Journal of Cell Science 108, 3171-3180 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
JCS4008

Functional assessment of white and brown adipocyte development and energy metabolism in cell culture

Dissociation of terminal differentiation and thermogenesis in brown adipocytes

Susanne Klaus*, Marina Ely, Dag Encke and Gerhard Heldmaier

Fachbereich Biologie/Zoologie, Philipps Universität Marburg, Karl v. Frisch Str., 35043 Marburg, Germany

*Author for correspondence

SUMMARY

We investigated the effect of insulin, triiodothyronine (T_3) and dexamethasone (a synthetic glucocorticoid) on differentiation, lipid metabolism and thermogenesis of preadipocytes isolated from white fat (WAT) and brown fat (BAT) from the Siberian dwarf hamster (*Phodopus sungorus*). Cell cultures from WAT and BAT were chronically treated with the above hormones alone or in any combination. After differentiation (day 8 or 9 of culture) we measured the following parameters: adipogenic index (number × size of adipocytes), protein content, lipolysis, cell respiration, and expression of the uncoupling protein UCP, which is unique to mitochondria of brown adipocytes. Insulin was the most important adipogenic factor for brown and white adipocytes and necessary for terminal differentiation, whereas dexamethasone alone completely inhibited differentiation. T_3 had no effect on adipogenesis in WAT cultures, but further increased insulin stimulated adipogenesis in BAT cultures. Basal lipolysis was higher in WAT than in BAT cultures except when dexamethasone was present, which stimulated lipolysis in both culture types to the same extent. T_3 had a pronounced dose dependent lipolytic effect on WAT cultures but very little effect on BAT cultures. Respiration rates were generally higher in differentiated adipocytes than in fibroblast like cells. T_3 had no effect on thermogenesis in WAT cultures but increased thermogenesis in BAT cultures, and this was further elevated by insulin. UCP expression in BAT cultures could be detected by western blot in insulin treated, T_3 treated and insulin + T_3 treated cultures with highest expression in the latter. These results imply a possible dissociation of terminal differentiation and thermogenic function of brown adipocytes. In WAT cultures there was also a low level of UCP detectable in the insulin + T_3 treated cultures. Immuno-fluorescence microscopy analysis revealed the presence of UCP in 10-15% of adipocytes from WAT cultures (in BAT cultures: 90%), indicating the presence of some brown preadipocytes in typical WAT deposits.

Key words: cell respiration, lipolysis, uncoupling protein, insulin, triiodothyronine, dexamethasone

INTRODUCTION

White adipose tissue (WAT) and brown adipose tissue (BAT) both play an important role in mammalian energy equilibrium because they are important for energy storage (WAT) as well as energy dissipation (BAT). The thermogenic i.e. energy dissipating function of brown fat is important for maintenance of body temperature in small and newborn mammals, and for arousal from the hypothermic state in hibernators. It is also the source of the so-called diet induced thermogenesis, which can be observed after overeating in several animal models (Rothwell and Stock, 1986). Brown fat is, in contrast to white fat, highly innervated and vascularised. Morphologically, brown adipocytes can be distinguished from white adipocytes by multilocular lipid inclusions and numerous, well developed mitochondria, whereas white adipocytes are characterized by one large lipid inclusion and very few mitochondria (reviewed by Néchad, 1986). Brown fat mitochondria are equipped with a unique protein, called uncoupling protein (UCP) which is a proton translocator in the inner mitochondrial membrane and functions as an uncoupler of the mitochondrial respiratory chain (reviewed by Nicholls et al., 1986; Ricquier et al., 1991; Klaus et al., 1991a). A UCP containing adipocyte is considered a brown adipocyte and it has repeatedly been reported that typical white fat deposits can contain UCP expressing fat cells (Lonçar, 1991; Cousin et al., 1992). Although white and brown adipocytes have quite different physiological functions, it is not yet clear if they represent truly different cell types or if a conversion from one type into the other is possible. In larger mammals an apparent conversion from BAT into WAT takes place (Casteilla et al., 1989), but so far there exists no strong evidence for a possible conversion from WAT into BAT.

