Impaired noradrenaline-induced lipolysis in white fat of aP2-*Ucp1* transgenic mice is associated with changes in G-protein levels

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In vitro experiments suggest that stimulation of lipolysis by catecholamines in adipocytes depends on the energy status of these cells. We tested whether mitochondrial uncoupling proteins (UCPs) that control the efficiency of ATP production could affect lipolysis and noradrenaline signalling in white fat *in vivo*. The lipolytic effect of noradrenaline was lowered by ectopic UCP1 in white adipocytes of aP2-*Ucp1* transgenic mice, overexpressing the UCP1 gene from the aP2 gene promoter, reflecting the magnitude of UCP1 expression, the impaired stimulation of cAMP levels by noradrenaline and the reduction of the ATP/ADP ratio in different fat depots. Thus only subcutaneous but not epididymal fat was affected. UCP1 also down-regulated the expression of hormone-sensitive lipase and lowered its activity, and altered the expression of trimeric G-proteins in adipocytes. The adipose tissue content of the stimulatory G-protein α subunit was increased while that of the inhibitory G-protein α subunits decreased in response to UCP1 expression. Our results support the idea that the energy status of cells, and the ATP/ADP ratio in particular, modulates the lipolytic effects of noradrenaline in adipose tissue *in vivo*. They also demonstrate changes at the G-protein level that tend to overcome the reduction of lipolysis when ATP level in adipocytes is low. Therefore, respiratory uncoupling may exert a broad effect on hormonal signalling in adipocytes.

Key words: adipocyte, ATP/ADP ratio, cAMP, catecholamine, hormone-sensitive lipase.

INTRODUCTION

Lipolysis in white adipose tissue is one of the key mechanisms engaged in the control of fuel partitioning. It is under complex hormonal control, the hormone-sensitive lipase (HSL; EC 3.1.1.3) being the target of both lipolytic and anti-lipolytic hormones [1]. While the action of catecholamines is the single most important stimulus for the activation of lipolysis that occurs through stimulation of the intracellular cAMP cascade, insulin represents an anti-lipolytic hormone inhibiting the cascade (for a review, see [2]). The activation of lipolysis may also depend on the energy status of fat cells [3]. Thus a decrease of intracellular ATP elicited in white adipocytes *in vitro* counteracted the stimulation of lipolysis by catecholamines [3,4]. In turn, incubation of isolated adipocytes with lipolytic hormones resulted in an up to 50 % decrease of their intracellular ATP level [5,6] and inhibition of the lipolytic process itself [5]. It has been suggested that these effects resulted from the uncoupling of oxidative phosphorylation by fatty acids accumulated within the adipose cell and might represent an artefact of experiments performed *in vitro* [5].

However, the efficiency of mitochondrial energy conversion and energy status of fat cells could also affect the lipolytic rate *in vivo*. Natural uncouplers of oxidative phosphorylation in mitochondria, the mitochondrial anion carriers, such as the adenine nucleotide translocator [7,8], and especially the uncoupling proteins (UCPs; for a review, see [9–11]) could be involved. In brown fat, four different UCP genes (UCP1, UCP2, UCP3 and UCP5) are expressed. Biochemical activitity of UCP1 and expression of its gene became stimulated by the sympathetic system during cold exposure of the organism and also under other circumstances associated with increased non-shivering thermogenesis. UCP1 is essential for the thermogenic function of this tissue [9,12,13]. In white fat cells, only the UCP2 and UCP5 genes are normally active [9,10,14]. However, both UCP1 [15–18] and UCP3 [17,19,20] can also be induced in white fat by treatments that reduce adiposity. Recent experimental data suggest that, similarly to UCP1, UCP2 and UCP3 can also enhance the proton leak across the mitochondrial inner membrane and decrease the formation of ATP during oxidative phosphorylation [11,21–24].

This study was designed to verify the hypothesis that UCPs can modulate lipolysis in adipose tissue *in vivo*. Transgenic mice [25] overexpressing the UCP1 gene from the aP2 gene promoter (aP2-*Ucp1*) in adipose tissue were used. These animals are resistant to the development of obesity induced by genetic or dietary factors, reflecting a lower accumulation of triacylglycerols in all fat depots except in gonadal (epididymal or periovarian) fat [25]. The differential effect of the transgene on the accumulation of adipose tissue in various anatomical locations is in accordance with the relatively low content of transgenic UCP1 in the gonadal fat [26,27]. The resistance to obesity results from respiratory uncoupling [28] and induction of mitochondrial biogenesis [27] in white but not brown fat [12,29], stimulation of thermogenesis [29] and depression of fatty acid synthesis *in situ* [26] in white fat, and elevation of metabolic rate of the animals [12]. The secondary objective was to test whether transgenic UCP1 could affect the content of G-proteins in adipose tissue, since activation of

Abbreviations used: UCP, uncoupling protein; aP2-*Ucp1* mice, transgenic mice overexpressing the UCP1 gene from the aP2 gene promoter; G_i, inhibitory G-protein; G_s, stimulatory G-protein; $G_i\alpha$, α-subunit of G_i; $G_s\alpha$, α-subunit of G_s; HSL, hormone-sensitive lipase; KRB buffer, modified Krebs–Ringer bicarbonate buffer.

