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Concerted Expression of the Thermogenic and Bioenergetic Mitochondrial Protein Machinery in Brown Adipose Tissue

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ABSTRACT

Brown adipose tissue (BAT) is specialized in non-shivering thermogenesis through the expression of the mitochondrial uncoupling protein-1 (UCP1). In this paper, we describe the relationship between UCP1 and proteins involved in ATP synthesis. By the use of BATIRKO mice, which have enhanced UCP1 expression in BAT, an increase in ATP synthase as well as in ubiquinol cytochrome *c* reductase levels was observed. Alterations in mitochondrial mass or variations in ATP levels were not observed in BAT of these mice. In addition, using a protocol of brown adipocyte differentiation, the concerted expression of UCP1 with ATP synthase was found. These two scenarios revealed that increases in the uncoupling machinery of brown adypocites must be concomitantly followed by an enhancement of proteins involved in ATP synthesis. These concerted changes reflect the need to maintain ATP production in an essentially uncoupling cell type. J. Cell. Biochem. 114: 2306–2313, 2013.

KEY WORDS: BROWN ADIPOSE TISSUE; UCP1; ATP SYNTHASE; MITOCHONDRIAL BIOENERGETICS

BAT is a unique tissue in dissipating large amounts of chemical energy as heat, serving as a thermogenic organ in placental mammals allowing the control of body temperature and weight regulation through cold- and diet-induced non-shivering thermogenesis respectively [Nicholls and Locke [1984]; Cannon and Nedergaard [2004]].

Brown fat cells contain a large number of mitochondria where chemical energy is dissipated to produce heat. In ordinary tissues, the proton gradient generated by fuel oxidation is the force which drives ATP synthase activity [von Ballmoos et al., 2009]. However, in BAT, this is disrupted by the uncoupling protein-1 (UCP1) located in the inner mitochondrial membrane [Nicholls et al., 1978; Azzu et al., 2010]. In the absence of UCP1, mice lose their ability to produce heat by non-shivering thermogenesis and exhibit a profound cold intolerance [Enerbäck et al., 1997].

Although BAT has been classically considered nonexistent or irrelevant in human adults, recent observations point out the existence of BAT depots along lifetime [Cypess et al., 2009; Virtanen et al., 2009; Van Marken Lichtenbelt et al., 2009]. This fact, together with several studies focused on BAT differentiation or transdifferentiation [Cinti, 2009; Vila-Bedmar and Fernández-Veledo, 2011] rises the potential targeting of BAT for the treatment of obesity related disorders.

Mitochondrial biogenesis is thought to be essential for the brown adipocyte differentiation program, since large amounts of ATP are required to maintain fully metabolically active brown adipocytes [Wu et al., 1999]. BAT has been classically considered a tissue with a

Abbreviations: AFABP, adipocyte-fatty acid binding protein; AMPK, 5′-adenosine monophosphate activated protein kinase; BAT, brown adipose tissue; IR, insulin receptor; ITT, insulin tolerance test; GTT, glucose tolerance test; UBQCR, ubiquinol cytocrome C reductase; UCP1, uncoupling protein-1; SIRT1, silent mating type information regulation 2 homolog; SOD2, superoxide dismutase-2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

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low expression and activity of ATP synthase [Ježek et al., 2004; Cannon and Nedergaard, 2008]. However, the relationship between coupling and uncoupling metabolism in this tissue has been a subject rs mention the opposite regulation of ATP synthase and UCP1 [Houstěk et al., 1988, 1995; Kramarova et al., 2008], whereas some others find a paradoxical increase in both coupling and uncoupling machinery [Douette et al., 2006; Navet et al., 2007].

Here, we now report in a mouse model with high expression of UCP1 in BAT (BATIRKO) and in a cellular model of brown adipocyte differentiation, that concurrent up-regulation of different proteins involved in uncoupling and ATP generation machinery occurs. This concerted mechanism in BAT assures that an essentially uncoupled tissue may concurrently generate phospho-linked energy to address its metabolic requirements.

MATERIALS AND METHODS

ANIMALS

BAT insulin receptor knock-out (BATIRKO) mice were generated by crossing UCP1-*Cre* and insulin receptor-exon 4 *loxP* mice (IR*loxP/loxP*), as described previously [Guerra et al., 2001]. IR*loxP/loxP* derived from C57/BL6 strain were created by homologs recombination using an insulin receptor gene-targeting vector with *LoxP* sites flanking exon 4 as previously described [Guerra et al., 2001]. BATIRKO mice were obtained in the genetic background of IR*loxP/loxP* animals for fourteen generations to assure that all BATIRKO mice had the same genetic background as its corresponding control animals (IR*loxP/loxP*). BATIRKO and IR*loxP/loxP* male mice from 6 to 8 months of age were used in this study.

GLUCOSE AND INSULIN TOLERANCE TESTS (GTT AND ITT)

GTT were performed on animals that had been fasted overnight, using an intraperitoneal glucose injection of 2 g/kg body weight. Blood glucose values were determined from whole blood obtained from the tail at 0, 30, 60, and 120 min, using an automatic monitor (Boehringer–Mannheim GmbH). ITT were performed on fed animals injected with 1 U/kg body weight of human insulin (Novo Nordisk A/B) into the peritoneal cavity. Blood glucose values were measured at 0, 15, 30, and 60 min using an automatic monitor. Results were expressed as percentage of initial blood glucose concentration.

