Expression of UCP3 in CHO cells does not cause uncoupling, but controls mitochondrial activity in the presence of glucose

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The proton-transport activity of UCP1 (uncoupling protein 1) triggers mitochondrial uncoupling and thermogenesis. The exact role of its close homologues, UCP2 and UCP3, is unclear. Mounting evidence associates them with the control of mitochondrial superoxide production. Using CHO (Chinese-hamster ovary) cells stably expressing UCP3 or UCP1, we found no evidence for respiration uncoupling. The explanation lies in the absence of an appropriate activator of UCP protonophoric function. Accordingly, the addition of retinoic acid uncouples the respiration of the UCP1-expressing clone, but not that of the UCP3-expressing ones. In a glucose-containing medium, the extent of the hyperpolarization of mitochondria by oligomycin was close to 22 mV

in the five UCP3-expressing clones, contrasting with the variable values observed with the 15 controls. Our observations suggest that, when glycolysis and mitochondria generate ATP, and in the absence of appropriate activators of proton transport, UCPs do not transport protons (uncoupling), but rather other ions of physiological relevance that control mitochondrial activity. A model is proposed using the known passive transport of pyruvate by UCP1.

Key words: glycolysis, membrane potential, mitochondria, pyruvate, reactive oxygen species, uncoupling protein (UCP).

INTRODUCTION

Several mitochondrial UCPs (uncoupling proteins) have been described so far (for reviews, see [1–3]). First identified in 1976 [4,5], UCP1 is expressed in the brown adipose tissue of mammals [6,7]. UCP2 is found in several cell types, and UCP3 is predominantly expressed in skeletal muscle [8,9]. Finally, UCP4 and the distantly related UCP5, also known as BMCP1 (brain mitochondrial carrier protein-1), have also been described [10,11]. The uncoupling activity of these proteins is a consequence of their protonophoric activity according to Mitchell's chemiosmotic theory [12]. UCP protonophoric activity in the mitochondrial inner membrane leads to the dissipation of the electrochemical gradient established by the respiratory chain. This promotes substrate oxidation and therefore expenditure of energy, which is used for thermogenesis in the brown adipose tissue of mammals. UCP1 acts as a regulated proton transporter [13] whose activity is promoted when thermogenesis is needed [14]. No clear evidence for a thermogenic role of UCP2 and UCP3 is available yet. In fact, the regulation of the expression of these genes, as well as results from mice in which the *Ucp2* gene or *Ucp3* gene has been invalidated, argue against a significant physiological role in the regulation of body temperature [15,16]. By contrast, UCP3 appears to be involved in drug-induced hyperthermia [17]. The lack of a role for these two proteins in energy expenditure precludes their consideration as possible targets for anti-obesity drugs, even though they are expressed in adults.

Mild uncoupling of mitochondria is believed to participate in the prevention of production of oxygen radicals by mitochondria [18]. Some data suggest that UCP2 and UCP3 are involved in such mechanisms [19–21], but their precise role is unclear, because UCPs belong to a family of mitochondrial transporters of anionic substrates, and some of these transporters partially uncouple mito-

chondria in the presence of fatty acids. The mechanism involves the permeation of the protonated fatty acid through the lipid phase of the membrane and, after its deprotonation on the matrix side of the inner membrane, the return of anionic fatty acid by the transporter [22]. Nevertheless, several studies suggest that uncoupling by any of the UCPs protects strongly against the consequences of mitochondrial hyperpolarization, such as ROS (reactive oxygen species) overproduction and Ca²⁺-mediated damage [23–25]. Mitigation of age-related damage has also been attributed to UCP2 [26].

We have compared the cellular bioenergetics of CHO (Chinese-hamster ovary) clones that express human UCP3 with those of the parental cell line, other control clones (CHO cells transfected for other purposes) and of a previously characterized UCP1 clone [27]. Basic bioenergetic principles predict that the relative influence of the uncoupling activity of the UCP should increase when mitochondria tend to the non-phosphorylating State 4. However, continuous ATP consumption is essential within cells, and therefore replenishment of ATP is continuously required and, consequently, at least a partial State 3 is maintained in mitochondria of living cells. However, State 4 can be created artificially by treating cells with an inhibitor of mitochondrial ATP synthase such as oligomycin, which was used in the present study.

Our observations led us to propose that the presence of UCP3 does not cause mitochondrial uncoupling within cells. This observation also applies to UCP1 and results from the absence of an appropriate activator. However, on the basis of our observations, we propose that the presence of UCP3 ensures a stable relation between oxidative phosphorylation and glycolysis. This could be dependent on the transport of an as yet unknown substrate across the mitochondrial inner membrane, for which the small amount of UCP3 present in some of our cellular clones, and *in vivo*, is sufficient.

Abbreviations used: AANAT, arylalkylamine N-acetyltransferase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHO, Chinese-hamster ovary; DHE, dihydroethidium; ROS, reactive oxygen species; UCP, uncoupling protein.

