Brown Fat UCP1 Is Specifically Expressed in Uterine Longitudinal Smooth Muscle Cells

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Until now, uncoupling protein 1 (UCP1) was considered as unique to brown adipocytes. It supports a highly regulated uncoupling of oxidative phosphorylation that is associated with diet as well as with non-shivering thermogenesis. Here we report that UCP1 is not specific to brown adipocytes and can be expressed in longitudinal smooth muscle layers. In the uterus, this conclusion was drawn from different convergent data. A specific antibody against mouse UCP1 revealed, in mitochondrial fractions, a protein with the same molecular weight as brown fat UCP1. Sensitive and specific reverse transcriptase-polymerase chain reaction detected a mRNA whose sequence was totally homologous to that of brown fat UCP1 mRNA. Antibody against UCP1 as well as a UCP1 antisense probe specifically stained uterine longitudinal smooth muscles. UCP1 was also expressed in longitudinal smooth muscle of digestive and male reproductive tracts but was never expressed in other types of smooth muscle, including those of arterial vessels. In uterine tract, UCP1 content was increased after cold exposure or β-adrenergic agonist treatment. It was also up-regulated during the postovulatory period after sexual cycle synchronization. Its content transiently increased during gestation and decreased markedly after birth. These regulations strongly argue about a role for UCP1 in thermogenesis as well as in relaxation of longitudinal smooth muscle layers.

The primary function of mitochondria is to supply the cell with energy as ATP. They are also involved in heat production and have other functions that are not directly related to energy transduction, such as redox state control and Ca2+ homeostasis. In all the functions, membrane potential and permeability play key roles. In specialized thermogenic cells, such as the brown adipocyte, increased proton conductance across the mitochondrial inner membrane was first postulated by Nicholls and Locke (3). The protein supporting this activity was later identified in mitochondrial inner membrane. A protein with the same molecular weight as brown fat UCP1. Sensitive and specific reverse transcriptase-polymerase chain reaction detected a mRNA whose sequence was totally homologous to that of brown fat UCP1 mRNA. Antibody against UCP1 as well as a UCP1 antisense probe specifically stained uterine longitudinal smooth muscles. UCP1 was also expressed in longitudinal smooth muscle of digestive and male reproductive tracts but was never expressed in other types of smooth muscle, including those of arterial vessels. In uterine tract, UCP1 content was increased after cold exposure or β-adrenergic agonist treatment. It was also up-regulated during the postovulatory period after sexual cycle synchronization. Its content transiently increased during gestation and decreased markedly after birth. These regulations strongly argue about a role for UCP1 in thermogenesis as well as in relaxation of longitudinal smooth muscle layers.

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The abbreviations used are: UCP, uncoupling protein; BAT, brown adipose tissue; IBAT, interscapular BAT; RT-PCR, reverse transcriptase-polymerase chain reaction; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; bp, base pairs.

The function of the different UCPs in the maintenance of body temperature has been debated (8). Until now, only UCP1 has been clearly linked to thermogenesis and energy balance. Firstly, physiological regulation during cold exposure and starvation are consistent with this function, whereas this is not the case for other UCPs. Secondly, transgenic ablation of brown fat using a transgene overexpressing toxin under the control of UCP1 promoter (UCP1-DTA mice) led to a decrease in energy expenditure, hypergia, and obesity (9). Thirdly, UCP1 knock-out mice failed to maintain body temperature after cold exposure (10). None of these phenotypes were observed in ucp3−/− and ucp2−/− mice (9, 10).

Nevertheless, two particular features of transgenic models could not be easily explained: (i) in UCP1-DTA mice, altered reproductive function was described and (ii) in ucp1−/− mice, the phenotype is more prominent in female compared with male mice (9–11). According to these phenotypes, we postulated and investigated the putative ectopic expression of UCP1 protein in the female reproductive tract.

