

Transgenic UCP1 in white adipocytes modulates mitochondrial membrane potential

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Abstract To test if mitochondrial uncoupling in white adipocytes is responsible for obesity resistance of the aP2-*Ucp* transgenic mice expressing ectopic uncoupling protein 1 (UCP1) in white fat, mitochondrial membrane potential ($\Delta\Psi_m$) was estimated by flow cytometry in adipocytes isolated from gonadal fat. Ectopic UCP1 (approximately 0.8 mol UCP1/mol respiratory chain) decreased the $\Delta\Psi_m$ and rendered the potential sensitive to GDP and fatty acids. These ligands of UCP1 had no effect on $\Delta\Psi_m$ in white adipocytes from non-transgenic mice, suggesting that the function of endogenous UCP2 in adipocytes was not affected. The results support the hypothesis that mitochondrial uncoupling in white fat may prevent development of obesity.

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Key words: Oxidative phosphorylation; Energy metabolism; Proton leak; Flow cytometry; Tetramethylrhodamine methyl ester

1. Introduction

A regulatable proton leak in mitochondria may be a general mechanism for controlling metabolic rates, energy balance and thermogenesis ([1]; for references see [2,3]). The mechanism of uncoupled thermogenesis is well characterised in mitochondria of mammalian brown fat [4], where it depends on the protonophoric function of uncoupling protein 1, UCP1 (for references see [4–6]). Protonophoric activity of UCP1 is influenced by fatty acids and purine nucleotides. Fatty acids stimulate protonophoric activity either by acting as a regulatory ligand of UCP1 [5,6], or by fatty acid cycling using UCP1 as a channel for the anionic form of the fatty acid [7]. Purine nucleotides, such as GDP, decrease protonophoric activity and halide anion transport mediated by UCP1 (for references see [4,6]). Brown fat is important for thermal homeostasis during cold exposure, and also for control of total energy balance in developing obesity (for references see [4]). However, other tissues [8,9] could substitute the latter function of brown fat.

Mitochondrial uncoupling in white adipocytes could affect adiposity and prevent obesity (see also Section 4, [10,11]). Such a mechanism was first suggested based on the obesity

resistance of the aP2-*Ucp* transgenic mice expressing ectopic UCP1 in white fat [8,12–14]. In agreement with this proposed function of ectopic UCP1, increased oxygen consumption was observed in fragments of white fat tissue of transgenic mice [14], and the metabolic rate of these animals was marginally elevated [8]. However, in spite of these findings, the protonophoric function of UCP1 in white adipocytes remains to be directly established.

In this report we document the ability of UCP1 to modulate the mitochondrial membrane potential ($\Delta\Psi_m$) in white adipocytes isolated from aP2-*Ucp* transgenic mice. Using flow cytometry in permeabilised cells, we demonstrate that ectopic UCP1 decreased $\Delta\Psi_m$ to the extent required for ‘uncoupled’ respiration, and rendered the $\Delta\Psi_m$ sensitive to regulatory ligands of UCP1. Compared to brown fat, relatively low amounts of UCP1 were required to elicit the effect in white adipocytes.

2. Materials and methods

2.1. Animals and tissues

Experiments were performed using 12-month-old female C57BL/6J mice, some of which were heterozygous for the aP2-*Ucp* transgene [12,14]. The animals were kept in a controlled environment (20°C; 12-h light/dark cycle) with free access to water and standard chow diet. Animals were killed by cervical dislocation under diethylether anaesthesia.