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brown-fat thermogenesis by cold exposure was associated with a functional desensitization of β-adrenergic responses in adipocytes and a decrease of the content of G-protein subunits [30–33], which stimulate (stimulatory G-protein, G_s) or inhibit (inhibitory G-protein, G_i) signal transduction along the cAMP cascade. Our results show that overexpression of UCP1 in white fat *in vivo* is associated with a suppression of the ATP/ADP ratio and a decrease in lipolytic responsiveness to noradrenaline in adipocytes. We present evidence for impaired stimulation of cAMP levels by noradrenaline and down-regulation of HSL in subcutaneous white fat of the transgenic mice, which was accompanied by changes in the expression of G-proteins in adipocytes.

EXPERIMENTAL

Animals

Male C57BL/6J control mice and their transgenic littermates, hemizygous for the aP2-*Ucp1* transgene [25,29,34], were kept in a controlled environment (20 °C with a 12 h:12 h light/dark cycle) with free access to water and a standard chow diet. At 6 months of age, animals were killed by cervical dislocation under anaesthesia. Epididymal and subcutaneous dorsolumbar white-fat depots [29] were dissected and used immediately for the isolation of adipocytes, or frozen and stored in liquid nitrogen for biochemical and RNA analyses (see below).

Isolation of adipocytes and cell-size measurement

Adipocytes were isolated according to Rodbell [35]. Modified Krebs–Ringer bicarbonate buffer (KRB buffer) was used, containing 118.5 mM NaCl, 4.8 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$,$7H_2O$, 25 mM $NaHCO_3$, 5 mM glucose and 4 % (w/v) BSA (fraction V), pH 7.4. Adipose tissue from epididymal and subcutaneous fat depots (1–2 g) was collected from four mice, minced with scissors and digested in 5 ml of KRB buffer containing 3 mg/ml type II collagenase (Sigma catalogue no. C-6885) while shaking at 37 °C for 45 min (epididymal fat) or 90 min (subcutaneous fat). The tissue was then filtered (250 μm) and floating adipocytes were washed three times in the KRB buffer in the absence of collagenase by centrifuging at 400 ***g*** for 1 min at 20 °C. Adipocytes were used for isolation of RNA (see below), measurements of lipolytic activity and quantification of adenine nucleotides and cAMP (see below). For morphometry, adipocytes were suspended at a 10 % (v/v) concentration in physiological buffered saline containing 0.5 % Crystal Violet. After 5 min of staining at room temperature, a drop of the cell suspension was placed on a glass slide using a siliconized pipette tip. An image of adipocytes that concentrated at the liquid/air interface was recorded at a $\times 200$ magnification using a Nikon Eclipse TE 300 inverse microscope equipped with a photographic camera. Pictures were digitalized and the diameter of 250–300 adipocytes in each type of preparation (see Figure 1, below) was determinated using AIDA 2.11 software (Raytest, Straubenhardt, Germany).

DNA and protein contents

The DNA content of isolated adipocytes was measured fluorometrically after digestion (at 56 °C, overnight) in 150 μl of a medium containing 20 mM Tris, 10 mM EDTA, 1 % (w/v) SDS and 50 μg/ml proteinase K [12]. Calf thymus DNA was used as