EXPERIMENTAL PROCEDURES

Animals—Experiments were performed on female OF 1 (IOPS ca) mice (IFAA-CREDO, L’Arbresle, France) weighing 26–30 g. Animals were housed in a controlled environment (12-h light/dark cycle and 21 °C) with free access to water and standard chow diet. During cold exposure, mice were caged individually at 4 °C for 6 days. To test the effect of β-adrenergic treatment, 0.5 μg/g of mouse/day of isoproterenol were injected intraperitoneally for 5 days.

To synchronize mice hormonally, one group received 5 units of intraperitoneal pregnant mare’s serum gonadotropin (to mimic follicle-stimulating hormone effect), and 46 h later, this group received 5 units of human chorionic gonadotropin (to mimic luteinizing hormone effect). The serum and uterus were immediately removed. The efficiency of hormonal treatment was confirmed by the measurement of sexual hormones by radioimmunoassay (Sorin Biomedica). The uteri were dissected free of adipose tissue and weighed; specimens were fixed in 3.6% formaldehyde-phosphate-buffered saline, pH 7, for histological analysis. The control group were kept in control medium for 24 h before the second injection, mice were killed by cervical dislocation after CO2 anesthesia. The serum and uterus were immediately removed. The efficiency of hormonal treatment was confirmed by the measurement of sexual hormones by radioimmunoassay (Sorin Biomedica). The efficiency of hormonal treatment was confirmed by the measurement of sexual hormones by radioimmunoassay (Sorin Biomedica).
Western Blot Analysis—Mitochondrial fractions were purified by differential centrifugation of tissue homogenates as described previously (12). 20 μg of protein from total homogenate or mitochondrial fractions were electrophoresed in 10% SDS-polyacrylamide gel and transferred (at 1 h) onto nitrocellulose. These membranes were systematically stained with Ponceau red to check that equal amounts of protein were analyzed. Overnight incubation with the same rabbit IgG against mouse UCP1 (UCP1 11A, 0.25 μg/ml) and washings were performed. Peroxidase activity of the second antibodies (diluted 1:8000) was revealed using the ECL kit and Hyperfilm ECL-TM. The blots were exposed for 2–10 min. Positive control was performed with 0.05–2 μg of BAT mitochondria purified from rat BAT.

Immunohistochemistry—After overnight fixation, tissues were dehydrated and paraffin-embedded. Sections (5–7 μm) were incubated for 1 h at room temperature with 0.5 μg/ml UCP1 11A. The second antibody coupled to alkaline phosphatase (1:200) was visualized using BCIP/NBT. Endogenous alkaline phosphatase activity was inhibited by levamisole. Slides were counterstained with nuclear red.

With the double labeling method, UCP1 was revealed using Texas Red-conjugated AffiniPure F(ab')2, fragment goat anti-rabbit IgG (H+L), diluted 1:100, as the second antibody. Monoclonal mouse anti-human smooth muscle actin, diluted 1:50, was used as a marker for actin, and fluorescein isothiocyanate-conjugated horse anti-mouse IgG (H+L), diluted 1:100, was used as the second antibody. Control experiments were performed using purified rabbit IgG and yielded no staining.

In Situ Hybridization—These experiments were performed on paraffin sections. All prehybridization, hybridization, and stringent washing steps were similar to our previously published method (13). Briefly, prehybridization was performed (3 h/42°C) in 80% formamide in 5× SSC and salmon sperm DNA (250 μg/ml). Prehybridized sections were hybridized for 15 min at 70°C and overnight at 42°C in 50 μl of the same solution containing only 40 μg of ssDNA and the DIG-labeled riboprobes (0.5 μg/ml for UCP2 and 0.1 μg/ml for UCP1). Stringent washes consisted of 30 min in 2× SSC (room temperature), 60 min in 2× SSC (50°C), 60 min in 0.2× SSC (50°C), and equilibration (5 min) in Tris-HCl buffer, pH 7.4.