2.2. Isolation of adipocytes

Adipocytes were isolated from gonadal fat according to the method of Rodbell [15]. Modified Krebs-Ringer bicarbonate buffer was used: 118.5 mM NaCl, 4.8 mM KCl, 2.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.1 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 5 mM glucose and 4% bovine serum albumin (fraction V; BSA), pH 7.4. Tissue (2–3 g) from four to five mice was digested (45 min shaking at 37°C) in 5 ml of the buffer containing 3 mg/ml collagenase type II (C-6885, Sigma), and filtered (250 µm). Adipocytes were washed and separated by centrifugation (1 min, 400 × g, 20°C; three times) in the absence of collagenase and BSA, and diluted by one volume of a medium containing 100 mM KCl, 10 mM Tris, 2 mM MgCl₂, 4 mM K₂HPO₄ and 1 mM EDTA (KCl buffer), pH 7.4.

2.3. Adipocyte differentiation in culture

Cells of the 3T3-L1 clonal line [16] were plated at a density of 8000 cells/cm² and grown in Dulbecco’s modified Eagle’s medium at 37°C in an atmosphere of 10% CO₂. The compositions of the growth medium and the differentiation medium (added to confluent cells 4–5 days after plating) have been described [17]. During the first 48 h after confluence, 2 µM dexamethasone was also present. Cultures used for experiments (12–14 days after confluence) contained 50–60% differentiated adipocytes.

2.4. Adipocyte permeabilisation

Freshly isolated adipocytes were permeabilised in 500-µl aliquots (5 min, on ice) using digitonin [18]. The optimal digitonin to cell protein ratio (0.05) was assessed by flow cytometry of cells stained with 1 µg/ml propidium iodide [19]. Permeabilised cells were washed twice (10 min, 10 000 × g, 4°C) in KCl buffer (pH 7.0), and the sedi-

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Abbreviations: UCP, uncoupling protein; aP2-*Ucp* mice, transgenic mice with synthesis of UCP1 from adipocyte lipid binding protein promoter; IFL, mean fluorescence; TMRM, tetramethylrhodamine methyl ester; BSA, bovine serum albumin; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\Psi_m$, mitochondrial membrane potential; KCl buffer, 100 mM KCl, 10 mM Tris, 2 mM MgCl₂, 4 mM K₂HPO₄ and 1 mM EDTA

mented material was resuspended in half of the original volume of the same buffer (pH 7.0). 3T3-L1 adipocytes were harvested using 0.05% trypsin and 0.53 mM Na-EDTA in physiological buffered saline. Cells were washed twice (5 min, $200\times g$, 20°C) in the buffered saline and suspended in KCl buffer (pH 7.4) to a final concentration of 0.3 mg protein/ml. The cells were treated with digitonin, sedimented by centrifugation (see above) and suspended in KCl buffer (pH 7.0).

2.5. Analysis of $\Delta\Psi_m$ by flow cytometry

Permeabilised cells were incubated for 6 min at room temperature in 250 μl of KCl buffer (pH 7.0) in the presence of 50 nM tetramethylrhodamine methyl ester (TMRM) and 10 mM succinate. The cell fluorescence was analysed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser. Excitation was at 488 nm and the fluorescence of TMRM was analysed with a 585-nm filter (bandwidth of 42 nm) and the photomultiplier set at 535 V. Approximately 5000 events per sample were acquired within a few seconds for analysis performed with CellQuest software (Becton Dickinson). The fluorescence intensity of TMRM (reflecting $\Delta\Psi_m$ [20,21]) was monitored on a four-order log scale and expressed as mean fluorescence (IFL). Several analyses were performed with each sample following addition of different compounds to the medium (additions were performed in approximately 1–2-min intervals). The values of IFL estimated under various experimental conditions were standardised relative to the values (IFL_{FCCP}) in the presence of 2 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The ratio IFL/IFL_{FCCP} was used to compare results obtained in different experiments.