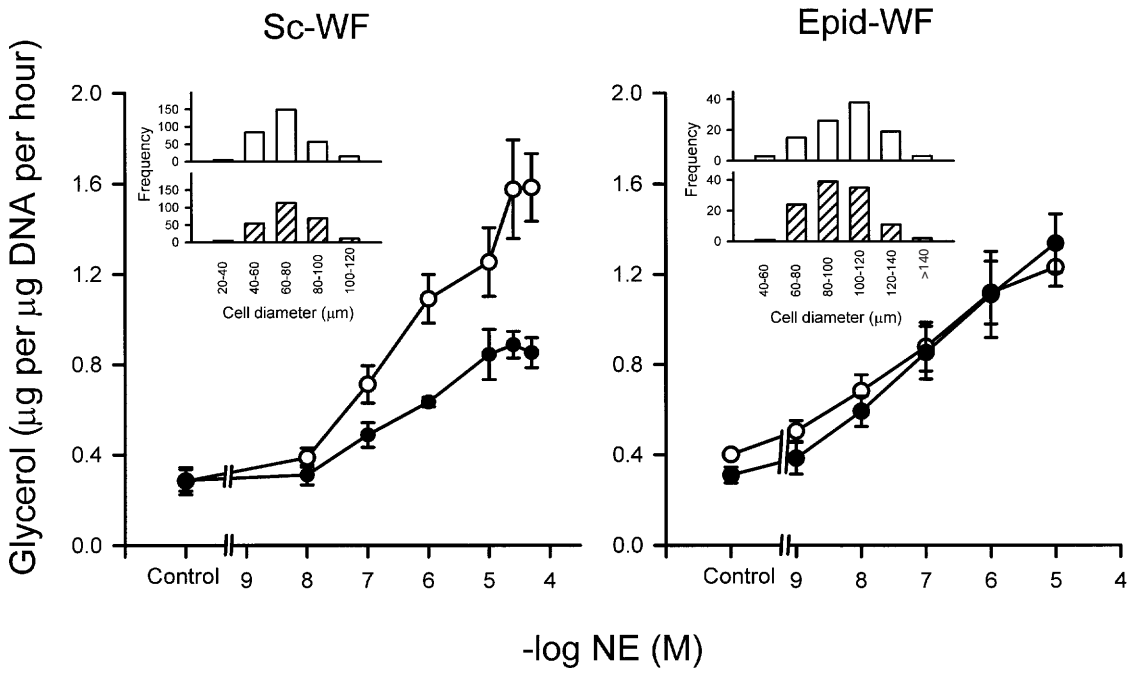

Figure 1 Lipolysis in isolated adipocytes and cell morphometry

Adipocytes isolated from subcutaneous white fat (Sc-WF) or epididymal white fat (Epid-WF) of control (○) or transgenic (●) mice were incubated in KRB buffer in the presence of adenosine deaminase and various concentrations of noradrenaline (NE; 10 nM–50 μM for subcutaneous fat and 1 nM–10 μM for epididymal fat), or in the absence of noradrenaline (Control). Glycerol release was assayed after 60 min of incubation at 37 °C and expressed relative to the DNA content of the sample. Values are means ± S.E.M. from four (Epid-WF) or three (Sc-WF) independent experiments. Inserts: diameters of adipocytes isolated from control (white bars) or transgenic (hatched bars) animals. Mean diameters of cells isolated from subcutaneous fat of control and transgenic mice were 55.2 ± 0.8 and 57.4 ± 0.7 μm, respectively. Corresponding values for epididymal fat were 80.3 ± 1.3 and 76.0 ± 1.1 μm. Only the latter difference between genotypes was statistically significant ($P < 0.03$). Cells isolated from epididymal fat were significantly larger than those from subcutaneous fat.

Table 1 Sequences of PCR primers

In the case of $G_s\alpha$, sense and antisense primers correspond to exons 1 and 6 of the gene. The primers were specific for both short and long forms of the $G_s\alpha$ transcript. In samples from white adipose tissue and isolated adipocytes, only a 426 bp PCR fragment was amplified, corresponding to the long variant. The primers did not detect the following splicing variants: NESP55, $XL_s\alpha$ and untranslated mRNAs containing the alternative exon 1A [63].

Gene	Sense primer (5′ → 3′)	Antisense primer (5′ → 3′)	cDNA GenBank accession no.
$G_s\alpha$	GCAGCGCGAGGCCAACAAAAAGA	CGCACTCCCTCATCCTCCCACAGA	NM-010309
β-actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	X03765

a standard. Protein concentration was measured using the bicinchoninic acid procedure [36] and BSA as a standard.

Lipolysis, lipolytic activity and cAMP measurements

Lipolysis was estimated by following the rate of glycerol release from isolated adipocytes. Aliquots (150 μl) of cell suspension (10 %, v/v) in KRB buffer (see above) were incubated in a 96-well dish for 60 min at 37 °C in 5 % CO_2/95 % air. The medium also contained 6 μg/ml type VIII adenosine deaminase (Sigma catalogue no. A-1030) and various concentrations of noradrenaline as described in the Results. Infranatant (100 μl) was collected and heated for 10 min at 70 °C to inactivate any enzyme released from the cells. Glycerol was assayed enzymically [37] using a Hewlett-Packard 8453 diode-array spectrophotometer. Lipolytic activity was measured in adipose-tissue extracts [38].

Content of cAMP in isolated adipocytes was estimated by radioimmunoassay using the cAMP [^{125}I] scintillation proximity assay system (Amersham Bioscience, Little Chalfont, Bucks, U.K.; kit code RDP 538). Cell suspension was diluted 60-fold in KRB buffer that also contained 100 μM 3-isobutyl-1-methylxanthine (an inhibitor of phosphodiesterase) and 1 μM noradrenaline and incubated for 10 min in 37 °C in a water bath with slow agitation. The liquid phase was extracted according to the manufacturer's instructions by trichloroacetic acid and diethyl ether.

Preparation of membrane fractions from adipose tissue

Frozen tissues were immersed in a buffer containing 50 mM Tris and 10 mM $MgCl_2$ (pH 7.4) and homogenized for 7 min on ice using a motor-driven Teflon/glass homogenizer. The homogenates were filtered through a silk cloth and centrifuged for 60 min at 4 °C and 100000 ***g***. The pellet containing all the adipose tissue membranes was resuspended in the homogenization buffer at a final protein concentration of 5–10 mg of protein/ml; aliquots were kept at −70 °C.