Adipocyte cell cultures are widely employed as model systems for terminal cell differentiation and numerous white preadipocyte cell lines have been studied for many years (reviewed by Cornelius et al., 1994). The first brown

preadipocyte cell lines have been characterized only very recently (Klaus et al., 1994; Kozak and Kozak, 1994). Although cell lines can be useful tools, they suffer the disadvantage that as they are immortalized they might differ considerably from tissue preadipocytes in regard to development and energy metabolism. Development of white as well as brown preadipocytes has therefore also been studied in stromal-vascular preadipocytes isolated from WAT and BAT of different species (Cornelius et al., 1994 and references within; Rehnmark et al., 1989; Casteilla et al., 1991; Klaus et al., 1991b; Champigny et al., 1992). Due to species and culture condition differences, results from the different studies even concerning only one given adipocyte type are occasionally quite controversial. To draw conclusions from comparisons of different studies concerning brown and white preadipocyte differentiation seems thus rather arguable. However, to our knowledge no extensive comparative studies have been performed on development and metabolism from preadipocytes isolated from WAT and BAT from the same animal and cultured in parallel under identical conditions.

There are numerous hormones and other factors which play a role in proliferation and differentiation of adipocytes (reviewed by Ailhaud, 1990; Ailhaud et al., 1992; Butterwirth, 1994). Insulin and T_3 were shown to be important for terminal differentiation in some adipocyte cell models (Grimaldi et al., 1982; Ailhaud, 1990; Hauner, 1990; Darimont et al., 1993). Glucocorticoids are also known to be involved in fat metabolism and adipocyte terminal differentiation, although their effects seem to be different, depending on the time of addition relative to the cell cycle (Xu and Björntorp, 1990). As the hormones mentioned have also been reported to be involved in brown fat thermogenic function (Park et al., 1989; Geloen and Trayhurn, 1990; Reiter et al., 1990) we decided to study the effect of chronic treatment with insulin, T_3 and dexamethasone (a synthetic glucocorticoid) on differentiation and development of energy metabolism of brown and white preadipocytes in cell culture.

We chose a comparative approach using stromal-vascular preadipocytes isolated from WAT and BAT of the Siberian hamster (*Phodopus sungorus*). This species has already proved an excellent system for the study of brown adipocyte differentiation (Klaus et al., 1991b). The Siberian hamster also possesses abundant white fat deposits and is prone to considerable seasonal changes in body fat, which makes it a good candidate for the study of white adipocyte development. Furthermore, we investigated the metabolic activity of adipocytes cultured in vitro by measuring cell respiration in order to relate these data to the physiological function of the two adipose types in vivo.

MATERIALS AND METHODS

Cell cultures

Inguinal white fat and several deposits of brown fat (axillar, suprasternal, interscapular, dorsal-cervical) from Siberian hamsters (*Phodopus sungorus*) aged 4 to 6 weeks and kept at thermoneutrality (23°C) were used for tissue cultures. Fat deposits from 4 to 6 animals of both sexes were pooled for every tissue culture. The stromal-vascular fraction was obtained after collagenase treatment as described by Klaus et al. (1991b). Cells were inoculated in Petri dishes (10 cm or 6 cm diameter) at approximately 1,500-2,000 cells/cm^2. Cells were grown at 37°C in air with 5% CO_2 content and 100% relative humidity in cell culture medium (50% modified Eagle's medium (Gibco/BRL) and 50% F12 Ham's F12 medium (Gibco/BRL) supplemented with $NaHCO_3$ (1.2 g/l), biotin (4 mg/l), Ca-panthotenate (2 mg/l), glutamine (5 mM), glucose (4.5 g/l) and Hepes (15 mM, pH 7.4), penicillin G (6.25 mg/l), and streptomycin (5 mg/l)). Until the third day of culture, the medium was supplemented with 10% fetal calf serum (FCS) then it was changed to 7% FCS. The medium was changed at days 1 and 3. Hormonal supplements were added at day 3 unless otherwise indicated. The medium referred to as standard culture medium was supplemented with 17 nM insulin and 1 nM T_3. At the indicated day of harvest, cell culture medium was aspirated and dishes with cells were directly frozen and kept at −20°C until further analysis.

