Existence of uncoupling protein-2 antigen in isolated mitochondria from various tissues

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Abstract Antibodies against *Escherichia coli*-expressed uncoupling protein-2 (UCP2) and uncoupling protein-3 (UCP3) were raised by operating the blotted proteins into the spleen of minipigs. The antisera reacted more intensively with the recombinant UCP2 and UCP3 than with uncoupling protein-1 (UCP1) isolated from brown adipose tissue. Moreover, *anti*-UCP2 and cross-reacting *anti*-UCP3 antibodies identified the presence of the UCP2/3 antigen in isolated mitochondria from rat heart, rat kidney, rat brain, rabbit epididymal white adipose tissue, hamster brown adipose tissue, and rabbit skeletal muscle. It has been concluded that UCP2 is expressed in these tissues (UCP3 in skeletal muscle); however their existence in mitochondria had not previously been demonstrated.

Key words: Anti-UCP3 antibody; Uncoupling protein 2; Uncoupling protein 3

1. Introduction

UCP2 was originally identified by means of mRNA detection and probes derived from EST libraries [1,2]. It appears to be ubiquitously expressed in human and mouse tissues. UCP3 was identified in the EST library [3] and by RT-PCR [4] and is predominately found in human skeletal muscle. Human UCP2 is 59% identical [1] and UCP3 is 57% identical [4] with UCP1 from brown adipose tissue mitochondria. The uncoupling function of UCP2 and UCP3 was inferred largely from their homology with UCP1. Recently, however, Jabůrek et al. [5] have demonstrated that purified, expressed UCP2 and UCP3 are functional uncoupling proteins in that they catalyze fatty acid-dependent proton flux in liposomes.

UCP2 maps with the quantitative trait *loci* for obesity on mouse chromosome 7 and the human insulin-dependent diabetes locus-4 on chromosome 11; consequently, it was proposed that UCP2 is a thermogenic protein regulated by diet [1,2]. Several findings support this view. Thus, starvation increased UCP2 and UCP3 mRNA levels in humans [6] and UCP2 in rats [7]; UCP2 and UCP3 mRNA increased with high-fat diet [8,9]; and UCP2 mRNA increased 5-fold in white fat of obese *ob*/*ob* and *db*/*db* mice [2]. Additional findings implicate UCP2 as an effector in leptin-regulated lipostasis, in which steady release of leptin by adipocytes stimulates UCP2 transcription via a family of leptin-OB receptors [10–12]. UCP2 involvement has also been proposed for the effect of thyroid hormone on resting metabolic rate [13,14]; however, this role has also been proposed for UCP3, based on its selectivity for skeletal muscle in humans [3,4,15]. The existence of UCP2 mRNA in spleen, macrophages, thymus, bone marrow [1] and Kupfer cells [16] has led to the speculation that UCP2 mediates the thermogenic response to inflammatory stimuli. Supporting evidence for a role in fever came from the increase of UCP2 mRNA by lipopolysaccharide in liver, muscle and WAT and by IL-1β and TNF in liver. Moreover, this mRNA response was prevented by the antipyretic, indomethacin [17]. It has been proposed that UCP2 also provides a defense mechanism against generation of reactive oxygen species [18].

These and numerous other studies have been based entirely on detection of mRNA levels using Northern blot techniques. It seemed important to establish that these proteins are synthesized and targeted to mitochondria of the respective tissues. Accordingly, we raised antibodies against pure UCP2 and UCP3 proteins that were expressed in *E. coli*. This paper presents an initial immunological screening for UCPs in intact, isolated mitochondria. The results confirm the existence of UCP2/UCP3 in a wide variety of rat, rabbit, and hamster tissues.

2. Materials and methods

Rat liver and rat kidney mitochondria were isolated from Whistar rats using standard procedures. Isolation buffer contained 250 mM sucrose, 10 mM Tris-MOPS, 0.1 mM Tris-EGTA, pH 7.4, and 0.5% BSA, which was omitted from the final wash. Rat brain mitochondria were isolated using Percoll density gradient centrifugation [19]. Rat heart mitochondria were isolated using trypsin digestion in 180 mM KCl, 5 mM Tris-Cl, 10 mM Tris-EDTA, pH 7.4, and differential centrifugation in 180 mM KCl, 5 mM Tris-Cl, pH 7.4, containing 0.5% BSA [20]. Rabbit skeletal muscle mitochondria were isolated from *M. flexor fasciilatae* as described [21]. Epididymal WAT was isolated from rabbit, and then the WAT mitochondria were isolated as described [22].