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EXPERIMENTAL

Construction of the expression vector for human UCP3

Total RNA from human skeletal muscle (Clontech) was reverse-transcribed with oligo(dT)₁₂₋₁₈. First-strand cDNA was subjected to 35 cycles of amplification using primers based on GenBank entry for human UCP3 (forward 180–199; reverse 1101–1121; accession number AF001787). After an initial cycle of denaturation at 94 °C for 1 min, PCR was carried out with the following cycle conditions: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 3 min with a post-incubation of 72 °C for 7 min. The 942-bp PCR fragment encodes the long form (UCP3_L) of human UCP3 which has the structure common to mitochondrial anion carriers with six transmembrane α -helices, and the term 'UCP3' will be used hereafter. This PCR fragment was isolated and ligated into the expression vector pcDNA3.1. The recombinant plasmid was sequenced on both strands by automated sequencing.

Generation of CHO cell lines stably expressing UCP-3

The CHO-K1 cells were from the A.T.C.C. The cells were routinely maintained at 37 °C in Ham's F12 medium (Invitrogen) supplemented with 10% (v/v) fetal-calf serum, 2 mM L-glutamine, 50 i.u./ml penicillin and 50 µg/ml streptomycin (Invitrogen) using a 5% CO₂ humidified incubator. A day before transfection, CHO-K1 cells (15 × 10⁶ cells) were seeded in a T225 (225 cm²) culture flask in complete Ham's F12 medium. For transfection, the cell layer was washed twice with OptiMEM® (Invitrogen) medium only supplemented with 1% Glutamax (Invitrogen) and then incubated for 5 h at 37 °C with the transfection solution prepared by mixing 25 µg of pcDNA3.1-UCP3_L plasmid and 225 µl of Lipofectamine® (Invitrogen) in a final volume of 25 ml of OptiMEM medium. Transfection was stopped by addition of 25 ml of complete Ham's F12 medium. After 48 h of culture, cells were trypsinized and seeded in flat-bottomed 96-well plates using a clonal dilution method in complete Ham's F12 medium supplemented with 800 µg/ml of geneticin (G418; Invitrogen). After 10 days of culture, 24 clones were selected, amplified first in a 24-well plate and then in a T75 (75 cm²) culture flask. Expression of UCP3_L for each clone was checked by immunofluorescence using a goat anti-UCP3 serum, and subsequently revealed by an anti-goat IgG serum coupled to cyanin 3 (Jackson Immuno-Laboratories).

Using the same technical approach, we produced several dozen CHO cell lines each stably expressing different recombinant proteins, particularly G-protein-coupled seven-transmembrane domain receptors or the melatonin-synthesizing enzyme serotonin N-acetyltransferase [28]. These cell lines served as negative controls for the UCP3 experiments.

The CHO cell line expressing UCP1 was previously described [27].

Cell culture

The CHO cell lines were grown in Ham's F12 (Life Technologies) medium supplemented with 10% self-inactivated bovine foetal serum, penicillin and geneticin at 37 °C with 5% CO₂. For glucose depletion, the cells were grown at confluence and the medium was replaced by Dulbecco's modified Eagle's medium without glucose and sodium pyruvate, but supplemented with 2 g/l galactose, 1% sodium pyruvate, penicillin and geneticin.

ATP and ADP levels

Cells were grown in 60-mm-diameter culture dishes in medium to confluence, washed in PBS and frozen in liquid nitrogen. A 250 µl

portion of 660 mM perchloric acid with 10 mM theophyllin was added to cold dishes and the cells were scraped off. Cells were broken by homogenization with a loose-fitting and a tight-fitting Dounce homogenizer and lysate was neutralized with 25 µl of 2.8 M K₃PO₄. Tubes were centrifuged at 10000 g for 20 min at 4 °C. The supernatants were injected into an Agilent 1100 series HPLC instrument connected to a Chromsep C₁₈ Microspher column (100 mm × 4.6 mm; granulometry, 3 µm; VARIAN). ATP and ADP were separated by isocratic gradient of 200 mM KH₂PO₄, pH 4.25 (buffer A) and water/methanol/acetonitrile (2:1:1, by vol.) (buffer B). ATP and ADP were detected by A₂₅₄, and the area under the curve was used as quantitative measurement of ATP or ADP.

Flow cytometry

The experiments were performed with an Epics Coulter XL4 (Beckman-Coulter) instrument using System II acquisition software. The cells were trypsinized and resuspended in their culture medium. Viability was checked by the use of propidium iodide.

Superoxide production was measured with the probe DHE (dihydroethidium; Sigma), according to the protocol derived from [29]. DHE was dissolved (5 mM) in DMSO and portions placed in microtubes under argon to avoid autoxidation. A fresh aliquot was used for each experiment, and the final concentration used was 1.25 µM in the culture medium. A twice-concentrated DHE culture medium was added to the cell suspension. When used, mitochondrial poisons were added at the same time. The red fluorescence of cells in this labelling mix was measured at several different times over a period of 30–60 min. At the end of some kinetic experiments a saturating dose of the uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; > 10 µM) was added to check that no quenching of the ethidium occurred [29].