Subsequently, bound probe was detected with alkaline phosphatase-conjugated antidiogoxigenin antibody (1:500 diluted) (Roche Molecular Biochemicals, catalog no. 1093274) and BCIP/NBT substrate. Sections were counterstained with nuclear red. The probes were prepared using the DIG RNA Labeling Kit (Roche Molecular Biochemicals, catalog no. 1-175-025) by in vitro transcription. A BamHI-EcoRI fragment (180 bp) of UCP1 cDNA was ligated into BS-Ad1. A 261-bp antisense riboprobe was synthesized using T3 RNA polymerase (Roche Molecular Biochemicals) from the start codon to position 584 for the murine UCP1 gene. A sense riboprobe was synthesized using T7 RNA polymerase (Roche Molecular Biochemicals) from the start codon to position 763 for the murine UCP1 gene. The riboprobes were hybridized at 70°C for several cycles, as indicated in the figure legends, followed by 7 min at 72°C. 1 μl of a 1:50 dilution of this PCR reaction was used in the nested amplification, using nested sense and antisense primers. The conditions were exactly the same as in the first PCR. In parallel, a 489-bp fragment of actin cDNA (from position 165 to 664) was amplified using sense 5'-CGAGGAGCCCAAGAGAAC-3' and antisense 5'-CTAGGAGCAGAGG-3' primers in the same conditions as the first UCP-1 amplification but with 25 cycles. Amplification products were run onto 1.5% agarose gel in 0.5× TBE (90 mla Tris, 90 mla boric acid, and 50 mla EDTA, pH 8) containing ethidium bromide. The molecular weights of the PCR product were estimated using the kilobase ladder (MW1; Life Technologies, Inc.) or the low molecular mass ladder (MW2; Eurogentec, Seraing, Belgium).

Materials—Purified rabbit IgG against mouse UCP1 (UCP1 11A) were purchased from Alpha Diagnostic International Company (San Antonio, TX). Monoclonal mouse anti-human smooth muscle actin (clone 1A4; M 0851), alkaline phosphatase-conjugated rabbit anti-mouse IgG (D-0314), BCIP/NBT (K-598) and levamisole (X-3021), and “antibody diluent” (S-3022) were all obtained from Dako Corporation (Carpinteria, CA). Alkaline phosphatase-conjugated AffiniPure F(ab')2, fragment donkey anti-rabbit IgG (H+L) (711 056-152) and Texas Red-conjugated AffiniPure F(ab')2, fragment goat anti-rabbit IgG (H+L) (111-076-045) were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-rabbit IgG, peroxidase-conjugated species-specific whole antibody (from donkey), Hybond™-P Transfer membranes (RPN 2020F), ECL revelation kit (RPN 2106), and Hyperfilm ECL-TM (RPN 310–3H) were obtained from Amersham Pharma Biotech. DIG RNA Labeling Kit and alkaline phosphatase-conjugated antidiogoxigenin antibody were obtained from Roche Molecular Biochemicals. Fluorescein isothiocyanate-conjugated horse anti-mouse IgG (H+L) was provided by Vector Laboratories, Inc. (Burlingame, CA). Pregnant mare's serum gonadotropin (Foligon) and human chorionic gonadotropin (Cloruban) were purchased from Intervet (Angers, France). Radioimmunoassay kits were obtained from Sorin Biomedica (Saluggia, VC, Italy).

RESULTS
To establish the specificity of UCP1 11A antibody, it was compared with an antibody directed against whole UCP1 purified from rat BAT known to detect UCP2 (15). Whereas the latter detected a positive signal both in brown fat and in thymus mitochondrial fractions (known to highly express UCP2), the UCP1 11A antibody only revealed one protein in BAT (Fig. 1). Negative results were also obtained with mitochondrial fractions purified from heart and skeletal muscles in which UCP3 is highly expressed (data not shown).