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Abbreviations: BAT, brown adipose tissue; BCIP, 5-bromo-4-chloro-3-indolylphosphate; DTT, dithiothreitol; EST, expressed sequence tag; IL-1β, interleukin 1β; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-MS, matrix-assisted laser dissorption/ionization mass spectroscopy; NBT, nitro blue tetrazolium; SLS, sodium lauroyl sarcosinate; TNF, tumor necrosis factor; UCP, uncoupling protein; WAT, white adipose tissue

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2.1. Expression of UCP2 and UCP3 in E. coli

Human UCP2 and human UCP3 open reading frames were amplified by PCR and inserted into the *Nde*l and *Not*l sites of the pET21a vector (Novagen). From DNA sequencing, the constructs are predicted to encode proteins with an amino acid sequence identical to the wild-type UCP2 or UCP3 proteins. Plasmids were transformed into the bacterial strain BL21 (Novagen). Transformed cells were grown at 30°C to $OD_{600} = 0.6$ and then induced with 1 mM IPTG at 30°C for 6 h. Cells from a 700 ml culture were lysed in a French Press in 20 ml lysis buffer (10 mM Tris pH 7, 1 mM EDTA, 1 mM DTT); the lysate was centrifuged at $27\,000 \times g$ for 15 min; and the pellet was resuspended in 20 ml lysis buffer and centrifuged at $1000 \times g$ for 3 min. One ml aliquots of the supernatant were centrifuged at $14\,000 \times g$ for 15 min in a microfuge. The resulting pelleted inclusion bodies were stored frozen at −70°C.

2.2. Extraction of UCP2 and UCP3 from inclusion bodies

The pelleted inclusion bodies (about 3 mg of protein) were suspended and washed two times in 10 mM Tris-Cl, 0.1 mM Tris-EDTA, pH 7.0. The washed pellet was pre-solubilized by 1.5 ml of 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.1 mM Tris-EDTA, pH 7.2, containing 0.3% sodium lauroylsarcosinate (SLS). After centrifugation at $14\,000 \times g$ for 2 min, the resulting pellet was solubilized in 0.75 ml of 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.1 mM Tris-EDTA, pH 7.2, containing 1.67% SLS and 1% octylpentaoxyethylene. Prior to immunization, the proteins were mixed with sample buffer, and PAGE and Western blots were performed as described below. About 100 mg of each protein was blotted onto nitrocellulose membranes and identified on duplicates with *anti*-rat UCP1 antibodies (from Karl Freeman, McMaster University, Hamilton, Ont., Canada), alkaline phosphatase-conjugated *anti*-rat IgG secondary antibodies, and BCIP/NBT tablets (Sigma).

2.3. Immunization

Healthy, 2 month old minipigs (two for each protein) were anesthetized with halothane, and the operative field was disinfected and depilated under sterile conditions. Laparotomy was performed in the left lateral mid-abdomen, and the spleen was elevated through the incision. A triangular piece of the membrane with antigen was inserted inside the spleen sheath so that the antigen was in direct contact with spleen tissue. The spleen was sutured and restored to the abdominal cavity, and the laparotomy was closed and disinfected. Animals received postoperative antibiotics for 6 days, and sutures were removed on the 8th day. Two of the immunized animals were re-operated after 3 weeks to receive an antigen booster. After another 3 weeks, the pigs were bled under general halothane anesthesia by cannulation of the vena carotida externa. Sera were collected and stored at −80°C.