The JC-1 probe (Molecular Probes) was dissolved in DMSO. Before each experiment, serial dilutions were made in the culture medium to generate the successive data points; the dilution ratio was 2 or 1.5, corresponding to a difference of 17.8 or 10.3 mV respectively. The red/green fluorescence ratio was obtained directly from the cytometer with the ratio parameter set to FL3/FL1, which was used to define the percentage of red-coloured cells in the population. The concentration of JC-1 leading to 50% of red-coloured cells was determined graphically.

Oxygen consumption measurements

Cells were trypsinized and resuspended in their medium. The oxygen consumption of the suspension was recorded with an O2k apparatus (Oroboros). Data were acquired using DatLab3 software and processed with the DatLab2 program, which allows the subtraction of the oxygen consumption due to the electrode. The basal respiration of cells was determined first in the absence of any addition. Oligomycin (0.5–1 µg/ml final concn.) was then added to determine the State-4 respiratory rate. Finally, increasing concentrations (1–20 µM) of CCCP were used to estimate the maximum (uncoupled) respiratory rate. In other experiments, increasing amounts of all-*trans*-retinoic acid were added to quantify its effect on cellular respiration. CCCP, oligomycin and retinoic acid were dissolved in DMSO/ethanol (3:1, v/v), a mixture that does not freeze when kept on ice.

Other experimental procedures

UCP3 was detected by Western blotting performed essentially as described in [30]. Signals were detected and quantified with a cooled CCD camera [GeneGnome apparatus; Syngene (U.K.)],

Figure 1 Expression of UCPs in CHO cellular clones

Top panel: Northern blot of total RNA isolated from different CHO cellular clones. Hybridization with three UCP cDNAs: *ucp1*, rat UCP1 cDNA probe; *ucp2*, mouse UCP2 cDNA; *ucp3*, human UCP3 cDNA. The names of the different clones are indicated at the top. Bottom panel: Western blots of mitochondrial proteins isolated from CHO cellular clones. The blots were probed with anti-UCP2 605 antibody, which cross-reacts with UCP3 and UCP1. Only the name (number) of UCP3-expressing clones is indicated at the bottom. Blot 1 is from hUCP3 to clone 19; blot 2 is from clone 20 to rat UCP1. For this second blot, two exposures were used, namely a long one from clone 20 to CHO-UCP1 and a shorter for the last lane to visualize the molecular mass of rat UCP1. Therefore, it is normal that the relative intensity of the bands does not correspond to the quantitative estimation given in the text.

Cambridge, U.K.; sold by Ozyme, St. Quentin-en-Yvelines, France). Northern blotting was performed as described in [30]. Data were analysed using Excel and, unless otherwise stated, Student's *t* test was used to test for statistical significance.

RESULTS

Expression of UCP3 in cellular clones

Five different clones were found to express UCP3 (Figure 1). Immunostaining of UCP3 in permeabilized cells revealed a punctate labelling, and confocal microscopy showed UCP3 immunoreactivity to be co-localized with that of cytochrome *c* (results not shown). This confirmed the mitochondrial localization of UCP3. UCP3 expression varied from one clone to another. Clone 13 had the highest expression and clone 30 the lowest. Quantification was undertaken using known quantities of recombinant UCP3 protein produced in *Escherichia coli*. Estimates of the amount of UCP3 present in mitochondrial preparations from the different CHO-UCP3 clones range from 90 ng to 1.2 mg/mg of mitochondrial protein. The physiological level of UCP3 in the muscle of mice was estimated to be 140 ng/mg of mitochondrial protein [31]. This value was obtained with a muscle mitochondria preparation. UCP3 distribution varies in different fibres [32], and it is likely that some mitochondria have significantly higher amounts of UCP3. Physiological levels of UCP1 in brown adipose tissue are much higher and range from 5 to 50 μ g/mg of mitochondrial protein [33]. Mitochondrial preparations from different sources (muscle or CHO cells) are not identical, so care is needed when comparing our values with values given for other sources [31,33]. It seems likely, however, that, in these CHO-UCP3 clones, UCP3 abundance ranges from values close to the physiological level of UCP3 in muscle (clone 30 or 10) to values lower than the expression level of UCP1 in brown adipose tissue. UCP1 expression in the CHO-UCP1 clone was estimated to be about

Figure 2 Production of superoxide measured with DHE

(A) A typical experiment: the red fluorescence of the ethidium produced inside cells was analysed by flow cytometry. The time-dependent increase in fluorescence (linear rate) was considered as a measure of the cellular superoxide production, ●, basal conditions (no addition); ○ or ◇, in the presence of CCCP; ◇, in the presence of 0.5–1 μ g/ml oligomycin. (B) Linear rates of fluorescence increase with time with the control cellular clone (AANAT) in the absence (basal) and presence of different inhibitors, and of various concentrations of the uncoupler CCCP. Results are mean values $\pm$ S.D. The number of experiments is shown on the histogram bars. In each experiment the basal rate was considered as the reference value (100) and other rates were expressed relative to it. The asterisks indicate values that were statistically different (*P* < 0.05) from the control (paired *t* test with non-transformed linear-rate values).