CELL CULTURE AND TREATMENTS

Fetal brown adipocytes were obtained from interscapular BAT of 20-day-old Wistar rat fetuses and isolated by collagenase dispersion as previously described [Lorenzo et al., 1988]. Isolated cells were plated in tissue culture dishes in MEM supplemented with 10% fetal bovine serum (FBS). Immortalization was performed as described previously [Benito et al., 1993]. The immortalized cell lines were maintained in DMEM supplemented with 10% FBS and antibiotics, at 37°C and 5% CO_2. To assess brown preadipocytes differentiation, cells were cultured for 10 days in 10% FBS-DMEM supplemented with 1 nM insulin, 3 μM rosiglitazone, 100 μM isobutylmethylxanthine (IBMX) and 1 μM dexamethasone (all from Sigma–Aldrich), as previously shown [Vila-Bedmar et al., 2010].

IMMUNOPRECIPITATION AND WESTERN BLOT

After protein content concentration determination from IR*loxP/loxP* and BATIRKO mice, total protein (20–40 μg) was resolved in SDS–PAGE gels for detection of IR. IR levels were routinely analyzed for corroboration of genotype. Alternatively, immunoprecipitation analyses were performed using equal amounts of protein (800 μg) for UCP1 detection. The immune complexes were collected on protein G-agarose beads (Roche Applied Sciences) and submitted to Western blot analysis. After SDS–PAGE, gels were transferred to Immobilon-P PVDF membranes (Merck–Millipore). Then, membranes were blocked with 5% non-fat dried milk or 3% bovine serum albumin; and incubated overnight with antibodies at 4°C. Antibodies anti-UCP1 (M-17) and anti-IRβ (sc-711) were from Santa Cruz. Antibodies against UBQCR (Rieske subunit) and ATP synthase (subunit d) were from Life Technologies. Anti P-AMPK Thr172, SIRT1 and PGC-1α antibodies were from Cell Signaling. Anti Hsp60 antibody was obtained from Enzo Life Sciences. Anti-β-actin was from Sigma–Aldrich. Immunoreactive bands were visualized using the ECL Western blotting protocol (GE Healthcare).

TWO-DIMENSIONAL ELECTROPHORESIS (2DE)

For 2DE, 500 μg of protein extracted from IR*loxP/loxP* or BATIRKO BAT was diluted in 350 μl of urea 8 M, CHAPS 2% (w/v), dithiothreitol 40 mmol/L, Bio-Lyte ampholyte 0.2% (Bio-Rad), and bromophenol blue 0.01% (w/v). The samples were loaded on immobilized gradient strips (pH 3–10), and isoelectric focusing was performed using a Protean immunoelectrophoresis cell system (Bio-Rad). After 16 h at 50 V for rehydration, we proceed with the first dimension. Before second dimension, the proteins from the strips were equilibrated with SDS and iodoacetamide treatment, and resolved on 10% SDS–PAGE gels. Silver-stained 2D gel electrophoresis gels were scanned, and digitized images were recorded and imported to PD-Quest. Analysis was performed matching the spots from at least four different gels of different animals for each group. For compensation for any variation in protein loading and development level of silver stain, spot quantity was normalized on the basis of the total staining density of the image.

ELECTRON MICROSCOPY ANALYSIS OF BAT AND MITOCHONDRIAL QUANTIFICATION

BAT samples were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), 2.5% glutaraldehyde grade I (Sigma) in 0.1 M sodium phosphate buffer (pH 7.3) for 4 h. Samples were post-fixed in 1% OsO_4 (Electron Microscopy Sciences) 1.5% $K_4[Fe(CN)_6]$ during 1 h, dehydrated with acetone and embedded in Epon-812 (Taab). Thin sections (60–70 nm) were obtained with an Ultracut E (Leica) ultramicrotome, stained with lead citrate and examined under a JEM-1010 transmission electron microscope (JEOL). BAT was obtained from two animals per group; at least 70 electron micrographs per animal were analyzed to measure mitochondrial and cytoplasmic areas.

TOTAL ATP CONTENT MEASUREMENT FROM BAT

The kit (ATP bioluminescence assay Kit HS II, Roche Applied Sciences) was used for ATP determination. We obtained BAT from IR*loxP*/*loxP* and BATIRKO mice. Briefly, after PBS washing, BAT were

homogenized in PBS in the presence of protease inhibitors. The tissue was totally disrupted by the use of an electrical homogenizer (ULTRA-TURRAX, Ika) at maximum speed. After that, the samples were centrifuged at 10,000*g* for 2 min at 4°C. The pellet was washed 3 times with PBS and then resuspended in 50 μl of cold ATP-lysis buffer (100 mM Tris, 4 mM EDTA, pH 7.75). Then 150 μl of boiling ATP lysis buffer were added and samples were incubated for 2 min at 99°C. After centrifugation at 10,000*g* for 1 min at 4°C pellets were discarded. For ATP measurement 50 μl of the samples were mixed with 50 μl of luciferase reagent accordingly with the manufacturer's instructions.

