270, 10342–10350
53. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mothia, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (2000) Cell 98, 115–124
54. Li, R., Luciakovka, K., Zaid, A., Betina, S., Fridell, E., and Nelson, B. D. (1997) Mol. Cell. Endocrinol. 128, 69–75
55. Enríquez, J. A., Fernández-Silva, P., Garrido-Pérez, N., López-Pérez, M. J., Pérez-Martos, A., and Montoya, J. (1999) Mol. Cell. Biol. 19, 657–670
56. Casas, F., Rochard, P., Rodier, A., Cassar-Malek, I., Marchal-Victorion, S., Wiesner, R. J., Cabello, G., and Wurtzaki, C. (1999) Mol. Cell. Biol. 19, 7913–7924
57. Ruiz de Oña, C., Morreale de Escobar, G., Escobar del Rey, F., and Obregón, M. J. (1991) Endocrinology 128, 422–432
58. Song, S., Adachi, K., Katsuyama, M., Sorimachi, K., and Oka, T. (2000) Mol. Cell. Endocrinol. 165, 189–198
Mitochondrial Biogenesis and Thyroid Status Maturation in Brown Fat Require CCAAT/Enhancer-binding Protein α

M. Carmen Carmona, Roser Iglesias, María-Jesús Obregón, Gretchen J. Darlington, Francesc Villarroya and Marta Giralt

J. Biol. Chem. 2002, 277:21489-21498.
doi: 10.1074/jbc.M201710200 originally published online April 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201710200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 31 of which can be accessed free at http://www.jbc.org/content/277/24/21489.full.html#ref-list-1