4% of that in the brown adipose tissue of mice housed at room temperature, which was used as a reference in our experiments (results not shown).

During the isolation procedure, other cellular clones were obtained that did not express UCP3. One of these expressed high levels of UCP3 mRNA, but UCP3 was not detectable in mitochondria. These clones (numbered) were used as negative controls, because they were cloned identically to the UCP3 clones. Other control clones (lettered) were used. They came from other transfection experiments and express recombinant proteins not relevant here. One of these clones expressing, AANAT (arylalkylamine N-acetyltransferase; also called 5-hydroxytryptamine N-acetyltransferase and serotonin N-acetyltransferase), was used as a standard throughout the study.

Superoxide production

DHE is oxidized into fluorescent ethidium by the superoxide ion. At low concentrations this probe can be used to monitor mitochondrial superoxide production [29]. To quantify the rate of superoxide production, we measured the slope of increase in red fluorescence with time (Figure 2A). Poisoning of mitochondria was used to check that DHE staining was consistent with what is known about mitochondrial superoxide production (Figure 2B). The reduction of respiratory rate, with an increase in the membrane potential obtained with oligomycin, led to an increase in DHE oxidation rate, as did the inhibition of respiration with a saturating dose of antimycin, which decreases the membrane

Figure 3 Production of superoxide in cellular clones

DHE fluorescence increase rates were measured as defined in Figure 2 in the glucose-containing medium. **(A)** Histogram of values for indicated CHO clones under basal conditions. These are relative values. In each experiment the control clone AANAT was used and its rate of production was considered as the 100 % value. The number of independent experiments for each clone is indicated on the histogram bars. **(B)** Comparison of the values shown in **(A)** for each type of clones and statistical analysis of the difference between the two populations of clones: control, $n = 8$; UCP3, $n = 5$. **(C)** Measurements made in the presence of oligomycin, comparison of the values and statistical analysis of the difference between the two populations of clones: control, $n = 15$; UCP3, $n = 5$.

potential. This excludes the possibility of the probe responding to membrane potential rather than to superoxide production rate. The results obtained with the uncoupler CCCP were complex, since low concentrations did not, or only moderately decreased, DHE oxidation, whereas high concentrations led to an increase. The increase at high CCCP concentration can be correlated with what was observed for cellular respiration: low concentrations of this uncoupler increased cellular respiration (genuine uncoupling effect), whereas above the optimal concentration (around $10 \mu\text{M}$ under our conditions) uncoupling still took place, but respiration declined, because the respiratory chain was inhibited. Accordingly, it was not surprising that high concentrations of CCCP produced an increase in ROS production. The lack of a significant decrease at the lowest CCCP concentrations ($1\text{--}5 \mu\text{M}$) suggests that, under basal conditions, mitochondrial superoxide production cannot be lowered by moderately increasing the electron movements in the respiratory chain (mild uncoupling). Concentrations leading to an almost maximal respiratory rate decreased DHE to only 80 % of the basal value; however, there is no proof for the mitochondrial origin of this residual production.

The rate of oxidation of DHE by cellular clones was determined in several experiments (Figure 3A), and the mean value of these determinations was used to compare the UCP3 clones with control

clones (Figure 3B). It should be emphasized that the statistical analysis [in Figures 3B, 3C, 4B (below), 5B and 5C (below)] refers to the number of independent clones used: five independent clones expressing UCP3 ($n = 5$) and from eight to 15 different controls ($8 \leq n \leq 15$). Since there is only one UCP1 clone, no statistical analysis is given for it. In the basal situation (mitochondria in State 3), the superoxide production rate appeared slightly lower in the UCP3 clones than in control clones, but this difference remained above the threshold of high statistical significance ($P = 0.076$; Figure 3B). This is worse if one considers exclusively the matched population of controls (clones 2–34). The population of controls showed significant heterogeneity in superoxide production rate. It is noticeable that the variability observed in the UCP3 clones is not correlated with the UCP3 expression level. These observations justify our approach of analysing a significant number of independent clones and not a reduced set of control and expressing cells. To improve the statistical significance, a larger collection of controls was used (for all the controls of Figure 1 and the original cell line, CHO-K1, $n = 15$). Addition of oligomycin was expected to enhance the difference between control and UCP clones. Surprisingly the opposite occurred (Figure 3C). In the experiments shown in Figure 2(B) extreme conditions changed the DHE oxidation rate according to the predictions concerning mitochondrial superoxide production. While these conditions affected mitochondria differently, they all caused a deterioration in cellular bioenergetics. Therefore the interpretation in terms of superoxide production rate is not necessarily straightforward. Moreover, this measurement might be complicated by the interplay between mitochondrial and non-mitochondrial superoxide production rates and the intervention of antioxidant defences. We therefore chose to measure hyperpolarization directly inside the cells after oligomycin addition, with the prediction that UCP would blunt the increase in membrane potential.