Morphological cell analysis

Adipocytes were counted directly in the dish with a phase-contrast microscope at a ×100 magnification with an ocular grid corresponding to a total surface of 1 mm^2. At least four randomly chosen fields were counted for each dish. Adipocytes could be distinguished from preadipocytes by the presence of visible lipid droplets. For better visualization, lipids were stained with O Red Oil and only cells positive for this stain were considered as adipocytes. Adipocyte size was measured at a ×500 magnification using an ocular scale. For each cell the width and the length were measured and multiplied in order to obtain a surface index (expressed in μm^2). Total cell number was assessed directly in aliquots of the suspended cells used for respiration studies, using a Thoma counting chamber.

Lipolysis

The amount of glycerol released into the medium was determined as an index for lipolytic activity of cells. Glycerol determination was performed using a kit from Boehringer Mannheim (No. 148 270) following the manufacturer's instructions, except that the test volume was scaled down to 600 μl. Samples of cell culture medium (50-100 μl) were drawn on the indicated days and frozen until measurement. The glycerol content of medium containing 7% FCS (approximately 10.5 mg/ml) was subtracted from each value.

Cell respiration

Respiration was measured with a Clark type electrode at 31°C. This temperature was chosen because at 37°C the electrodes were not very stable and showed considerable drift. Cell medium was aspirated and cells from one 10 cm dish were suspended in 1 to 2 ml of cell culture medium (without FCS) containing 5 mM EDTA, which detaches cells from the bottom. The culture medium used for respiration was saturated with air, containing 5% CO_2. The cell suspension (1 ml, which was gently pipetted several times to ensure detachment of cells) was immediately filled into the reaction chamber of the Clark electrode, and oxygen consumption was continuously monitored on a chart recorder. After a few minutes, isoproterenol (0.1 μM) was added for measurement of β-adrenergic stimulation of cell respiration. Finally, maximum i.e. uncoupled respiration rates were obtained by addition of an uncoupler (FCCP, 0.1 μM). For each cell preparation we thus obtained values for unstimulated, stimulated and uncoupled respiration. For calculation of oxygen consumption, the medium was considered to contain 433 nmol O/ml. An aliquot of the cell suspension was used for cell counting (see above). Protein content was determined by the method of Bradford. Cells were recovered after the experiment and frozen for further analysis (e.g. western blot).

Western blot analysis

For western blot analysis total cell homogenates were prepared by sonification of cells in PBS. A 20 μg sample of total protein was used for SDS-PAGE and immuno-blotting using a polyclonal antibody against Siberian hamster UCP as described (Klaus et al., 1991b),