MITOCHONDRIA ISOLATION AND MEASUREMENT OF CYTOCHROME *C* OXIDASE ACTIVITY

Mitochondria from 9 month old IR*loxP*/*loxP* and BATIRKO mice were isolated as follows. Briefly, the animals were sacrificed and the interscapular BAT was dissected and homogenized in a solution containing sucrose 250 mM, Tris–HCl 5 mM, EDTA 2 mM at pH 7.4 with a glass homogenizer using a Teflon pestle. The homogenate was centrifuged at 8,500*g* for 10 min. The fat layer and supernatant were discarded, and the pellet was resuspended in a solution containing sucrose 250 mM, Tris–HCl 20 mM, BSA 0.2% at pH 7.2 and centrifuged again at 700*g* for 10 min. The supernatant was transferred to a clean tube and centrifuged again at 8,500*g* for 10 min and the resulting mitochondrial pellet was resuspended in PBS and stored at -20°C. At the time of use, mitochondria were frozen and thawed three times in liquid nitrogen and centrifuged 5 min at 700*g* and the pellet was discarded. Cytochrome *c* oxidase activity was assayed at 550 nm. Twenty micrograms of mitochondria protein were added to 1 ml of isosmotic medium containing 50 mM KH_2PO_4, pH 7 and 0.1 μM reduced cytochrome *c*. Fresh cytochrome *c* solution was reduced by adding some crystals of sodium dithionite. Measurements were performed in a Thermo Electron Corporation spectrophotometer at 30°C with a stirrer. The reaction was followed for 3 min and the rate of the reaction was calculated as the slope of the linear change in absorption. Conversion to enzyme activity was achieved using the Lambert–Beer equation taking 21.84 $mM^{-1}\,cm^{-1}$ as the value of molar extinction coefficient.

STATISTICAL ANALYSES

Statistically significant differences between mean values were determined using paired Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

BAT FROM BAT-SPECIFIC INSULIN RECEPTOR KNOCKOUT MICE (BATIRKO) IS ENRICHED IN UCP1

As we previously described [Guerra et al., 2001], BATIRKO mice showed a reduction in BAT size as observed in the image as well as in the quantitation of BAT weight (Fig. 1A). The increase in UCP1 expression (Fig. 1B) was probably as a compensatory mechanism due to the reduction in functional tissue. As expected, BATIRKO mice lacked the insulin receptor in BAT (Fig. 1C). These mice were glucose intolerant as showed in the glucose tolerance test (GTT) (Fig. 1D) but analysis of insulin tolerance test (ITT) discarded insulin resistance (Fig. 1E). Then, we sought to investigate the major changes in the BAT proteome related to UCP1 overexpression in these animals.

PROTEOMIC ANALYSIS OF BAT FROM BATIRKO MICE

The 2-DE analysis of BAT proteomes from IR*loxP*/*loxP* versus BATIRKO mice led to the identification of some proteins differently expressed between these two groups. We observed a total repression of the fatty acid binding protein in BATIRKO compared with IR*loxP*/*loxP* mice. Furthermore, in BAT from BATIRKO we detected an increase in proteins involved in metabolic processes, such as the glycolytic enzyme triose-phosphate isomerase. On the basis of UCP1 increase in BAT, we expected modifications in mitochondrial proteins involved in the electron transport chain and ATP synthesis. We identified an increase in both ATP synthase subunit-d and ubiquinol–cytochrome *c* reductase (UBQCR). Moreover, the levels of adenylate kinase 1 increased compared with the IR*loxP*/*loxP* mice. Also a significant increase of superoxide dismutase 2 (SOD2) was identified in BAT proteomes from BATIRKO mice. On the other hand we found a diminished expression of adipocyte–fatty acid binding protein (AFABP) (Fig. 2, Table I and Supplementary Table I).

BAT FROM BATIRKO MICE DOES NEITHER INCREASE MITOCHONDRIAL CONTENT NOR ATP LEVELS

In order to determine whether the observed changes in mitochondrial proteins were due to an increase in mitochondrial density in BAT, two different approaches were carried out. First, we analyzed the levels of a mitochondrial content marker protein such as Hsp60 in total protein extracts from BAT. We observed a slight, although non-significant increase in the levels of Hsp60 in BATIRKO mice (Fig. 3A). Our second approach was the determination of mitochondrial density by electron microscopy. We observed a slight increase, but not statistically significant, in mitochondrial content in BATIRKO mice in comparison with IR*loxP*/*loxP* mice (Fig. 3B). In addition, no further differences in mitochondrial morphology between both IR*loxP*/*loxP* and BATIRKO mice were revealed by electron microscopy analysis (Fig. 3B). To assess if changes observed in BAT proteome affected ATP production, we measured the intracellular ATP levels in the tissue. We observed no difference in ATP content in BAT comparing IR*loxP*/*loxP* versus BATIRKO mice (Fig. 3C). Cytochrome *c* oxidase activity was found to be diminished in mitochondria isolated from BAT of BATIRKO mice (Fig. 3D).