Hyperpolarization in the presence of oligomycin

The red shift in the fluorescence of the JC-1 probe [34] was studied by flow cytometry. Incubation of cells with increasing concentrations of JC-1 showed that the appearance of a red fluorescence was highly dependent upon the concentration of JC-1 present in the medium (Figure 4A). Accordingly, it was possible to determine with good precision the concentration at which 50 % of the cells show red fluorescence. As expected, this red shift occurred at a lower concentration in the presence of oligomycin (Figure 4A, C₁) than in the basal conditions (Figure 4A, C₂), because mitochondrial ATP synthase inhibition by oligomycin led to the hyperpolarization of mitochondria. Using the Nernst Law, we estimated the variation of the mitochondrial membrane potential. It was not possible to determine a concentration at zero potential (cells treated with an excess of CCCP or antimycin + CCCP), so an absolute measurement of membrane potential could not be made.

The increase in mitochondrial membrane potential due to oligomycin is shown for the different cellular clones (Figure 4B). Firstly, the values (in mV) remained within the value ($\approx 40 \text{ mV}$) we obtained with isolated mitochondria during a transition from State 3 to State 4 [35]. The population of UCP3-expressing clones differed significantly from the controls in two ways: (1) the mean value was about 4 mV lower than the value observed with control cells; (2) the homogeneity of the values with the UCP3 clones contrasted with the heterogeneity of controls. In UCP3 clones, hyperpolarization ranged from 21.3 to 22.5 mV, whereas values between 12.2 and 32.3 mV were observed for controls. The statistical significance was largely due to this homogeneity, and the Fisher test gave a P value lower than 0.01.

Figure 4 Measuring hyperpolarization of mitochondria with the JC-1 probe

(A) Fluorescence measurement with successive dilutions of JC-1. The ratio of red to green fluorescence calculated by the cytometer was the parameter used to define a cell as green or red. The percentage of red cells was plotted against increasing JC-1 concentration. The curve shows the mean values $\pm$ S.D. for three experiments. Open symbols represent the result under basal conditions (no addition in the cell culture medium), and closed symbols represent the results obtained when oligomycin ($0.5 \mu\text{g/ml}$) was present in the medium. The concentration of the JC-1 probe at which 50% of the cells appeared as red (C_1 or C_2) was determined graphically. Here C_2 corresponds to the concentration in the basal state and C_1 to the concentration in the presence of $0.5 \mu\text{g/ml}$ oligomycin. The hyperpolarization in mV was obtained with the use of the Nernst Law:

$$\text{Hyperpolarization (mV)} = 58 \times \log (C_1/C_2)$$

(B) Statistical analysis of the difference between the two populations of clones: control, $n = 15$; UCP3, $n = 5$. The number of independent determinations for each clone varied from 1 to 10, with a mean value of 3.47. The result of the t test is shown with the hypothesis of independent variation in controls and the UCP3 population of clones. This is justified because the Fisher test gave a P value of 0.0013. (C) Values obtained for a selected number of clones in the glucose-containing medium. The number of determinations is shown on the histogram bars. (D) Values obtained with these same clones, but in the galactose-containing medium; $n = 3$ for all clones.

The use of a galactose medium made CHO cells entirely dependent upon mitochondrial respiration [36]. Two controls and two UCP3 clones as well as the UCP1-expressing clone were studied in the presence of galactose versus glucose. We chose a pair of controls that showed a stronger hyperpolarization than the UCP clones (Figure 4C). Since not all controls showed such a characteristic (Figure 4B), this is a biased choice, but it mimics the situation that would be expected if the controls are more coupled than the UCP clones. This difference disappeared in the galactose medium, and all clones showed almost identical values for the oligomycin-induced hyperpolarization (Figure 4D). These values were remarkably high, being almost twice the values estimated in the presence of glucose. This result should be viewed with caution. Accurate determination of mitochondrial hyperpolarization is dependent on an unchanged permeability of the

Figure 5 ATP/ADP ratio

(A) The ATP/ADP ratio was measured for cells growing in glucose medium or in galactose medium (Gal.) without further addition (basal). Before collection for ATP/ADP measurement, cells were treated either with $0.5 \mu\text{g/ml}$ oligomycin final (+ Oligo) or with $10 \mu\text{M}$ CCCP (+ CCCP). The number of independent experiments is indicated above the histogram bars. (B) Glucose-containing medium: statistical analysis of the difference between the two populations of clones: control, $n = 15$; UCP3, $n = 5$. The number of independent determinations for each clone varied from 4 to 23, with a mean value of 5.5. (C) Same clones in the galactose-containing medium; the number of independent determinations for each clone varied from 2 to 3, with a mean value of 2.5.

plasma membrane between the two conditions (basal or with oligomycin present). JC-1 cytosolic concentration is likely to result from the balance between passive entry and active extrusion by ATPases such as the multidrug resistance protein Pgp. Therefore, when the ATP level collapses (galactose medium plus oligomycin), extrusion is no longer possible and the cytosolic concentration increases much more than it might when glycolytic ATP is present (glucose medium plus oligomycin). Therefore, hyperpolarization values might be overestimated, and this would be much more severe in the galactose medium. In contrast with this, we found no reason to suspect that the comparison of values obtained with different clones in the same medium would not be legitimate.