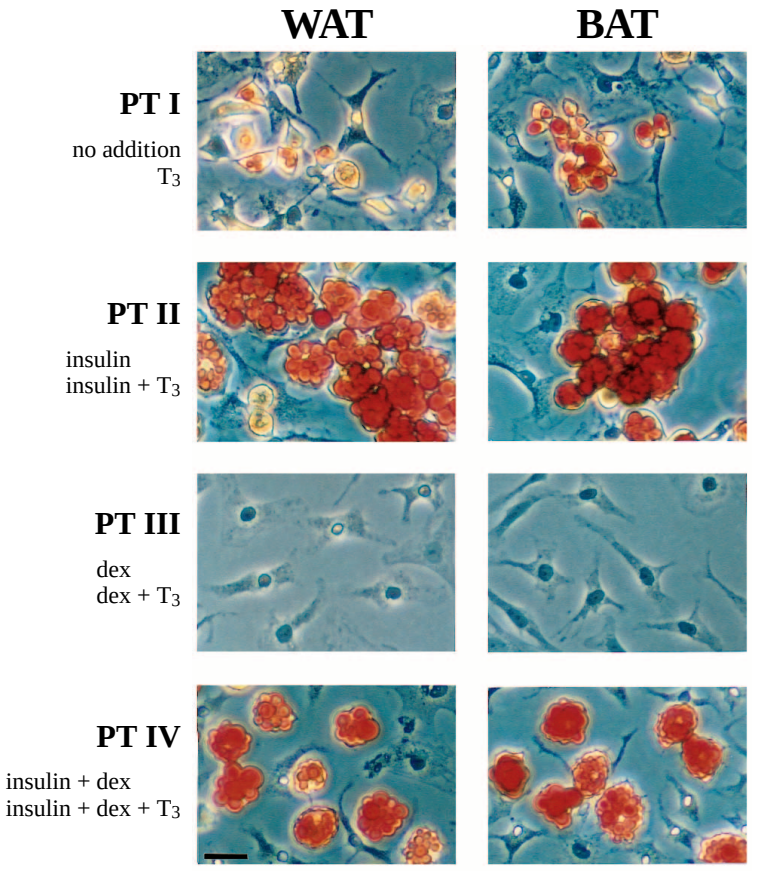

Fig. 1. Microphotograph of cultured stromal-vascular cells isolated from white adipose tissue (WAT) and brown adipose tissue (BAT). Preadipocytes were cultured from day 3 in medium containing 7% FCS and the indicated supplements. At day 10 of culture cells were stained with O Red Oil which specifically stains neutral lipids. Four different phenotypes (PT) were obtained depending on hormonal supplements. Bar, 40 μm.

except that proteins were blotted on Hybond-C membrane (Amersham) and an enhanced chemoluminescence (ECL) western blotting detection system (Amersham, Germany) was used with a horseradish peroxidase-conjugated second antibody according to the manufacturer's protocol. Signals were quantified by densitometrical scanning of the films.

Indirect immuno-fluorescence microscopy

Suspensions of the stromal-vascular fraction of brown or white fat tissues were dropped into wells of a multiwell-coverslip. After 2 hours samples were covered with cell culture medium and cells were cultured as indicated above. At day 8 or 9 coverslips were washed with ice-cold PBS and fixed in ice-cold 95% ethanol/5% acetic acid for at least 10 minutes. Coverslips were washed 3 times in cold PBS. Cells were then permeabilized consecutively at –20°C in 50% acetone, 100% acetone, and 50% acetone for 1 minute each. The following steps were performed at room temperature unless otherwise indicated. After washing in PBS cells were incubated with 20% FCS in PBS for 40 minutes. After the PBS wash samples were incubated with the first primary antibody (chicken serum against LPL, 1:1,000, diluted with IIF dilution buffer (0.5% bovine serum albumin, 1% gelatin, 0.1% Tween-20) for 1 hour in a moist chamber. Coverslips were washed 3 times in PBS. Cells were incubated with second antibody (FITC-conjugated goat anti-chicken IgG, 1:10 (DAKO GmbH, Hamburg, Germany) diluted with IIF buffer) for 1 hour in a moist chamber. After 3 washes in PBS antibodies were fixed with 95% ethanol/5% acetic acid at –20°C for 20 minutes. Then this procedure was repeated for a second primary antibody (rabbit serum against UCP, 1:1,000 diluted in IIF buffer) and second antibody (rhodamine-conjugated swine anti-rabbit IgG, 1:40 diluted in IIF dilution buffer). After 3 washes in PBS samples were stained with the DNA-specific dye DAPI (10 μg/ml, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) for 2 minutes. The coverslips were mounted in 50% glycerol, 2.5% DAPCO (1,4-diazabicyclo-2.2.2-octan, Merck, Darmstadt, Germany) for stabilization of fluorescence. Samples were examined using a Zeiss Axiophot microscope equipped with fluorescence optics (40/0.9 neofluar objective).