CONCURRENT INCREASE OF COUPLING AND UNCOUPLING MITOCHONDRIAL MACHINERY ALONG BROWN PREADIPOCYTE DIFFERENTIATION

In order to corroborate that coupling and uncoupling machinery changed in the same direction, brown preadipocytes were submitted to a differentiation protocol. Along the differentiation process there was an increase in UCP1, as well as in mitochondrial proteins from the electronic transport chain such as UBQCR, or involved in ATP synthesis such as ATP synthase subunit-d (Fig. 4A). In addition, SOD2 expression was increased during the first days of differentiation, although it remained stable during the latest phase in which coupling

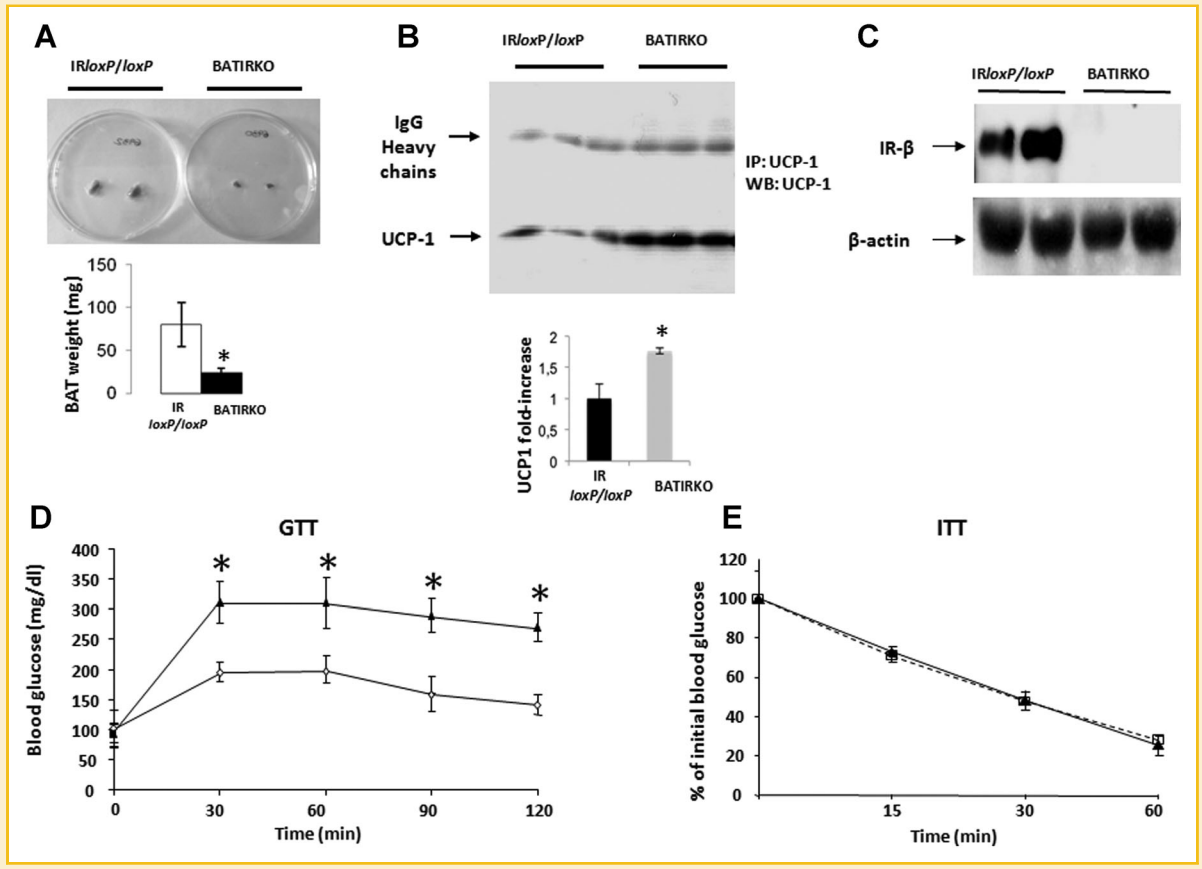

Fig. 1. A: BAT size comparing IR*loxP/loxP* versus BATIRKO mice. The graph shows BAT weight, expressed in milligrams (mg) of five animals per genotype. BAT from BATIRKO mice shows an increase in UCP1 protein content. B: Representative immunoprecipitation and Western blotting against of UCP1 in the BAT from IR*loxP/loxP* and BATIRKO mice. The plot represents the fold-increase in the quantitation of UCP1 protein levels comparing IR*loxP/loxP* versus BATIRKO mice. Data are presented as means ± SD. $^{*}P < 0.05$ compared with IR*loxP/loxP* mice. C: Western blotting showing the levels of insulin receptor comparing several IR*loxP/loxP* and BATIRKO animals. D: Glucose tolerance test from IR*loxP/loxP* and BATIRKO animals (n = 5). Results are expressed as mean blood glucose concentration ± SD. $^{*}P < 0.05$ comparing the different time points from IR*loxP/loxP* versus BATIRKO mice. (□) Represents IR*loxP/loxP* mice and (▲) represents BATIRKO mice. E: Insulin tolerance test comparing IR*loxP/loxP* and BATIRKO mice (n = 5). Results are expressed as the percentage of initial blood glucose concentration ± SD (▲) with continuous line represents IR*loxP/loxP* mice and (□) with dashed line represents BATIRKO mice.

and uncoupling machinery increased. Other proteins involved in mitochondrial biogenesis also augmented such as SIRT1 and PGC-1α (Fig. 4A). Rapamycin treatment was able to block this differentiation protocol, as previously shown [Vila-Bedmar et al., 2010], and under rapamycin exposure neither proteins involved in uncoupling nor coupling metabolism were increased following the same differentiation protocol (Fig. 4B).