Western blotting and quantitative immunoblot analysis

Membrane fractions (80 μg protein) prepared from adipose tissue of control and transgenic mice were solubilized in the presence of 2.5 % (w/v) SDS and 2.5 % (w/v) mercaptoethanol for 5 min at 95 °C and then analysed on 10 % acrylamide/0.26 % bisacrylamide gels containing 0.1 % (w/v) SDS as described in [30] or on 12.5 % (w/v) acrylamide/0.0625 % (w/v) bisacrylamide gels containing 0.1 % (w/v) SDS and 6 M urea [32]. The resolved proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell), and the blots were blocked with 4 % (w/v) BSA for 1 h and incubated with G-protein-specific primary antisera for 2 h at room temperature. Preparation of primary rabbit anti-$G_s\alpha$ (against the α-subunit of G_s; CS3), anti-$G_i\alpha$1,2 (against α-subunits of G_i1 and G_i2; SG1), anti-$G_i\alpha$3 (against the α-subunit of G_i3; I3B) and anti-Gβ (against the β_1-subunit of G-protein; BN3) antisera has been described previously in detail [30,39]. After three 10 min washes in Tris-buffered saline (TBS; 10 mM Tris/HCl and 150 mM NaCl, pH 8.0) containing 0.03 % (w/v) Tween 20, the secondary goat anti-rabbit IgG labelled with alkaline phosphatase (Sigma) was applied for 1 h. Blots were washed three times in TBS/Tween for 10 min and antigens were visualized using 5-bromo-4-chloro-3-indolyl phosphate (100 μg/ml) and Nitro Blue Tetrazolium (200 μg/ml) dissolved in 100 mM Tris/HCl/100 mM NaCl/5 mM $MgCl_2$ (pH 9.0) [40]. The immunoblots were scanned (Astra 610P; UMAX Data Systems, Hsinchu, Taiwan) and quantitatively analysed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RNA analysis

HSL mRNA was quantified in total RNA isolated from adipose tissue using Northern blots and a 477 bp PCR fragment of mouse HSL cDNA [41] as a probe. The DECAprime II random priming DNA labelling kit (Ambion, Austin, TX, U.S.A.; catalogue no. 1455) was used, and the radioactive signals were evaluated by the Bioimage Analysing System (BAS-5000, Fuji, Tokyo, Japan). Transfer efficiency and the presence of equivalent amounts of ribosomal RNA in the sample were checked by UV shadowing at 256 nm. Quantification of the transcripts for $G_s\alpha$ was performed by real-time quantitative two-step reverse transcriptase PCR using LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). Total RNA was isolated using TRIzol Reagent (Life Technologies, Gaithersburg, MD, U.S.A.). RNA was treated with RNase-free DNase. cDNA was amplified by reverse transcription with poly-T primer using Moloney murine leukaemia virus reverse transcriptase (Life Technologies) followed by real-time quantitative PCR using LC-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals; catalogue no. 2239264) with primers specific for mouse $G_s\alpha$ and β-actin (Table 1). The PCR cycling profile consisted of an initial 10 min activation at 95 °C, followed by 35 cycles of annealing (15 s, 94 °C), extension (5 s, 62 °C) and denaturation (20 s, 72 °C), with melting curve analysis for the final step. Levels of β-actin were used to correct for inter-sample variation.

Determination of ATP and ADP

Flash-frozen tissues or adipocytes stored in liquid nitrogen were extracted by homogenization in 6 % (w/v) perchloric acid (1 ml of solution/200 mg of tissue or 200 μl of cells). After centrifugation for 10 min at 4 °C and 10000 ***g***, the supernatants were neutralized carefully with 0.4 M triethanolamine/1.8 M KOH and pH was adjusted to 6–7. ATP and ADP were determined using the HPLC procedure [42]. Separation and quantification was performed using a Hewlett-Packard HP 1100 system with a

diode array detector. Calibration was performed using the appropriate standards.

Statistics

A two-way ANOVA with post-hoc multiple comparisons was used as described in [29]. Statistical significance was evaluated using the Student's t test. All the comparisons were judged to be significant at $P < 0.05$.

RESULTS

Minor effects of mouse genotype on the morphology of adipocytes isolated from white fat

It is known that the cellular composition of subcutaneous fat is more heterogeneous than that of epididymal fat, with the former tissue containing a relatively large proportion of endothelial, fibroblastic and blood vessel cells, and that the heterogeneity of subcutaneous fat is even more pronounced in aP2-*Ucp1* than in control mice [25]. Therefore, to assess the various effects of transgenic UCP1 (see below) in adipocytes isolated from subcutaneous and epididymal fat pads, it was important to characterize the morphology of the isolated cells. In all cases, more than 95% of cells were typical unilocular adipocytes (results not shown) and cells isolated from epididymal fat were larger than those from subcutaneous fat, whereas no major effect of genotype on the diameter of the isolated adipocytes could be detected (Figure 1). Thus morphometric analysis revealed that the transgene had no large impact on either the morphology or the diameter of the isolated cells.