Putative UCP1 expression in the uterus was investigated using the specific UCP1 11A antibody and RT-PCR technology. As shown in Fig. 2A, this antibody (used in Western blot experiments with uterine protein fractions) detected a positive signal at the same molecular weight as that detected in brown fat mitochondria. This signal was stronger in proteins from mitochondrial fractions than in total protein fractions. This is consistent with the mitochondrial localization of the protein. To establish the identity of this signal with the highest specificity...
and sensitivity, nested RT-PCR was performed. As expected, no signal was observed in control RT-PCR nor in NIH3T3 or 10T1/2 cell lines used as negative controls (Fig. 2B and data not shown). In contrast, a single band at the expected size (198 bp) was obtained from uterine RNA (Fig. 2B). Its cloning and sequencing confirmed the perfect homology with the published mouse UCP1 mRNA sequence (16). As shown in Fig. 2B, the signal intensity of the RT-PCR product was proportional to the number of PCR rounds.

To determine the cell type expressing UCP1 gene, immunocytochemistry experiments were performed on the reproductive tract. As shown in Fig. 3, the uterus was clearly stained with anti-UCP1 antibody (Fig. 3B). No staining was observed when the UCP1 antibody was omitted (data not shown). The use of antibody against smooth muscle actin isoform demonstrated that the strong positive fields correspond to longitudinal layers of smooth cells (Fig. 3A). Surprisingly, no labeling was observed in circular smooth cells (Fig. 3B). The co-localization was confirmed by fluorescent double labeling (Fig. 3, C and D). Examination at a higher magnitude revealed clear signals longitudinally organized into smooth muscle cells (Fig. 3E). We never observed contaminating brown fat cells. The same antibody was unable to detect any signal in cardiac or skeletal muscle cells strongly expressing UCP3 nor in the different population of macrophages known to highly express UCP3 (data not shown). To confirm the nature of the detected protein, in situ hybridization experiments were performed and gave identical results (Fig. 3G). The detection of UCP2 mRNA by cross-hybridization of the probe could be excluded because signals obtained with the UCP2 antisense probe were quite different and did not correspond to circular muscle layers (Fig. 3H).

The examination of other tissues containing smooth muscle (Fig. 4) demonstrated that in male reproductive, urinary, and digestive tracts, UCP1 11A antibody specifically stained the smooth muscle cells of the longitudinal muscle layer. No signal could be detected in circular layers, including those of large arterial vessels (Fig. 4P).

Considering the large number of tissues in which UCP1 expression was detected, we focused our investigations on the uterine tract. Cold and adrenergic induction is a characteristic feature of the UCP1 gene among the UCP family. To test for similar regulation, we performed Western blot analysis of mitochondrial fractions from mice exposed to cold for 6 days or treated with isoproterenol, a β-adrenergic agonist. In both cases, treatments enhanced the signal detected by antibodies (Fig. 5, A and B). These changes were confirmed by histological examination of the uterus from control animals compared with isoproterenol-treated mice (Fig. 3, E and F, respectively). The increase in uterine UCP1 content was also observed after ovulation in mice synchronized by hormonal treatment (Fig. 5C).

Finally, we investigated changes in UCP1 content during...
gestation (Fig. 6). UCP1 content in mitochondrial fractions increased during the beginning of gestation and reached its maximal value during the last third of gestation. This content abruptly decreased after birth to return to the control value.