DISCUSSION

The most significant finding of this study is the fact that both uncoupling and coupling mitochondrial machinery of BAT or brown adipocytes are concurrently enhanced. BAT clinical relevance is increasingly recognized, as recent findings show the existence of BAT depots and resident cells in adult humans [Cypess et al., 2009; Van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009]. Other studies point to the possibility of BAT-related therapies for the treatment of diabetes and obesity related disorders [Cypess and Kahn, 2010; Langin, 2010; Nedergaard and Cannon, 2010].

In this study, the glucose intolerant phenotype of BATIRKO mice was confirmed. These mice showed unaltered insulin sensitivity, suggesting that the defect in glucose tolerance is dependent on insulin secretion. BATIRKO mice presented an altered glucose stimulated insulin secretion defect as it was previously demonstrated [Guerra et al., 2001]. The use of BATIRKO mice, which show a severe BAT lipoatrophy and enhanced UCP-1 expression, revealed a twofold increase of the mitochondrial electron transport chain protein UBQCR as well as in the ATP synthase. Similar levels of ATP between IR*loxP/loxP* and BATIRKO mice were observed. Surprisingly, cytochrome *c* oxidase activity was reduced in BATIRKO mice; this might indicate a diminished mitochondrial oxidative capacity, although we cannot discard an increase in ATP synthase activity. In fact, some authors report the possibility of compensatory regulation between different mitochondrial respiratory complexes [Havlíčková et al., 2012; Kovářová et al., 2012]. Several evidences suggest that extramitochondrial ATP synthase could play a role in the generation of ATP in different cell types [Ravera et al., 2009; Panfoli et al., 2011]. However, we could not observe any difference in ATP synthase localization

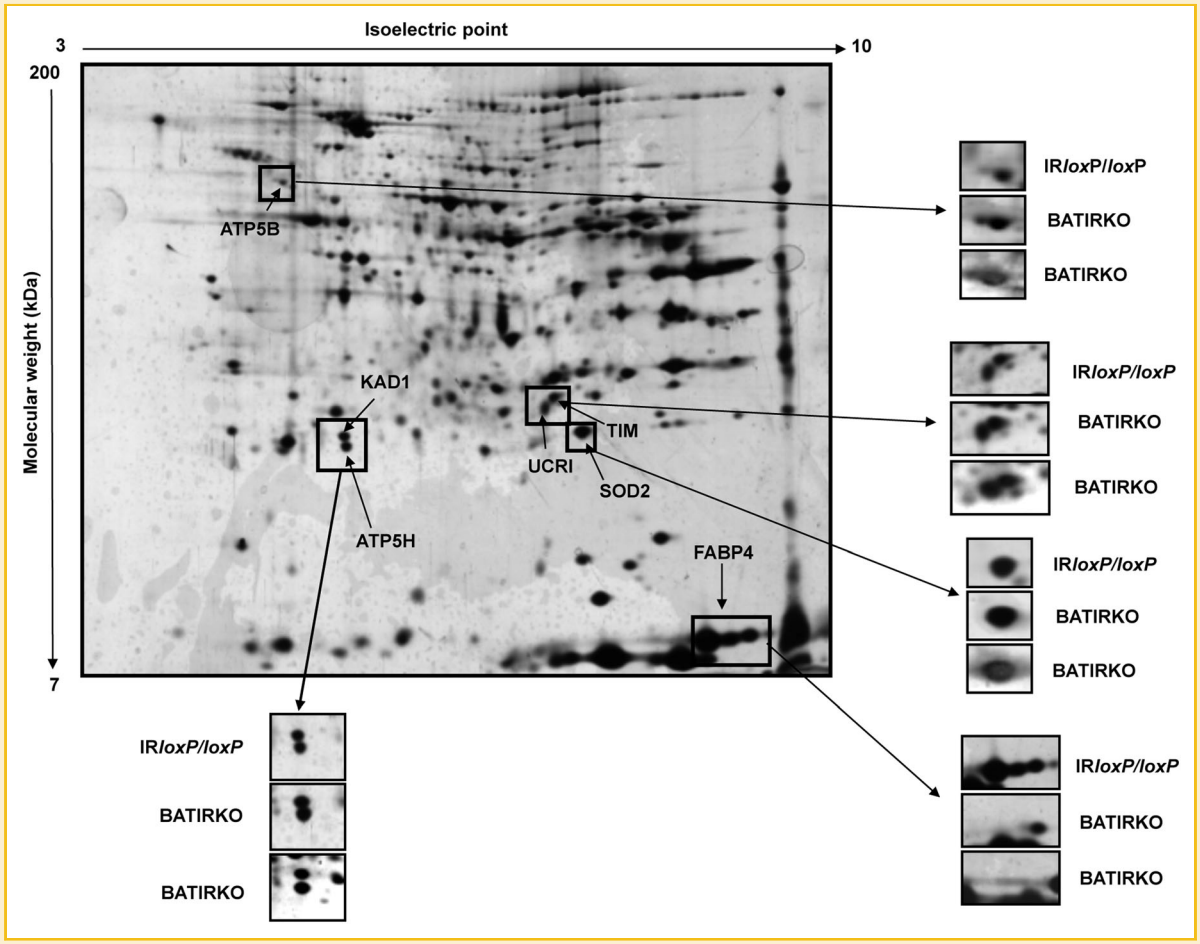

Fig. 2. Representative 2DE gel of BAT proteome obtained from IR*loxP*/*loxP* mice. The inset indicates the location of the determined proteins that changed comparing with BATIRKO mice. The different images shown outside the gel represent the spot intensity observed in different animals from BATIRKO mice compared with IR*loxP*/*loxP* mice.