Depression of ATP/ADP ratio by UCP1 in white fat

Ectopic UCP1 in white fat of transgenic aP2-*Ucp1* mice was expected to decrease ATP synthesis in mitochondria. Therefore, the ATP and ADP contents in adipose tissue of control and transgenic mice were estimated. In subcutaneous fat of the transgenic mice, both ATP content and ATP/ADP ratio tended to be lower than in control mice, but only the latter difference was statistically significant (Table 2). No effect of the transgene on either the content of adenine nucleotides or the ATP/ADP ratio was noticed in epididymal fat (results not shown). The effect of the transgene was confirmed by measurements of the ATP/ADP ratio in collagenase-prepared adipocytes from subcutaneous fat, which indicated a lower ratio in transgenic than in control mice (Table 2). In the case of adipocytes from epididymal fat, the ratio was not affected (results not shown). The differential effect of the transgene on the ATP/ADP ratio in subcutaneous and epididymal fat was in accordance with the approx. 2–3-fold higher expression of the transgene in the subcutaneous fat depot detected at both the mRNA [26] and protein [27] levels.

Table 2 Quantification of adenine nucleotides

Concentrations of ATP and ADP were estimated in neutralized extracts from freeze-clamped subcutaneous white fat ($n = 12$–17) of control (+/+) and transgenic (tg/+) mice, and in adipocytes ($n = 4$) isolated from this tissue. Values are means ± S.E.M. The tissue contents of the nucleotides are expressed in nmol of nucleotide/g of tissue, whereas contents of the nucleotides per adipocyte were not determined (N.D.). Asterisks indicate statistically significant differences between genotypes. Within the same genotype, only the difference between ATP/ADP ratio between the tissue and adipocytes of the transgenic mice was statistically significant.

Sample	Genotype	ATP	ADP	ATP/ADP ratio
Tissue	+/+	179 ± 19	46 ± 4	3.9 ± 0.19
	tg/+	137 ± 17	50 ± 5	2.83 ± 0.19*
Adipocyte	+/+	N.D.	N.D.	3.04 ± 0.93
	tg/+	N.D.	N.D.	1.32 ± 0.2*

Table 3 Stimulation of cAMP levels by noradrenaline in adipocytes

cAMP content was measured in adipocytes prepared from subcutaneous fat of control (+/+) and transgenic (tg/+) mice before (Basal level) and after (Noradrenaline-stimulated) incubation with 1 μM noradrenaline (see the Experimental section). Values are means ± S.E.M. from seven experiments. Asterisks indicate statistically significant differences between genotypes.

	cAMP level (pmol of nucleotide/μg of adipocyte DNA)		
Genotype	Basal level	Noradrenaline-stimulated	Induction (-fold)
+/+	2.6 ± 0.42	4.22 ± 0.48	1.68 ± 0.1
tg/+	2.59 ± 0.09	3.02 ± 0.29*	1.25 ± 0.11*

Depression of noradrenaline-induced lipolysis by UCP1 in adipocytes isolated from white fat

Lipolysis was measured at different noradrenaline concentrations as the rate of glycerol release, and expressed per μg of adipocyte DNA (Figure 1). The maximum rate of noradrenaline-stimulated lipolysis in adipocytes isolated from subcutaneous fat was significantly (approx. 2-fold) lower in transgenic than in control animals. On the other hand, in epididymal fat, noradrenaline stimulated the release of glycerol to a similar extent in both genotypes and lipolysis was more sensitive to stimulation by noradrenaline compared with subcutaneous fat (as illustrated by the effects of the low noradrenaline concentrations tested; see Figure 1). In neither fat depot did the transgene affect the basal level of lipolysis or the concentration of noradrenaline that induced half-maximum activation of lipolysis (the EC_{50} value). The morphometric results described above excluded the possibility that the differential effect of the transgene on noradrenaline-stimulated lipolysis in the two fat depots reflected differences in cell size or other heterogeneity of isolated cells. Therefore, the depression in lipolytic responsiveness to noradrenaline in subcutaneous fat could be related to the change in ATP/ADP ratio induced by the transgene in adipocytes (see above).

Impaired stimulation of cAMP levels by noradrenaline in adipocytes from transgenic mice

To examine the basis for the mitigation of the lipolytic responsiveness to noradrenaline in subcutaneous fat of the transgenic mice, the effect of noradrenaline on the content of cAMP was measured in collagenase-prepared adipocytes. The content of cAMP was similar in adipocytes isolated from subcutaneous fat of both control and transgenic mice (Table 3) and incubation of these cells in the presence of 1 μM noradrenaline and the inhibitor of phosphodiesterase (3-isobutyl-1-methylxanthine; see the Experimental section) resulted in an increase of cAMP levels. This induction was significantly less in transgenic than in control mice (Table 3). No effect of the transgene on the induction of cAMP by noradrenaline could be detected in adipocytes isolated from epididymal fat (results not shown). These data indicate that the differential suppression of the lipolytic responsiveness by transgenic UCP1 in subcutaneous and epididymal fat could be related to impaired induction of cAMP by noradrenaline in the former fat depot.