ATP/ADP ratio

The ATP/ADP ratio is a measure of energy potential inside cells. As expected, it changes when mitochondrial poisons are used (Figure 5A). In the presence of glucose, oligomycin decreases the ATP/ADP ratio, as does the concentration of uncoupler CCCP, producing a maximal stimulation of respiration. Both indicate that mitochondria increase the ATP/ADP ratio further than glycolysis alone. In the presence of galactose, oligomycin caused a collapse

of the ATP/ADP ratio, which confirmed that, in this medium, ATP comes mainly from mitochondria [36]. It should also be noted that the galactose medium led to a higher ATP/ADP ratio than glucose and, moreover, the internal nucleotide content was increased in this medium: for control clones ($n = 14$), values in arbitrary units (area under peak) were 2097 ± 409 in galactose medium versus 1392 ± 170 in glucose medium ($P = 0.003$; 2231 ± 155 versus 1377 ± 315 , with the five UCP3 clones). CHO cells remained viable for days, but did not divide in the galactose medium. Therefore it is likely that their energy requirement is reduced, which may explain the higher ATP/ADP ratio. This experiment (Figure 5A) compares the control clone AANAT, used throughout the study as a standard for transfected cells containing no UCP, and UCP3 clone 30, which showed a marked difference compared with the control. It should be recalled that this is a clone expressing a low level of UCP3 (Figure 1). Comparison of the different clones in the presence of glucose or galactose showed that a small decrease, close to being statistically significance, was observed with UCP3 clones in glucose medium (Figure 5B). No correlation with UCP3 expression level was observed (results not shown). This decrease was no longer apparent when cells were grown in the galactose medium (Figure 5C).

Oxygen consumption of cells

Our earlier attempts with CHO clones expressing either UCP2 or a tagged version of UCP3 failed to show any increase in oxygen consumption (uncoupling) reproducibly associated with UCP expression (results not shown). However, we undertook this study (Figure 6A) using the same biased collection of clones as described in Figure 4(C). No increase in respiratory rate was observed in the presence of oligomycin. In the presence of an uncoupler, a lower respiratory activity in the UCP1 clone as well as in the UCP3 clone with the high expression level (clone 13) was observed. Therefore no energy waste due to mitochondrial uncoupling was recorded in these clones, but the presence of a significant amount of a UCP could be suspected to decrease mitochondrial activity and/or content rather than to increase mitochondrial energy expenditure. Of course, this decrease in the maximal respiratory rate results in a poorer respiratory control ratio, but it is not evidence for the increase in the proton conductance of the mitochondrial inner membrane. As observed previously, the effect of oligomycin on respiration showed that, in the glucose-containing medium, mitochondria contribute significantly to ATP production.

The lack of uncoupling observed, even with the UCP1 clone, was not unexpected, since intracellular conditions were believed to maintain UCP1 in an inhibited state [24]. To trigger UCP1 activity, we used a potent activator of its proton transport activity: all-*trans*-retinoic acid [37]. The addition of this compound to the culture medium led to an increase in the respiratory rate of the UCP1 clone (Figure 6B). This increase was of greater amplitude in the presence of oligomycin, verifying our prediction concerning the effect of this drug with regard to UCP activity. In both the absence or presence of oligomycin, the respiration rate reached the same value when stimulation was maximal. This value is about half that obtained by the use of a chemical uncoupler (results not shown). UCP3 clones and controls showed no increase in respiration due to retinoic acid. The small increase observed (Figure 6B) is a time-dependent process independent of the retinoic acid addition.

DISCUSSION

Many reports in the literature now support the hypothesis that UCP3 (or UCP2), like UCP1, allows a proton return through the

Figure 6 Oxygen consumption by cells

(A) Oxygen consumption (J_{O_2}) of cells resuspended in their culture medium. Abbreviations: basal, no addition; oligo, $0.5 \mu g/ml$ oligomycin; cccp, maximum rate in the presence of an optimal concentration of the uncoupler CCCP. The histograms of the values obtained with the five different clones are presented according to the order shown above the 'oligo' condition (AANAT and UCP1 B5-4, six experiments; others, five experiments). (B) Effect of retinoic acid on cellular respiration: concentration response of respiratory activity of cells resuspended in their culture medium (J_{O_2}) according to the all-*trans*-retinoic acid concentration in the medium. Relative units are shown, the oxygen consumption under basal conditions for each preparation was taken as a reference value = 1. Grey closed symbols, UCP1 B5-4 clone in the absence (circles; means for three experiments) or presence (squares; means for four experiments) of oligomycin. Other clones were studied in the presence of oligomycin. Open symbols, control clones: $\circ$, AANAT (two to five experiments); $\square$, clone 17 (one experiment); black closed symbols, UCP3 clones: $\blacksquare$, UCP3 clone 30 (three experiments); $\bullet$, UCP3 clone 13 (four experiments).