(data not shown). Thus, the increase of ATP synthase could be attributed to a compensatory mechanism, in order to moderate the decrease in ATP synthesis expected from UCP1 overexpression in BATIRKO mice. Furthermore, we found an up-regulation in the glycolytic enzyme triose-phosphate isomerase, and the cytosolic isoform of adenylate kinase. These changes suggest that BAT sustains the ability of increasing ATP, apart from mitochondria, by substrate-level phosphorylation. In fact, pointing to the idea that an increase of enzymes involved in uncoupling metabolism concomitantly define an increase of the protein involved in ATP synthesis (ATP synthase and substrate-level phosphorylation). More importantly, the changes in the ATP synthesis machinery occurred in the absence of insulin signaling in BAT from BATIRKO mice, indicating that UCP-1 and ATP synthesis enzymes could be enhanced by activation of the sympathetic innervations [Ribeiro et al., 2010]. However, we cannot exclude other possibilities such as IGF1 and BMP7 signaling that

TABLE I. Identified Proteins by Mass Spectrometry or by Comparison With the Machset Gel From Mouse BAT in the Expasy Proteomics Server

Protein name	Abbreviation	pI/MW (kDa)	Ions scores	Accession number	Fold-increase
Triose-phosphate isomerase	TIM	7.08/27	12	UmProt: P17751	$1.3 \pm 0.1^{\#}$
Ubiquinol cytochrome *c* reductase (RIESKE subunit)	UQCRFS1	8.91/29.6	32	UniProt: Q9CR68	$2.0 \pm 0.2^{\#}$
ATP synthase (F_0 subunit d)	ATP5H	5.52/18.6	20	UniProt: Q9DCX2	$2.0 \pm 0.3^{\#}$
ATP synthase (F_i subunit β)[a]	ATP5B	5.1/46.0	[a]	UniProt: P56480	$2.0 \pm 0.1^{\#}$
ATP–AMP transphosphorylase	KADI	5.67/21.0	35	UniProt: Q9R0Y5	$2.0 \pm 0.2^{\#}$
Adipocyte fatty acid binding protein 4	FABP4	8.55/14.6	63	UniProt P04117	Disappearance[#]
Superoxide dismutase 2	SOD2	7.15/22.4	29	UniProt: P09671	$1.2 \pm 0.1^{\#}$

This table also includes the fold-increase obtained in the different proteins and the protein name, abbreviation, and the accession number from the UniProt server. The fold-increases are the ratio of at least three different samples from IR*loxP*/*loxP* versus BATIRKO mice, expressed as means ± SD.

[a]http://www.expasy.org/swiss-2dpage/viewer.

[#]$P < 0.05$.