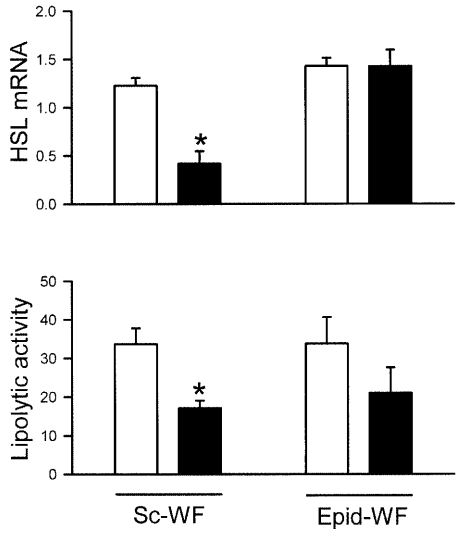

Figure 2 Expression of the HSL gene in adipose tissue and lipolytic activity in tissue extracts

Upper panel: the HSL transcript levels (arbitrary units) were estimated in total RNA isolated from subcutaneous (Sc-WF) and epididymal (Epid-WF) white fat of control (white bars) or transgenic (black bars) mice using Northern blots. Lower panel: lipolytic activity was assayed in tissue extracts and expressed as nmol of cholesteryl oleate hydrolysed/mg of protein. Values are means ± S.E.M. ($n = 5$–6). Asterisks indicate statistically significant differences between genotypes.

Table 4 Relative levels of $G_s\alpha$ and $G_i\alpha$ proteins in adipose tissue

The contents of selected G-protein subunits detected in subcutaneous and epididymal white fat of transgenic mice using immunoblotting (see Figures 3 and 4) are expressed as percentages of the amounts found in the corresponding white fat of control mice (100%). Values are means ± S.E.M. ($n = 6$). Asterisks indicate significant differences between transgenic and control animals. N.D., not detectable.

	G-protein content (% of control)			
Fat depot	$G_s\alpha$	$G_i\alpha1$	$G_i\alpha2$	$G_i\alpha3$
Subcutaneous white fat	184 ± 12*	28 ± 5*	51 ± 6*	14 ± 6*
Epididymal white fat	N.D.	92 ± 7	99 ± 7	49 ± 5*

Changes of HSL gene expression and lipolytic activity induced by UCP1 in white fat

In order to further characterize the mechanism of UCP1-induced alteration of the lipolytic action of the catecholamine, expression of the gene encoding HSL was analysed on Northern blots with total RNA isolated from fat depots of transgenic and control mice. Lipolytic activity of adipose tissue extracts was also estimated (Figure 2). Similar levels of both the HSL transcript and lipolytic activity were found in subcutaneous and epididymal fat of control animals. The presence of transgene resulted in a 3-fold decrease of the HSL mRNA and a 2-fold decrease of enzyme activity in subcutaneous fat. In epididymal fat, no statistically significant differences between the two genotypes in either the HSL mRNA levels or the lipolytic activity could be detected. These results document a down-regulation of the HSL gene by ectopic UCP1 in white fat.

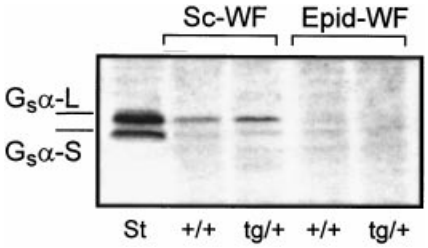

Figure 3 Detection of $G_s\alpha$ in adipose tissue using immunoblotting

Membrane preparations (80 μg of protein/lane) isolated from subcutaneous (Sc-WF) and epididymal (Epid-WF) white fat of control (+/+) or transgenic (tg/+) mice were analysed as described in the Experimental section. Myocardial membranes (50 μg of protein/lane) were used as a standard (St). $G_s\alpha$-S and $G_s\alpha$-L indicate the short and long isoforms of $G_s\alpha$.

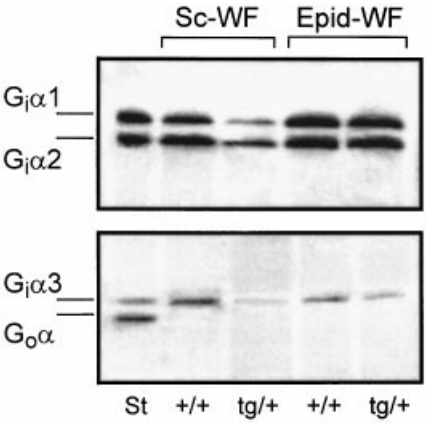

Figure 4 Detection of $G_i\alpha$ in adipose tissue using immunoblotting

Membrane preparations from subcutaneous (Sc-WF) and epididymal (Epid-WF) white fat of control (+/+) or transgenic (tg/+) mice were analysed as in Figure 3. Brain microsomes (50 μg of protein/lane) were used as a standard (St). Because of the known cross-reactivity between $G_i\alpha3$ antibody (I3B) and the G_o subunit [30], a strong band corresponding to G_o appears ahead of the $G_i\alpha3$ band in brain microsomes, which are highly endowed with G_o.