RESULTS

Adipogenesis

In order to evaluate the effect of different hormones on white and brown adipocytes in culture, we supplemented the cell culture medium (containing 7% FCS) from day 3 onward, with insulin (17 nM), T_3 (1 nM), and/or dexamethasone (10 μM). This experimental design resulted in 8 different groups: (1) no addition (–); (2) insulin (I); (3) T_3 (T3); (4) dexamethasone (Dex); (5) insulin + T_3 (I+T3); (6) insulin + dexamethasone (I+Dex); (7) T_3 + dexamethasone (T3+Dex); (8) insulin + T_3 + dexamethasone (I+T3+Dex).

In preliminary experiments, medium supplemented with charcoal treated FCS or non treated FCS was compared in order to evaluate a possible T_3 contamination of FCS. However, no difference in either cell morphology or respiration could be detected between the two FCS types (not shown). The different hormonal treatments resulted in 4 different phenotypes, both in BAT and WAT cultures (Fig. 1). In phenotype I clusters of rounded cells with very little lipid accumulation were located amongst fibroblast like cells. This corresponds to the groups with no addition or only T_3 addition (groups 1 and 3). We shall refer to these cells as poorly differentiated adipocytes. Phenotype II was characterized by clusters of fully differentiated, mature adipocytes with numerous, big lipid droplets. This corresponded to the groups with insulin and

insulin + T_3 addition (groups 2 and 5). In phenotype III no apparent terminal differentiation took place, cells resembled fibroblast like precursor cells with no lipid inclusion (termed non differentiated cells). This was the case in cells treated with dexamethasone and dexamethasone + T_3 (groups 4 and 7). Finally, phenotype IV was characterized by numerous single adipocytes which were not arranged in the typical cluster pattern, but rather singled out. This corresponded to the groups treated with insulin + dexamethasone and insulin + dexamethasone + T_3 (groups 6 and 8). Taken together these results mean that T_3 had no influence on the phenotype of cultured WAT and BAT cells; insulin, however, was important for lipid accumulation, i.e. acquirement of a terminally differentiated phenotype. Dexamethasone completely inhibited adipocyte differentiation when added alone (Table 1) and together with insulin it still prevented the formation of the typical cluster pattern of differentiated adipocytes.

We investigated more closely the different adipogenic actions of the different hormones (Table 1). The total number of lipid containing cells (including both poorly and fully differentiated adipocytes) was significantly higher when cultures were treated with insulin (100 and 91 adipocytes/mm^2 in WAT and BAT, respectively) than in the cultures treated only with T_3 or without hormonal supplements. In this respect no difference was found between cultures of BAT and WAT. Nevertheless, in phenotype II there was a difference in size between white and brown adipocytes. As can be seen in Table 1, adipocytes from cultured WAT were significantly bigger than adipocytes from BAT cultures when treated with insulin or insulin + T_3. Interestingly, when insulin was present, T_3 had a positive effect on adipocyte size in BAT cultures but not in WAT cultures. T_3 alone had no effect on either adipocyte size or number. As adipocyte size correlates with lipid accumulation, we defined an adipogenic index by multiplying the number of lipid containing cells with the adipocyte size. The adipogenic index is thus an indicator for the adipose conversion of a given cell culture. Fig. 2 shows the adipogenic index of cell cultures from WAT and BAT treated with different hormones. In both cell culture types the adipogenic index was very low in the absence of insulin. In the presence of insulin the adipogenic index of WAT cultures was twice as high as that of BAT cultures. T_3 had no influence on WAT cultures, but increased the adipogenic index of BAT cultures by about 45% in the presence of insulin. When dexamethasone was present in addition to insulin, the adipogenic index of both adipocyte culture types was the same. Protein content increased with differentiation state and in both cultures the protein content was significantly elevated only in phenotype II cultures, i.e. after treatment with insulin or insulin + T_3 (Table 1). This result corroborates the importance of insulin for terminal differentiation.