2.6. Evaluation of UCP1 and cytochrome content

UCP1 and mitochondrial cytochrome contents were estimated in crude membrane fractions separated by centrifugation ($10^5\times g$, 30 min) from homogenates of gonadal fat [14]. UCP1 was quantified by immunoblotting as described in detail before [14], using rabbit antiserum against isolated [22] mouse UCP1. The immune complexes were detected using ^{125}I -labelled whole donkey antibody against rabbit IgG (Amersham), and radioactivity was evaluated using PhosphorImager SF (Molecular Dynamics). Quantification was performed [14] using standard isolated mouse UCP1 [22]. Cytochromes were measured in membrane fractions solubilised in the presence of 2% *n*-dodecyl β -D-maltoside (Sigma). Reduced–oxidised difference spectra [23] were recorded using a Hewlett-Packard 8453 diode-array spectrophotometer. In order to improve sensitivity, a pseudo-dual-wavelength arrangement was used. Spectra were recorded at 2-s intervals for 3 min in 2-ml samples (in a stirred cuvette, at 25°C , and 0.1 mg protein/ml). Dithionite was added 40 s after the start of the measurement. A typical cytochrome spectrum appeared. Cytochrome content was calculated using published absorbance coefficients and wavelength pairs for cytochrome *a+a₃* ($\Delta\epsilon_{605-590} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to eliminate the effect of light scatter around 630 nm; see [24]), cytochrome *b* ($\Delta\epsilon_{563-577} = 17.9 \text{ mM}^{-1} \text{ cm}^{-1}$ [23]), and cytochrome *c+c₁* ($\Delta\epsilon_{552-540} = 19.0 \text{ mM}^{-1} \text{ cm}^{-1}$ [23]). The detection limit was 10–20 pmol for different cytochromes. Protein concentration was measured in the solubilised samples (see above) using the bicinchoninic acid procedure [25] and BSA as a standard.

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test. All tests were judged to be significant at $P < 0.05$.

3. Results

3.1. High sensitivity of the measurement of $\Delta\Psi_m$ in permeabilised adipocytes

A sensitive technique was required for measurements of UCP1 function in white fat mitochondria in mice (see Section 4). Therefore, $\Delta\Psi_m$ was estimated by flow cytometry using a fluorescent dye, TMRM, in adipocytes permeabilised by digitonin (see Section 2). The dye accumulates in mitochondria in response to $\Delta\Psi_m$ and this process is reflected by an increase of the fluorescence [20,21]. A model experiment was performed using 3T3-L1 adipocytes differentiated in cell culture (Fig. 1).

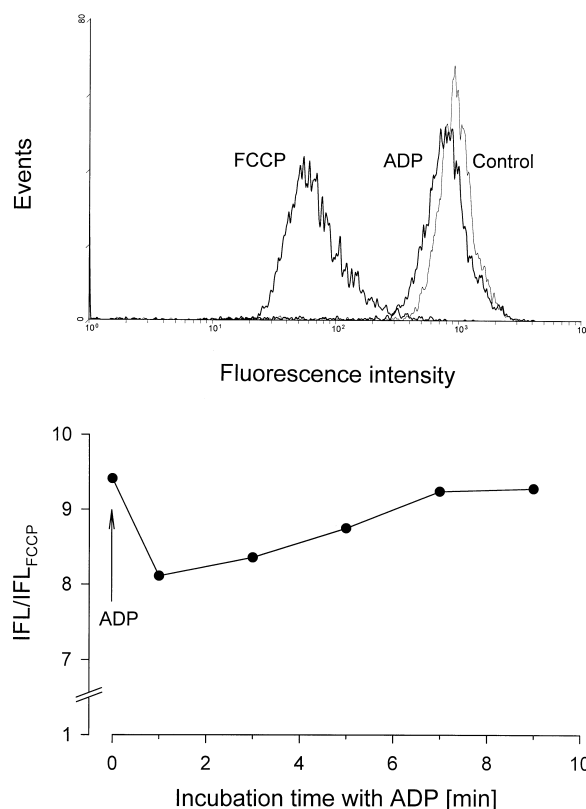


Fig. 1. Changes of the TMRM fluorescence during phosphorylation of ADP in 3T3-L1 cells. Cells differentiated in cell culture were permeabilised with digitonin and incubated for 6 min at 25°C in the presence of 50 nM TMRM, 10 mM succinate and 2 mg BSA/ml (see Section 2). Fluorescence was recorded before and after the addition of ADP (final concentration 30 μM). Upper graph, reading of fluorescence intensity before (Control) and 1 min after ADP addition. Fluorescence after addition of FCCP (final concentration 2 μM) is also shown. Lower graph, quantification of the fluorescence signal (IFL/IFL_{FCCP}; see Section 2) during phosphorylation of ADP.