Changes of G-protein levels induced by UCP1 in white fat

The levels of $G_s\alpha$ and $G_i\alpha$ subunits were evaluated in the membrane fractions isolated from adipose tissue depots of control and transgenic mice. The relative contents (Table 4) of various G-protein subunits were determined by densitometric scanning of the immunoblots (Figures 3 and 4). Whereas two isoforms of $G_s\alpha$ were well resolved by conventional PAGE (Figure 3), $G_i\alpha$ proteins could be separated only on high-resolution gels containing urea in addition to SDS (Figure 4). The long but not the short isoform of $G_s\alpha$ was detected in membranes prepared from subcutaneous fat and its content almost doubled in transgenic animals as compared with the controls (Table 4). No detectable $G_s\alpha$ isoforms were found in epididymal fat (Figure 3). The content of all three isoforms of $G_i\alpha$ protein ($G_i\alpha1$, $G_i\alpha2$ and $G_i\alpha3$) was substantially decreased in subcutaneous white fat of transgenic mice compared with control animals (Table 4). No such dramatic differences between the genotypes were observed in epididymal fat. The membrane fractions prepared from epididymal fat depots of transgenic animals contained a lower amount of $G_i\alpha3$ than those from control mice, but the contents of the two major $G_i\alpha$ proteins ($G_i\alpha1$ and $G_i\alpha2$) did not differ between the two genotypes (Table 4).

It was investigated whether the effect of ectopic UCP1 on G-protein levels, detected in the membrane fractions prepared from whole tissue, reflected the expression of G-protein genes in adipocytes. Levels of the transcripts for $G_s\alpha$ that are known to be much higher than those of $G_i\alpha$ in adipocytes [43] were measured in subcutaneous adipose tissue as well as in adipocytes isolated from this fat depot ($n = 5$–6). In both whole tissue and isolated cells the levels of the $G_s\alpha$ transcript were significantly higher in the transgenic (0.61 ± 0.05 and 1.75 ± 0.35 arbitrary units in tissue and adipocytes, respectively) than in control (0.36 ± 0.07 and 0.49 ± 0.07 arbitrary units in tissue and adipocytes, respectively) mice and this difference was greater in the isolated cells (3.6-fold) than in whole tissue (1.7-fold). These results confirmed the idea that the change of $G_s\alpha$ gene expression in adipocytes was responsible for the differences in the $G_s\alpha$ protein content observed in subcutaneous white fat of the two genotypes.

DISCUSSION

This study documents that transgenic UCP1 can decrease the ATP/ADP ratio and the rate of the noradrenaline-induced lipolysis, and affect the expression of G-proteins *in vivo*. These effects could be observed in subcutaneous and not in epididymal fat, reflecting the higher content of transgenic UCP1 in the former fat depot [26,27]. Importantly, most of the results have been documented in both adipose tissue and collagenase-prepared adipocytes. Our results are in agreement with previous studies showing the effect of UCP1 [44,45], UCP2 [23] and UCP3 [21] on the ATP/ADP ratio *in vivo* and suggest a role for UCPs in the modulation of lipolysis and hormone signalling in adipocytes.

Our present data strongly support the hypothesis that noradrenaline-induced lipolysis in adipose tissue may be modulated by the energy status of fat cells. This hypothesis is based on previous findings from experiments *in vitro* showing a reciprocal relationship between the content of intracellular ATP and noradrenaline-induced lipolysis in adipocytes (see the Introduction). A similar mechanism may operate under physiological conditions. Thus in rats, lipolysis could be stimulated very little by noradrenaline, either in epididymal fat, during anaerobiosis, or in interscapular brown fat, after cold acclimation of the animals [3]. Under both situations, mitochondrial ATP production is expected to decrease, due to diminished activity of the respiratory chain, or due to the activation of UCP1 [44]. In normal human subjects fasted overnight, adrenergic stimulation of lipolysis in white fat is not functional [46], whereas a very-low-calorie diet used as part of a weight-reduction program results in an increase in noradrenaline sensitivity of lipolysis (for references, see [1]) and increased sympathetic stimulation of white fat [47]. In rats, fasting results in a reduction of ATP [6] as well as the HSL mRNA level in adipocytes [48]. Importantly, the down-regulation of the HSL gene is transient and could be detected only at the beginning of the fasting period [48].

The resistance against obesity is associated with low lipolytic responsiveness to noradrenaline in white fat of the aP2-*Ucp1* mice [25] but with increased sensitivity of the β-adrenergic/cAMP/protein kinase A signalling pathway in mice over-expressing the winged helix/forkhead transcription factor gene *FOXC2* in adipose tissue [49]. The somehow paradoxical occurrence of the low lipolytic responsiveness in the obesity-resistant aP2-*Ucp1* mice is not without precedent. Targeted disruption of HSL did not induce obesity [50] and reduction of the lipolytic responsiveness to noradrenaline, due to ablation of perilipin in mice, resulted in resistance to diet-induced obesity [51]. Therefore, the attenuation of lipolytic responsiveness in subcutaneous fat to noradrenaline may contribute to the mitigation of obesity in aP2-*Ucp1* mice. Since fatty acid synthesis is also reduced in subcutaneous but not in epididymal fat of aP2-*Ucp1* mice [26] it is conceivable that the ATP/ADP ratio in adipocytes may modulate the activity of two major pathways of lipid metabolism that are critical for lipid accumulation in adipocytes. Low accumulation of subcutaneous but not epididymal fat counteracts development of obesity in the aP2-*Ucp1* mice [25].