**DISCUSSION**

The main results of this study are that UCP1 expression can no longer be considered as restricted to brown adipocytes and that in the tissues tested, it is specifically expressed in longitudinal smooth muscle layers. Atypical expression of UCP1 had already been described in liver and muscles (17, 18). These observations have been attributed to cross-detection between the different UCPs (17, 19). The existence of other UCPs, and particularly, the broad range of UCP2 tissular expressions further confirmed the exclusive occurrence of UCP1 gene expression in brown adipocytes. To exclude any false interpretation, we carefully validated the different tools and techniques we used. The specificity of the UCP1 11A antibody was confirmed by a variety of data. Firstly, this antibody is unable to detect UCP-like proteins in thymus or muscle, whereas other antibodies do. Secondly, these results were confirmed by alternative methods using RNA detection, such as RT-PCR or in situ hybridization. In the latter case, the experiment on the uterus using UCP2 antisense probe excludes any cross-detection with UCP2. Finally, the sequence of the RT-PCR fragment definitively establishes that UCP1 expression is not unique to brown adipocytes but can occur in smooth muscles. The striking feature of this atypical expression of UCP1 is that, in all tissues examined, it only takes place in longitudinal smooth muscle layers.

To define the physiological importance of such expression, we focused our investigations on the uterine tract. Indeed, among positive tissues, this one is easy to sample, and the relative importance of the different muscle layers is well known according to physiological situations (20, 21). In this tissue, the UCP1 content in the whole uterus could be estimated to be 1000-fold lower than that detected in brown fat mitochondria. This ex-
plains the lack of UCP1 detection by classic methods such as Northern blotting (22). A major feature of the UCP1 gene is its induction by low temperatures and β-adrenergic agonist treatment (23, 24). Both treatments strongly induced the protein in the uterus as well. These results confirm the identity of the detected protein. This strongly suggests that the uterine expression of UCP1 could be involved in non-shivering thermogenesis and is also consistent with the higher sensitivity of \( \text{ucp}^1 \) females to cold exposure (10). A complementary role could be an involvement in the changes in body temperature that naturally occur during the menstrual cycle after ovulation (25). This is indeed reinforced by the postovulatory increase of UCP1 content in uterine mitochondria.

In the uterus, the longitudinal and circular smooth muscle layers control the progression and/or retention, respectively, of the fetus. Indeed, the uterus must expand enormously during pregnancy to accommodate the growing fetus and to support it through sustained muscle tone without generating propagated contractions (20). During this time, adrenergic effects are opposite between the two uterine smooth muscle layers; the contractile effect via the action on α1 receptor and the relaxing effect via β-adrenergic receptor predominate until term in the circular and longitudinal myometrium, respectively. After pregnancy, adrenergic stimulation is down-regulated by different mechanisms (21). These changes are remarkably consistent with the localization of UCP1 expression and its changes after β-adrenergic stimulation and during pregnancy. The uterine role of UCP1 could consist of an effect on cellular energetic processes, inducing an inactive and quiescent contractile state in the positive smooth cells. This body of proof is conclusive enough for us to assume that UCP1 via its uncoupling property could control the mitochondria functions of longitudinal smooth muscle cells. Among these functions, ATP synthesis is the most obvious, but it does not exclude the involvement of UCP1 in reactive oxygen species metabolism and signaling pathways (6). This, in turn, could alter contractile properties of longitudinal smooth muscle layer. Its expression in these cells could partly be involved in the different contractile properties of uterine muscle layers that permit the maintenance of the fetus during the gestation and then its expulsion (20). This agrees with the reported reproductive disturbances of UCP1-DTA mice (11). It is noteworthy that, in this physiological situation, UCP1 action could be facilitated by the increase of blood fatty acids occurring during gestation that stimulates the uncoupling activity of UCP1.

Altogether, this report demonstrates that UCP1 is not specific to brown adipocytes. In addition, our data suggest that as well as its involvement in thermogenesis after cold exposure or during the sexual cycle, it may play a role in other functions such as contraction. Our data agree well with uterine biology and open new perspectives, which must be closely investigated, concerning the physiological and pathophysiological importance of UCP1 in numerous contractile tracts. Indeed, by analogy, we can reasonably assume that UCP1 may similarly participate in the contractile properties of the urinary, digestive, and reproductive tracts. This indirectly emphasizes the importance of mitochondria and their coupling in these tissues.

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