mitochondrial inner membrane. The physiological importance of this process certainly differs according to the UCP considered. This is a quantitatively important uncoupling of respiration, leading to thermogenesis for UCP1 in brown adipose tissue of mammals. In contrast, the role(s) of the proton transport catalysed by UCP3 (or UCP2) remains obscure. However, many studies link UCP3 or UCP2 activity to the control of ROS production [19–21]. Accordingly, it was shown that the presence of any of the UCPS has a protective effect against damage linked to ROS [23–25]. Knowledge of UCPS led to the conclusion that this is due to their protonophoric activity, which would prevent mitochondrial ROS production by a mechanism known as mild uncoupling. However, what is more difficult to reconcile with this hypothesis is the fact that UCPS also seem to protect against added radicals [26]. Mitochondrial superoxide production is governed by the reduction state of the coenzymes of the respiratory chain [38]. This reduction is increased by direct inhibition of the respiratory chain or indirectly by the hyperpolarization associated with the reduced State 4 rate of respiration, which can be caused either by a severely reduced

ATP demand or by oligomycin. We confirmed that the DHE staining protocol used followed these predictions (Figure 2B) and, in agreement with the literature [29], we consider our protocol of DHE staining to be a measurement of superoxide production rate within cells. UCP activity cannot prevent superoxide production when inhibition of the respiratory chain occurs, but was expected to operate when the State 4 rate was reached in the presence of oligomycin. This proved not to be the case (Figure 3C). Moreover, UCP activity could not be detected by direct measurement of the respiratory rate (Figure 6A). This lack of activity was also noted with studies of UCP1, a protein whose physiological function is thermogenic uncoupling. Lack of UCP1 activity could be explained by two factors: inhibition of the transport activity by endogenous levels of nucleotides, and lack of an activator of this transport. Addition of retinoic acid, a direct activator of the protonophoric activity of UCP1, was able to override inhibition and triggered a significant increase in the respiratory rate of CHO-UCP1 cells (Figure 6B). Note that UCP1 expression in this CHO-UCP1 cell line is about 4 % of that found in the brown adipose tissue used as a reference, and is roughly estimated to be around 1 $\mu\text{g}/\text{mg}$ of mitochondrial protein. However, when maximally stimulated by retinoic acid, it could increase the State 4 rate of respiration several times, and reached a respiratory rate that was about half of the maximal uncoupled rate in the presence of CCCP (Figure 6). Various reports indicate that expression of UCP3 in muscle [31], or of UCP2 in other organs [30], is more than two orders of magnitude lower than that of UCP1 in brown adipose tissue. These values are approximately one-tenth to one-fifth of the amount of UCP1 present in the mitochondria of this CHO-UCP1 cell line. If UCP1 and UCP3 have similar properties in terms of velocity of proton transport, *in vivo* maximally stimulated UCP3 would permit an energy expenditure of limited importance, but not negligible, when compared with the resting State 4 of mitochondrial respiration.

The fact that retinoic acid could not activate respiration of CHO-UCP3 cells was not unexpected since our previous studies with yeast mitochondria expressing UCP3 also demonstrated this lack of effect [37]. However, regulation of UCP3 is sometimes thought to be identical with that of UCP1 [39–41], and results in contradiction of this view obtained with recombinant yeast [37] have been ascribed to artefacts due to overexpression or inappropriate folding in the yeast mitochondria [33,42]. We therefore explored the effect of retinoic acid on the CHO-UCP3 cells (Figure 6B). No activation was observed in clone 30, where UCP3 is expressed in a mammalian cell line at levels similar to those of natural abundance in muscle. This argues for distinct affinities of these two UCPs for lipid ligands, as suggested by previous conclusions concerning the yeast expression system [37]. Superoxide itself, or products derived from superoxide attack on phospholipid membranes, have been proposed as activators of UCPs [40,43–45]. The experiments reported in Figures 3(C) and 6(B) involved prolonged exposure to oligomycin and to the superoxide generated by mitochondria under these conditions (Figure 3B). In this respect the UCP3 present inside CHO cells did not respond in a manner that might have been predicted from these studies.

The present report confirms that the UCP uncoupling activity is dependent upon two different mechanisms, namely (1) the expression of the gene, and (2) the direct activation of the protonophoric activity [14]. With the aim of increasing energy expenditure in man, maximization of UCP3 uncoupling activity would require both stimulation of transcription of the *Ucp3* gene, for which in-depth analysis of the human *Ucp3* gene promoter is required [46–48], and the presence of appropriate activators of the protein itself. In this respect it is important to recognize that, while the

present study confirms that UCP3 is not in itself able to cause uncoupling, it does not exclude the possibility that UCP3 could behave as an uncoupler if appropriate activators are provided, and its physiological relevance should be judged by the nature of such activators. Induction of the thermogenic function of UCP3 seems feasible [17], but activators devoid of adverse side effects remain to be discovered.