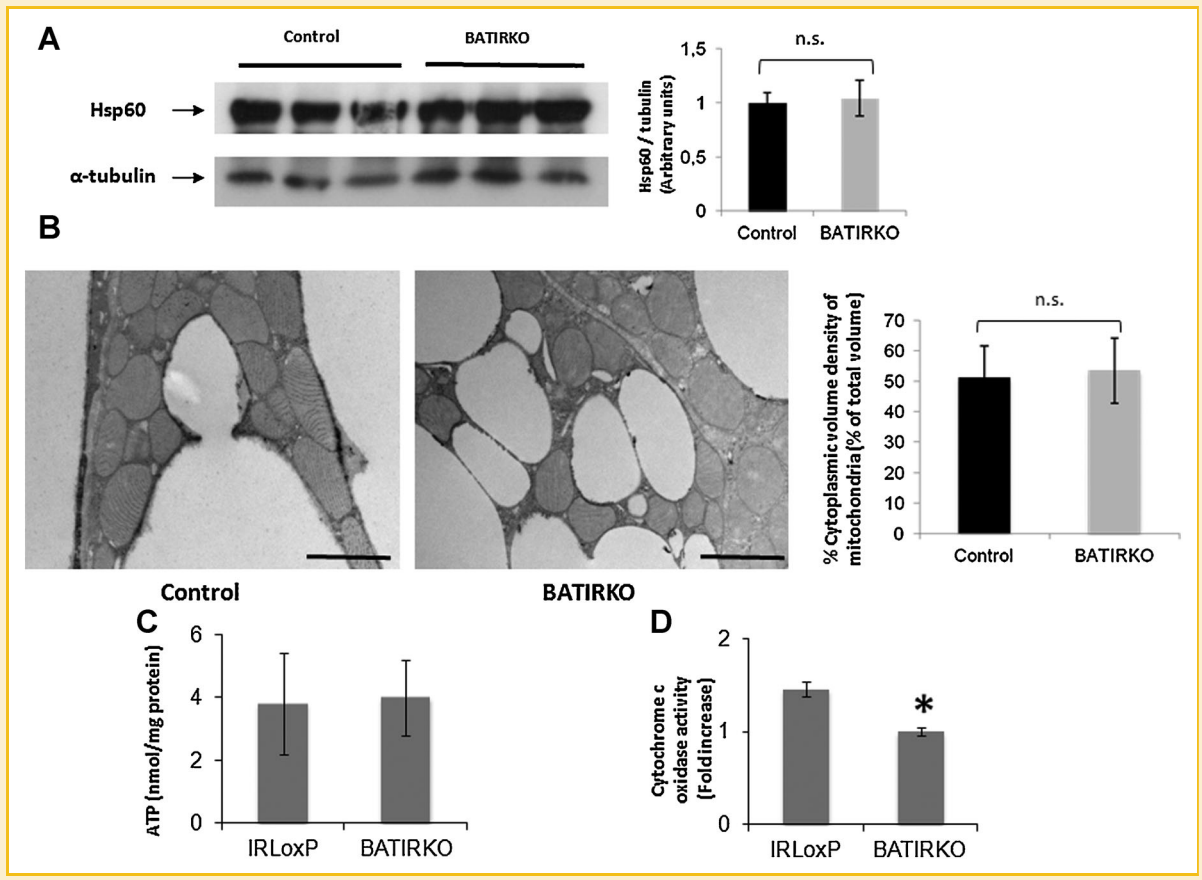

Fig. 3. Similar mitochondrial and ATP content in BATIRKO and IR*loxP*/*loxP* mice. A: Blots from BAT protein, Hsp60 were used as a mitochondrial content marker. B: Representative electron micrographs from BAT samples of IR*loxP*/*loxP* mice (Control) and BATIRKO, bars represent 1 μm. On the right, mitochondrial density quantitation results represented as proportion of cytoplasmic area occupied by mitochondria of electron micrographs analyzed, and expresed as mean ± SD. C: ATP levels expressed as nmol/mg of protein in BAT tissue from BATIRKO and IR*loxP*/*loxP* mice. D: Plot indicating cytochrome *c* oxidase activity expressed in μmoles/min/mg tissue comparing IR*loxP*/*loxP* (two animals) versus BATIRKO mice (two animals). Results are expressed as mean cytochrome *c* oxidase activity ± SD. $^{*}P < 0.05$ comparing IR*loxP*/*loxP* mice versus BATIRKO mice.

could account for the coordinated increase of both UCP-1 and ATP synthase. Another study also showed that in BAT both uncoupling and coupling metabolism could be increased after cold-induced UCP1 expression [Navet et al., 2007], as well as in yeast overexpressing UCP1 [Douette et al., 2006]. In contrast, other authors previously observed a reduction in ATP synthase along the induction of UCP1 in BAT, either during cold adaptation of mice, or BAT ontogenesis [Houstěk et al., 1988, 1995; Kramarova et al., 2008]. However, our observations arise from a mouse model with a chronic overexpression of UCP1 in BAT, in which a compensation of the coupling metabolism could be expected to sustain ATP production and viability in the long term. BAT mitochondria from UCP1 knockout mice have fully energized mitochondria as a consequence of UCP1 ablation. Surprisingly, ATP synthase activity was found unaltered compared with control mice [Matthias et al., 1999]. This, together with our results, supports the concept that the switch on of the bioenergetics mitochondrial machinery is concurrent with the activation of the uncoupling mitochondrial machinery in BAT.

Along with UCP1 and ATP synthase, we found SOD2 levels slightly increased in BAT of transgenic mice. UCP1 drives mitochondrial proton leak, subsequently reducing the mitochondrial potential [Nicholls et al., 1978; Azzu et al., 2010]. This would be diminishing the reduced state of the mitochondrial respiratory chain and electron leak form superoxide radicals as proposed by [Korshunov et al., 1997]. On the other hand, other group has shown that an increase of UCP1 expression produces an adaptive response of the cell by increasing respiration [Douette et al., 2006], thus an increase in superoxide production should be expected. The net result of the increase of UCP1 and coupling machinery might thus explain the slight increase in the levels of SOD2.

As revealed by the expression of the mitochondrial marker Hsp60 as well as by electron microscopy, changes in BAT mitochondrial proteins between IR*loxP*/*loxP* and BATIRKO mice were not associated with changes of mitochondrial density in the tissue. Therefore, protein increase from the respiratory chain and ATP synthase is part of an adaptive mechanism independent from mitochondrial biogenesis.

We observed similar results in a cellular model of UCP1 increase. Throughout brown preadipocyte differentiation, UCP1 is increasingly expressed. Paradoxically, and accordingly with our in vivo data, we also found an increase in the levels of different proteins involved in the generation of ATP during brown adipocyte differentiation. Proteins involved in mitochondrial biogenesis were also increased, as well as phosphorylation of the energetic sensor AMPK, probably