In the presence of succinate, addition of ADP induced a drop in the mean fluorescence intensity (IFL), which returned to the original level after about 7 min (Fig. 1, lower graph). Subsequent addition of FCCP resulted in an even greater decrease in the fluorescence intensity (Fig. 1, upper graph), reflecting collapse of the $\Delta\Psi_m$. The duration of the ADP-induced decrease of fluorescence was proportional to the amount of added ADP, and separately performed respiratory measurements indicated that the changes of fluorescence resulted from phosphorylation of ADP (not shown). The results of this experiment demonstrate that the technique reliably monitors relatively small changes in the $\Delta\Psi_m$ associated with phosphorylation of ADP in white fat mitochondria (see Section 4).

3.2. Membrane potential in white fat mitochondria is modulated by ectopic UCP1

Using the technique described above, we studied the effect of ectopic UCP1 on $\Delta\Psi_m$ in gonadal fat. Fig. 2 illustrates the TMRM fluorescence in permeabilised adipocytes from control and transgenic mice, and the response of the fluorescence intensity to addition of different agents. In adipocytes from non-transgenic mice incubated in the presence of substrate

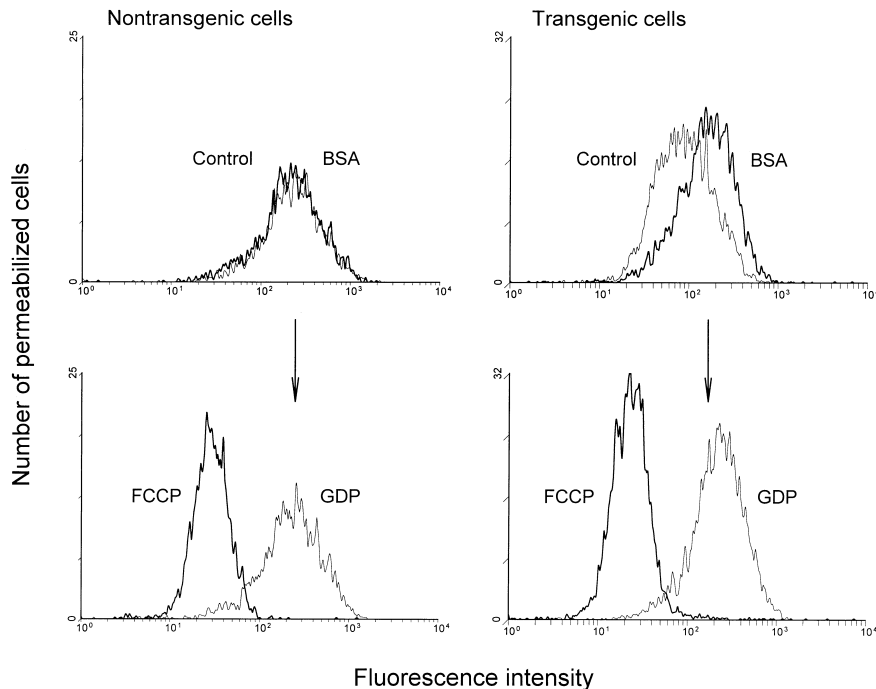


Fig. 2. TMRM fluorescence in adipocytes isolated from gonadal fat of non-transgenic and transgenic mice. Permeabilised cells were incubated with TMRM and succinate before cytofluorimetric analysis (see Section 2). Fluorescence was recorded before (Control) and after subsequent additions of BSA (final concentration 2 mg/ml; upper graphs), GDP (final concentration 0.5 mM; lower graphs) and FCCP (final concentration 2 μ M; lower graphs). Arrows indicate IFL (mean fluorescence) after the addition of BSA. Data from one typical experiment.