UCPs need activation for significant uncoupling to be observed (Figure 6B). However, could a marginal uncoupling be present and be revealed by indirect measurements such as ROS production or the ATP/ADP ratio? Whenever differences seemed to corroborate the existence of a marginal uncoupling (Figures 3B and 5B), they appeared under conditions that were not ideally suited to emphasize its consequences, and changes in the experimental parameters to make it more influential had the opposite effect (Figures 3C and 5C). It is noticeable, for example, that when cells were shifted to a galactose-containing medium, where ATP production is entirely dependent upon mitochondria, these differences vanished (Figures 4C, 4D, 5B and 5C). Consequently, either these differences are of no significance (note *P* values slightly above 0.05), or they stem from another UCP activity, distinct from uncoupling.

All the UCP3 clones shared the same oligomycin-induced hyperpolarization value, which is about 4 mV lower than the mean for the heterogeneous population of controls. Therefore, in a glucose-containing medium, the presence of UCP3 ensures that mitochondria remain 22 mV below the State-4 potential value. This remains true for all clones, although UCP3 expression varies. This suggests that the amount of protein present is not the limiting factor, which does not fit with an 'uncoupling activity' directly proportional to UCP abundance as long as the respiratory chain can compensate for the proton leak induced by the UCP. Instead it suggests that the transport of a limited amount of a substrate somehow controls mitochondrial hyperpolarization. For this, a small amount of the UCP would be sufficient, and increasing it further has no effect. According to the Nernst Law, independently of the starting potential, an increase of 22 mV in the potential would increase 2.4-fold the ratio of the internal to external concentrations of an ion distribution according to mitochondrial membrane potential. Accordingly, we would like to suggest that UCP3 controls the concentration of a critical ion inside mitochondria. The purpose of this would be to maintain an equilibrium between anaerobic ATP production and oxidative phosphorylation in the glucose-containing medium. This hypothesis would fit with the fact that, *in vivo*, UCP3 is expressed mainly in glycolytic fibres [32]. What could this critical compound be? Several reports show that UCP1 can behave as an anion uniporter for many synthetic compounds [49], as well as for chloride [12,50] or pyruvate [49,51]. Pyruvate would afford the simplest explanation, by which the presence of such a uniporter could create an autonomous mitochondrial negative feedback by a membrane potential-driven substrate withdrawal, therefore limiting mitochondrial polarization and ROS production. Previous reports in the literature proposed that the pyruvate transport by UCPs might be relevant to physiology [51,52]. In these reports it was considered that the pyruvate uniporter out of mitochondria through UCP would create a futile cycle, since pyruvate is taken up into mitochondria by a pyruvate/proton symporter. This results in the entry of one proton per cycle and would appear as an uncoupling pathway in mitochondria. This was not observed here. The validity of the model presented (see Scheme 1) is therefore dependent upon a secondary hypothesis, presumably a compartmentalization of pyruvate metabolism, which would make cycling impossible. The present study cannot provide evidence for this. Opposing both hypotheses (cycling and withdrawal) is the fact that the endogenous nucleotide (ATP)

Scheme 1 Hypothesized uniport activity of UCP and metabolism of glucose

Cellular metabolism in the presence of glucose is divided into three steps: (1) glycolysis from glucose to pyruvate and possibly lactate; (2) the transport of pyruvate into mitochondria by a transporter common to all cells (proton pyruvate cotransport or pyruvate hydroxide antiport); and (3) the conversion of pyruvate into ATP [Krebs cycle (tricarboxylic acid cycle) and oxidative phosphorylation]. The latter process exerts a negative feedback on glycolysis – ‘the Pasteur effect’. If, as has been suggested by other authors (see the text), UCP acts as a passive pyruvate uniporter, then the mitochondrial membrane potential will lead to extrusion of pyruvate out of mitochondria. This withdrawal of substrate will create negative feedback on step (3). It is suggested here that some compartmentalization occurs and that lactate dehydrogenase removes the pyruvate coming out of mitochondria from the cytosol by converting it into lactate. This would prevent futile cycling of pyruvate. $\Delta\psi$ is the membrane potential.

concentrations are expected to inhibit all types of transport by UCPs. However, this nucleotide inhibition is complex [42] and, according to the conditions (organs), UCP1 appeared to be active [35] or inactive [24] *in vivo*. Therefore a partial activity of UCP at least cannot be ruled out. Moreover, whereas some authors have shown nucleotide inhibition of proton transport by UCP2 or UCP3 [39–41], others were unable to detect it [37]. Finally, although applied to proton transport, it is clear that this inhibition can be overcome (Figure 6B).

In contrast to mild uncoupling, this substrate-dependent control may be extremely sensitive to flux conditions: it would be operational at relatively high flux. The non-ohmic behaviour of the mitochondrial inner membrane means that a low respiratory activity is able to maintain high polarization of the membrane. Therefore this substrate-dependent control would not be as efficient under State-4 conditions. This would explain why, in the presence of oligomycin, the differences due to the presence of UCP vanished, whereas mild uncoupling would have predicted that they should increase.

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