2.4. Western blots

Proteins were separated on Laemmli SDS-PAGE gels with 12% acrylamide, using either a minigel Mighty Small II (Hoefer) or 15 cm gels on a Protean IIxi (Bio-Rad). Kaleidoscop and prestained M_r standards (both Bio-Rad) were run on each gel. Proteins were transferred to nitrocellulose membranes either using a tank blotter TE22 (Hoefer, CA, USA, 120 mÅ, 1 h) or in a semidry blotter (E and K Scientific Products Inc., with 0.8 mÅ per 1 cm^2 for 1 h). The transfer buffer contained 50 mM Tris-Cl, 400 mM glycine, 0.35 mM SDS, 20% methanol pH 8.3. After transfer, nitrocellulose sheets were blocked overnight by 5% dry fat-free milk in a washing buffer, containing 20 mM Tris-Cl, 500 mM NaCl, 0.05% Tween 20, pH 7.5. Immunological detection was performed using *anti*-UCP2 or *anti*-UCP3 immune sera as the primary antibodies, diluted 500 times for sera from twice-operated pigs, or 10 to 100 times for once-operated pigs. Antisera were incubated with antigen in washing buffer containing 2–5% dry fat-free milk for 1 h, followed by three 10 min incubations in washing buffer containing 2–5% dry fat-free milk. Detection was achieved using *anti*-pig IgG (whole molecule) antibodies conjugated with alkaline phosphatase (Sigma), diluted 20 000 times in washing buffer, incubated for 1 h, washed three times in washing buffer, and developed using BCIP/NBT tablets (Sigma). Western blots were photographed using a Canon Power Shot Pro70 digital camera. MALDI-MS was performed on a Bruker-Franzen BIFLEX instrument after in-gel cleavage by endoproteinase Lys-C.

3. Results

3.1. Selectivities of anti-UCP antibodies

Fig. 1A contains representative Western blots of each of the UCPs reacted with each of the three antibodies. The reaction of *anti*-rat UCP1 antibodies with UCP2 and UCP3 is too weak to see in the blot, illustrating the difficulty of tracking these proteins with *anti*-UCP1 antisera. Thus, the broad sensitivity of the *anti*-rat UCP1 antibodies is UCP1 ≫ UCP2 or UCP3. The *anti*-UCP2 antibodies reacted in the order UCP2 > UCP3 ≫ UCP1, and the *anti*-UCP3 antibodies reacted in the order UCP3 ≈ UCP2 ≫ UCP1. We were interested in whether the new antibodies were selective relative to UCP1; accordingly, the gels were loaded with roughly equal amounts of UCP2 and UCP3 and a 5-fold excess of UCP1 (Fig. 1B). On this basis, we conclude that *anti*-UCP2 and UCP3 antibodies cross-react with each other, but react very poorly with UCP1. The Western blots shown are representative of 10 or more experiments with each combination.

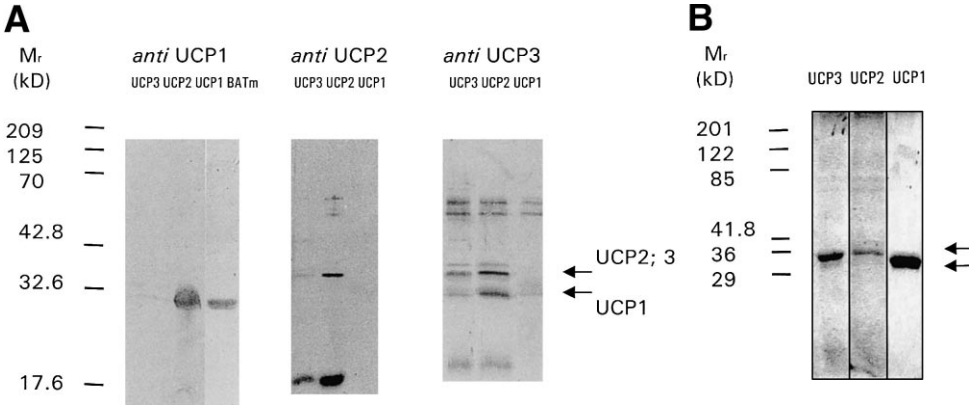

Fig. 1. A: Immunoreaction of *anti*-UCP1, *anti*-UCP2 and *anti*-UCP3 antibodies with UCP proteins. The upper row of labels designates the antisera or antibodies used in the Western blots. The second row of labels identifies the antigens. Blots were developed as described in Section 2. Molecular mass (M_r) in kDa is based on the positions of Kaleidoscop standards (Bio-Rad). These results are representative of 10 or more blots with each antibody/antigen combination. B: Typical migration of UCP1, UCP2 and UCP3 on PAGE. A gel run parallel to the Western blots is shown after silver staining and digitalization by an electronic camera. Molecular mass (M_r) in kDa is based on the positions of Kaleidoscop and low M_r standards (Bio-Rad).