(Fig. 2, left upper graph), the addition of BSA (which can bind free fatty acids in the medium and thus inhibit the protonophoric activity of UCP1 [5,6]) had no significant effect on the fluorescence intensity. Similarly, subsequent addition of GDP (the inhibitory ligand of UCP1 [6]) did not cause a shift in TMRM fluorescence (Fig. 2, left lower graph). Thus, all three fluorescence histograms were superimposed. Finally, FCCP addition collapsed the $\Delta\Psi_m$, as reflected by a shift of the fluorescence histogram towards lower intensities (Fig. 2, left lower graph).

In adipocytes from transgenic mice (Fig. 2, right part), lower TMRM fluorescence intensity was observed compared with control mice (Fig. 2, upper graphs). This difference indicates relatively lower values of $\Delta\Psi_m$ in the mitochondria of all mature adipocytes in gonadal fat of transgenic mice. The addition of BSA resulted in the increase of the fluorescence intensity in all the cells (Fig. 2, right upper graph), although the $\Delta\Psi_m$ was still lower compared with non-transgenic mice. Subsequent addition of GDP (Fig. 2, right lower graph) induced a further increase of the fluorescence to levels similar to those observed in non-transgenic mice. Addition of FCCP collapsed the membrane potential (Fig. 2, right lower graph).

The use of the IFL/IFL_{FCCP} ratio (see Section 2) allowed for a quantitative comparison of the $\Delta\Psi_m$ in adipocytes from non-transgenic and transgenic mice (Fig. 3). Statistical evaluation of the data confirmed that $\Delta\Psi_m$ in non-transgenic mice was independent of the presence of BSA or GDP. However, in mitochondria of transgenic mice, both BSA and GDP had a significant and additive effect on the IFL/IFL_{FCCP} ratio (and hence on $\Delta\Psi_m$). In the presence of both BSA and GDP, the magnitude of the $\Delta\Psi_m$ was similar in mice of both genotypes.

3.3. A relatively low amount of UCP1 is able to decrease $\Delta\Psi_m$ in white fat cells

In order to correlate the energisation of white fat mitochondria with the content of ectopic UCP1, the amount of UCP1 was quantified using immunoblotting (for details, see [14], and Section 2). UCP1 could be detected only in the membranes from white fat of the transgenic mice (Fig. 4, compare lanes 2 and 3). UCP1 content (4.30 ± 0.83 μ g/mg protein, corresponding to 0.13 ± 0.03 nmol UCP1/mg protein; $n=5$) was

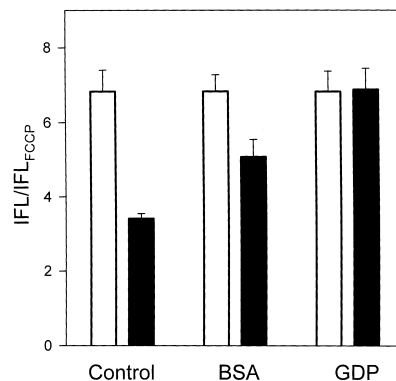


Fig. 3. Quantification of TMRM fluorescence in adipocytes from gonadal fat. Adipocytes were isolated from non-transgenic (empty bars) and transgenic (full bars) mice and analysed as described in Fig. 2. All the differences in transgenic mice were statistically significant, as well as the differences between adipocytes from non-transgenic and transgenic mice in the absence of BSA and GDP (Control), or in the presence of BSA only. All the other differences were not statistically significant. Data are means $\pm$ S.E.M. from six independent experiments.