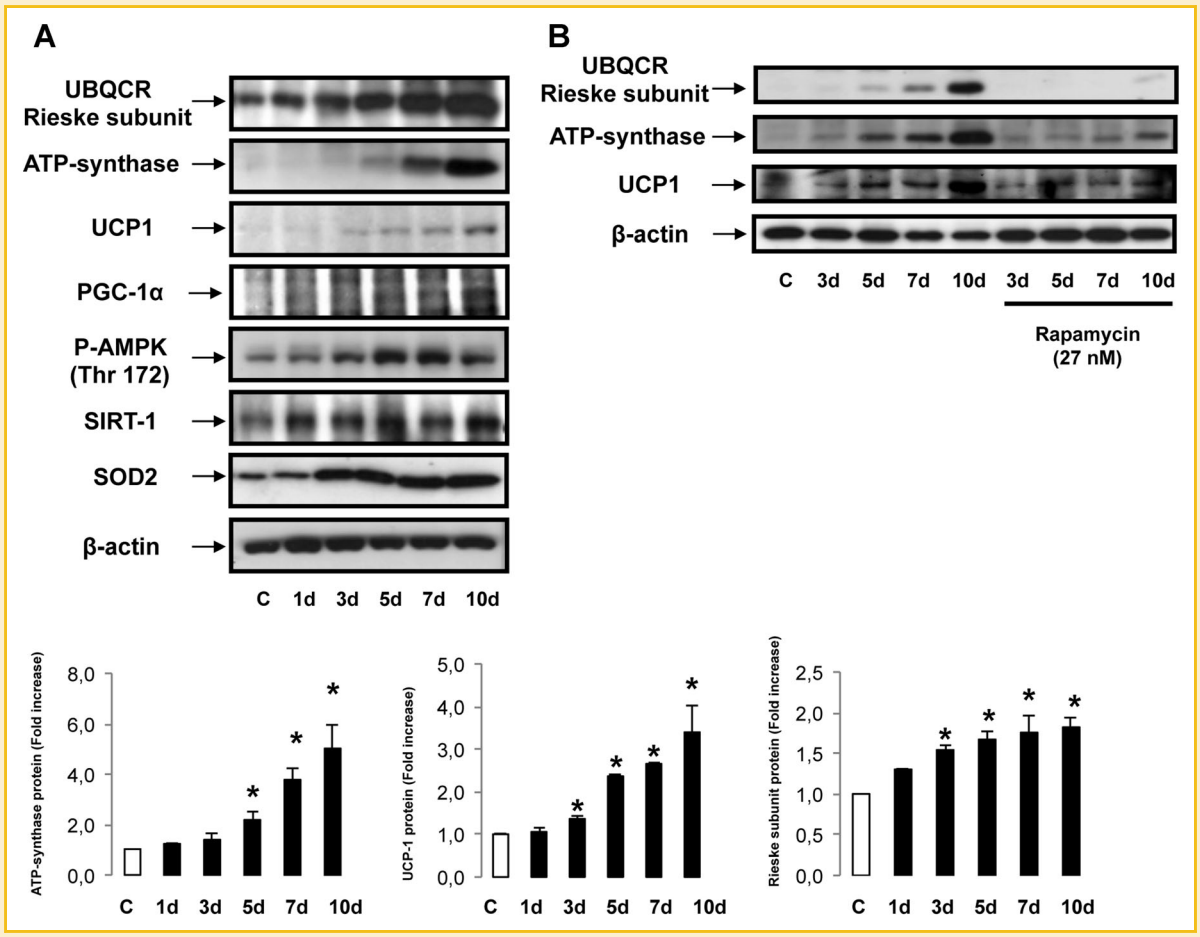

Fig. 4. Concomitant increases of coupling and uncoupling proteins during brown fat differentiation. A: Representative blots from brown adipose cells submitted to a differentiation protocol for different times as previously described in material and methods. A densitometric analysis represented as the fold-increase of ATP synthase, UCP-1 and Rieske subunit proteins ± SD along differentiation. $^{*}P < 0.05$ comparing the different days of differentiation versus control. B: Effect of rapamycin treatment (27 nM) on UBQCR, ATP synthase and UCP1 expression throughout the differentiation process.

indicating a rise in the AMP/ATP ratio through the differentiation program. As the uncoupling machinery increases through differentiation of brown adipocytes, less ATP should be produced from metabolic fuel [Wu et al., 1999]. An increase in AMP/ATP ratio was also observed after norepinephrine-induced activation of UCP1 in isolated brown adipocytes [Pettersson and Vallin, 1976]. Our observations point to a concurrent increase of the coupling machinery for ATP production along the differentiation program, indicating that this cell type may need to counteract the increase in uncoupling metabolism by increasing ATP synthesis for cell maintaining. Accordingly, AMPK has been recently proposed as a key regulator of brown adipogenesis, since inhibition of AMPK pathway blocks the differentiation of brown adipocytes [Vila-Bedmar et al., 2010]. Similarly, rapamycin blocks brown preadipocyte adipogenesis, probably by the inhibition of insulin signaling required towards mTORC1 as previously described [Cho et al., 2004; Vila-Bedmar et al., 2010].

In conclusion, the most important characteristic of BAT is its capacity for uncoupling metabolism and fuel oxidation. This unique feature makes this tissue a suitable target or tool for therapy against obesity related disorders. However the molecular mechanisms governing both coupling and uncoupling processes are not fully understood. Our study points out the activation of the bioenergetics mitochondrial machinery that generates ATP to maintain the cellular energetic status as an event that takes place in parallel to the upregulation of uncoupling machinery in certain physiological and pathophysiological conditions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

TABLE SII. Data Obtained From Mascot Analysis of Protein Identification Summarizing Score or Ion Score and Sequence Coverage.