Further studies are required to find out whether the efficiency of mitochondrial energy conversion may alter lipid metabolism in white fat and adiposity under physiological conditions. It has to be established whether the depression of the ATP content in white adipocytes by adrenergic agonists [5,52] is mediated by UCP2, or by an other protein that induces proton leak in mitochondria, and whether such protonophores could be activated by intracellular fatty acids which are liberated during catecholamine-induced lipolysis of endogenous triacylglycerols (see the Introduction and [5,7,52]). Results from our laboratory indicate induction of UCP2 expression in white adipose tissue of fasted mice (K. Bardová, P. Flachs, M. Rossmeisl and J. Kopecký, unpublished work).

The lack of ATP may affect lipolytic responsiveness to noradrenaline in adipocytes on several different levels. It has been shown that adenylate cyclase in cardiomyocytes required ATP for effective stimulation by isoproterenol [53]. Our experiments demonstrated mitigated induction of cAMP levels by noradrenaline in subcutaneous fat but not in epididymal fat of the transgenic mice, reflecting the differential effect of the transgene on the ATP/ADP ratio in the two fat depots. These data indicate that the attenuation of lipolytic responsiveness to noradrenaline in transgenic mice results, at least in part, from the impaired formation of cAMP. However, a previous study also suggested that a different site of control, located beyond cAMP formation and depending on ATP synthesis, would become rate limiting to the activation of lipase in white fat during anaerobiosis or due to cold acclimation [3]. Those authors speculated that cAMP-dependent phosphorylation of HSL that activates this enzyme was abolished when ATP concentration in adipocytes was low, and that the phosphorylation relied on intramitochondrial rather than cytoplasmic ATP [3]. Moreover, it was demonstrated that translocation of the lipase from cytosol to the surface of lipid-storage droplets, which represents the major consequence of the phosphorylation of the lipase, required ATP [54]. Our data suggest that either the transcription of the HSL gene, or the stability of its transcript, may decrease following a drop of ATP level in adipocytes. The factors modulating HSL gene expression are largely unknown. There are at least five alternative first exons for HSL in mouse adipose tissue. Multiple *cis*-elements are located upstream of the exons, suggesting differential transcriptional control of HSL in response to various physiological stimuli [55].

Our findings that the membrane preparations from subcutaneous fat of transgenic mice contained a significantly higher amount of $G_s\alpha$ and a lower amount of $G_i\alpha$ protein subunits compared with control animals strongly suggest a link between mitochondrial energetics and the content of trimeric G-proteins in adipocytes. The differences in $G_s\alpha$ levels reflected those in the levels of the $G_s\alpha$ mRNA, in both subcutaneous white fat and isolated adipocytes. No such genotypic differences were found in preparations derived from epididymal fat where the expression of transgenic UCP1 was relatively low and the ATP/ADP ratio in adipocytes was not changed.

As we showed previously, activation of brown-fat thermogenesis (and UCP1) in cold-exposed animals, and desensitization

of β-adrenergic responses in adipocytes, were associated with a depression of G-protein levels [30–33]. Moreover, stimulation of brown adipocytes by β_3-adrenergic agonists, which mimics the activation of brown fat by cold, resulted in a complete elimination of insulin-stimulated glucose uptake. The resistance to insulin was explained by the effect of the β_3-adrenergic agonists on protein kinases in adipocytes [56]. However, it has been also shown that both $G_i\alpha 2$ [57] and intracellular ATP [58] were required for insulin signalling. Therefore, our results suggest that both desensitization of β-adrenergic responses and insulin resistance during sympathetic over-stimulation of brown adipocytes have a common cause. The most important factor may be the alteration of G-protein levels in adipocytes, resulting from the activation of UCP1 and the consequent decrease of the intracellular ATP/ADP ratio.

Besides the ATP/ADP ratio, reactive oxygen species may also represent a link between mitochondria and G-proteins in various tissues, since formation of reactive oxygen species in mitochondria is probably depressed by UCPs ([59] and references therein) and some of the G-proteins are activated by reactive oxygen species [60]. The mechanism underlying the effect of mitochondrial energetics on the expression of various G-proteins in adipose tissue deserves further investigation. Recent studies underlined the crucial importance of $G_s\alpha$ protein in mediating hormonal signalling in adipocytes in both humans [61] and mice [62], in particular for the lipolytic action of noradrenaline [61], as well as involvement of the maternal allele of the $G_s\alpha$ gene in obesity [61,62].

Taken together, our results support the idea that the energy status of adipocytes modulates the lipolytic action of catecholamines and may affect expression of G-protein subunits *in vivo*. UCPs and other protonophores that decrease adiposity by increasing energy expenditure and by mitigating lipogenesis could also depress the catecholamine responsiveness of lipolysis due to lowering mitochondrial ATP synthesis in adipocytes. Changes at the G-protein level may represent a secondary response to the alteration of energy status that tends to compensate for the depression of lipolysis and exerts a broad influence on hormonal signalling in adipocytes.

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