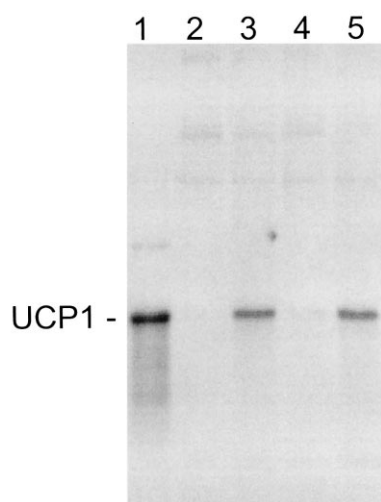


Fig. 4. Immunodetection of UCP1 in adipose tissue. The following samples were analysed: 1, mitochondria (5 μ g protein) isolated from brown fat of cold (4°C , 12 days)-acclimated mouse (containing 93 μ g UCP1/mg protein); 2 and 3, membrane fractions (35 μ g protein) isolated from gonadal fat; 4 and 5, adipocytes (35 μ g protein) isolated from gonadal fat, and permeabilised with digitonin. 1, 2, and 4, non-transgenic mice; 3 and 5, transgenic mice.

approximately 30% lower than that found in mitochondrial fractions isolated from 9-month-old transgenic male mice [14]. Importantly, UCP1 could be also detected in mature adipocytes isolated from transgenic animals (Fig. 4, compare lanes 4 and 5). Spectrophotometry indicated that the membrane fractions isolated from gonadal fat of the transgenic mice contained 0.34 ± 0.04 , 0.35 ± 0.07 , and 0.31 ± 0.06 nmol/mg protein of cytochromes $a+a_3$, b , and $c+c_1$, respectively ($n=5$). Based on the contents of cytochromes $a+a_3$ and $c+c_1$ (with 1 mol of each a , a_3 , b , c , and c_1 present in 1 mol of mitochondrial respiratory chain), the UCP1/mitochondrial respiratory chain ratio was around 0.8.

4. Discussion

The principal finding of this report is that ectopic UCP1 expressed in mitochondria of white adipocytes of obesity-resistant *aP2-Ucp* transgenic mice could modulate the $\Delta\Psi_m$. Ectopic UCP1 rendered $\Delta\Psi_m$ sensitive to fatty acids and purine nucleotides, while $\Delta\Psi_m$ in adipocytes of normal animals was not affected.

Isolation of intact mitochondria from white adipose tissue is difficult, because mature adipocytes contain relatively few mitochondria and mitochondrial functions are altered by fatty acids during isolation. Phosphorylating mitochondria have been isolated [26] from adipose cells released by collagenase from rat epididymal fat. However, this approach required large quantities of tissue, and could not be used in mice. In this study, isolated adipocytes were also used as a starting material, but instead of isolating mitochondria, cells were permeabilised by digitonin, and $\Delta\Psi_m$ was measured by flow cytometry using a fluorescent dye. Model experiments with 3T3-L1 adipocytes (Fig. 1) showed that the method, as in other types of cells, detects relatively small changes (approximately 15–25 mV; see [27,28]) of $\Delta\Psi_m$ during phosphorylation of ADP. Furthermore, the sensitivity of the flow cytometry allowed us to perform measurements on approximately 5000

adipocytes (corresponding to 50 mg tissue at the beginning of the procedure; not shown). Therefore, the method is suitable for analysis of $\Delta\Psi_m$ in white adipocytes from small tissue specimens, and for studying effects of compounds which cannot cross the plasma membrane of intact adipocytes.