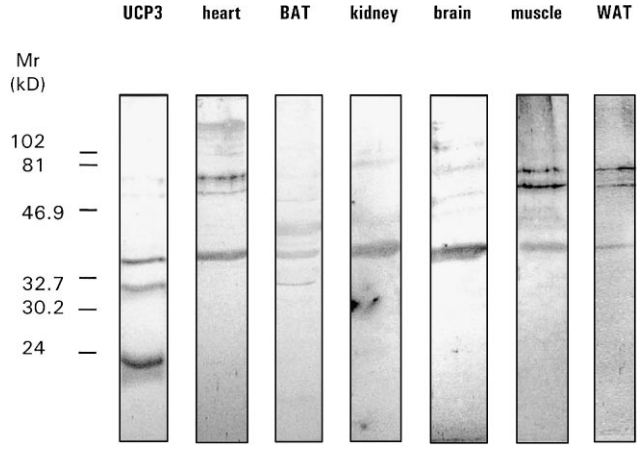

Fig. 2. Western blot analysis of mitochondrial proteins using *anti*-UCP3 antibodies. Western blots were performed on whole mitochondrial extracts using *anti*-UCP3 antisera in dilutions from 1:10 to 1:50. *UCP3*: SLS-solubilized *E. coli* inclusion bodies containing recombinant UCP3; *heart*: rat heart mitochondria; *BAT*: hamster BAT mitochondria; *kidney*: rat kidney mitochondria; *brain*: rat brain mitochondria; *muscle*: rabbit *M. flexor fasciilatae* skeletal muscle mitochondria; *WAT*: rabbit epididymal white adipose tissue mitochondria. Molecular mass (M_r) in kDa is based on the positions of Kaleidoscop standards (Bio-Rad).

UCP2 and UCP3 migrate at 33–35 kDa. The other bands visible in the Western blot were not visible in Coomassie-blue stained gels and most likely arose from the presence of *anti-E. coli* protein immunoglobins in the sera. To establish the correct identification, the putative UCP bands (marked by the arrow in Fig. 1A) were identified by MALDI-MS to be UCP2 (by 32.7% coverage of its identical sequence) or UCP3 (by 40.7% coverage of its identical sequence).

3.2. Existence of UCP2/3 antigen in isolated mitochondria

Fig. 2 contains Western blots of mitochondria isolated from various tissues and stained with *anti*-UCP3 antibodies, which recognize both UCP2 and UCP3 (Fig. 1A). Mitochondria from rat heart, rat kidney, rat brain, hamster BAT, rat epididymal WAT, and rabbit skeletal muscle contained readily detectable amounts of UCP2/3. The Western blots shown are representative of three to five experiments with each tissue.

4. Discussion

Previous evidence for the existence of UCP2 and UCP3 proteins in mitochondria was largely inferred from mRNA levels [1–4,6–11,13–17,23–27]. Because the postranslational fate of the proteins was unknown, it remained to be established that they were synthesized and localized to mitochondria. Our results now confirm the existence of UCP2/3 protein in mitochondria of heart, skeletal muscle, kidney, brain, WAT and BAT. A previous report also identified UCP2 antigen in WAT [12]; however, no documentation of the antibodies was provided. Antibodies against a conserved peptide sequence in murine and human UCP2 were reported to detect UCP2 in liver of ob/ob mice [27] and in yeast expressing UCP2 [28]. *Anti*-UCP1 antibodies were used to detect UCP2 in Kupfer cells [16] and fetal liver [29].

In view of their high level of sequence identities, it is not surprising that the *anti*-UCP antibodies do not discriminate well against each other. This may not detract seriously to their usefulness in studying tissue distribution, because mRNA studies indicate that UCP2 and UCP3 coexpress solely in skeletal muscle in human tissues and in skeletal muscle and BAT in rat tissues [3,4].

The work of many laboratories has led to considerable progress in understanding control of expression of UCP2 and UCP3 (reviewed in [30–32]) and in understanding their transport function on a biophysical level [5,33]. The major challenges remain to deduce their physiological role in the cell and the mechanisms used to regulate them.

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