Measurements of TMRM fluorescence showed that the $\Delta\Psi_m$ in mature white adipocytes from transgenic mice was lower than that from non-transgenic mice. Absolute values of $\Delta\Psi_m$ have not been estimated. However, the decrease in the $\text{IFL}/\text{IFL}_{\text{FCCP}}$ ratio induced by ectopic UCP1 (Figs. 2 and 3) was larger than that caused by phosphorylation of ADP (Fig. 1). This suggests that depression of $\Delta\Psi_m$ by UCP1 exceeded 25 mV (see above) and could result in uncoupled thermogenesis in white fat cells. Our previous immunoblotting experiments [14] indicated that the molar ratio of UCP1/cytochrome oxidase in mitochondria was close to 0.7 in epididymal fat of 9-month-old (chow diet-fed) *aP2-Ucp* transgenic mice. Data in this paper, obtained with older transgenic female mice, indicated a similar molar ratio (0.8) between UCP1 and the respiratory chain. Brown fat mitochondria contain at least three molecules of UCP1/molecule of cytochrome oxidase (for references see [14,22]), and it has been suggested [22] that each of three coupling sites of the mitochondrial respiratory chain must contain one molecule of UCP1 in order to collapse the $\Delta\Psi_m$. Experiments in this report indicate that substoichiometric amounts of UCP1 are sufficient to collapse the $\Delta\Psi_m$ and induce uncoupling.

UCP1 is regarded as the key marker of mature brown fat cells. However, UCP1 might also be expressed in white adipocytes of normal animals and activate energy dissipation. In this respect, the inducibility of UCP1 gene expression in white fat depots by β_3 -adrenergic agonists (slimming drugs) has been discussed (for references see [29]). Other candidate genes for thermoregulatory uncoupling in white fat, UCP2 [10,30] and UCP3 [31–33], have been discovered recently. The UCP2 gene is highly expressed in white fat cells and in cells of the immune system, and, to a lower extent, also in other tissues [10,30]. A positive correlation was found between the resistance to obesity in mice and the expression of UCP2 in white fat [10,11], and this link could exist in humans as well [34]. The UCP3 gene is transcribed in brown fat and muscle [31–33]. However, β_3 -adrenergic agonists can also induce its expression in white fat [31]. Both UCP2 and UCP3 decreased $\Delta\Psi_m$ when expressed in yeast [10,11,31]. However, their effect on adiposity in white fat might depend on some other biochemical activities (e.g. involvement of UCPs in regulation of lipid metabolism [35]; see also [36–38]). The results of the present study indicate that UCP2 (and UCP3) could affect adiposity by thermoregulatory uncoupling in white fat.

BSA and GDP affected $\Delta\Psi_m$ only in transgenic animals. Since UCP2 is endogenously expressed in white adipose tissue [10,30], the results suggest that protonophoric activity of UCP2 in white fat mitochondria is not sensitive to purine nucleotides or free fatty acids. This conclusion is compatible with the lack of the GDP effect on proton leak in brown fat mitochondria isolated from animals with a disrupted UCP1 gene [9,39], and also activity of UCP2 expressed in the heterologous yeast system [10]. Alternatively, UCP2 may have lower affinity to fatty acids than UCP1. Therefore, UCP2 may not be active in permeabilised adipocytes, where the amount of fatty acids may be sufficient to affect only UCP1. In accordance with such a possibility, mitochondrial UCP2 is sup-

posed to be sensitive to GDP in cells of the immune system, where it is involved in control of hydrogen peroxide production [37], and in muscle mitochondria where it probably mediates thermoregulatory uncoupling [2]. Further experiments are required to understand the control of UCP2 activity in white fat.

The present results are an important complement to data previously obtained from the aP2-*Ucp* transgenic mice, a unique model for studying the effect of white fat mitochondria on adiposity. They support the hypothesis that mitochondrial uncoupling in white adipocytes may prevent development of obesity. Assuming that UCP1 is synthesised in a population or subpopulation of white fat cells, and/or that proton leak in white fat mitochondria may be affected by other mitochondrial transporters, the demonstration of a UCP1-mediated decrease of $\Delta\Psi_m$ in white adipocytes is relevant for the biology of adipose tissue.

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