Lipolysis

Basal lipolytic activity was distinctly different in BAT and WAT cultures. WAT cultures supplemented with insulin and T_3 released over 5 times as much glycerol into the medium as corresponding BAT cultures (Table 1). When adipocyte differentiation was inhibited by the addition of dexamethasone, there was no glycerol release at all, which means that lipolysis was restricted to differentiated adipocytes. (Negative values indicate that there was even a slight uptake of glycerol from the culture medium in these cases.) As the number of adipocytes was different depending on culture conditions, we

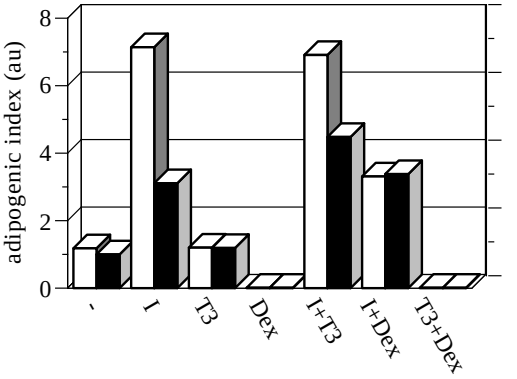

Fig. 2. Adipogenic index (number × size of adipocytes) from cultured stromal-vascular cells isolated from white adipose tissue (open bars) and brown adipose tissue (filled bars). Preadipocytes were cultured from day 3 in medium containing 7% FCS and indicated hormonal supplements (see text for abbreviations). Size and number of adipocytes were measured at day 8 of culture and adipogenic index was calculated from the mean values listed in Table 1.

Table 1. Effect of culture medium supplements on adipogenesis and lipolysis

	Total cell number (per mm^2)		Number of adipocytes (per mm^2)		Adipocyte surface index (μm^2)		Total protein content (mg/10^6 cells)		Glycerol released into medium (μg/plate)	
	WAT	BAT	WAT	BAT	WAT	BAT	WAT	BAT	WAT	BAT
None	246±23	241±44	57.7±3.8	52.2±4.5	275±18	258±32	0.67±0.06	0.63±0.08	78±18	21±10
I	275±21	294±40.3	100.6±5.3*	91.2±2.9*	957±68*	460±22*,†	0.97±0.06*	0.93±0.07*	250±30*	92±32*,†
T3	268±21	239±23.1	50.7±4.8	59.2±5.9	321±30	274±20	0.66±0.03	0.74±0.05	111±29	36±11*,†
Dex	199±15	215±25	0	0	-	-	0.55±0.06	0.54±0.11	–6±6*	–27±8*
I+T3	295±23	281±24	102.3±5.2*	85.5±5.2*,†	913±39*	706±38*,†	1.02±0.06*	1.05±0.12*	280±26*	53±12*,†
I+Dex	304±46	275±47	75.6±4.1*	74.8±5.3*	591±30*	611±40*	0.59±0.06	0.54±0.11	587±46*	583±37*
T3+Dex	189±30	213±37	0	0	n.d.	n.d.	0.62±0.09	0.63±0.18	–27±19*	–15±19
I+T3+Dex	229±23	212±20	84.6±7.4*	53.6±10.0	n.d.	n.d.	0.66±0.04	0.64±0.07	1205±99*	748±103*

Cell number and size index was obtained as indicated in the text. Protein content was determined by a Bradford assay. All values are means ± s.e.m. from multiple experiments (at least 5 different cell cultures) from day 8 or 9 of culture.

*Value significantly different (P<0.05) from non-treated control.

†Value of BAT culture significantly different (P<0.05) from corresponding WAT culture. n.d. not determined.

calculated the glycerol released per adipocyte which is shown in Fig. 3. White adipocytes released consistently more glycerol than brown adipocytes, unless dexamethasone + insulin were supplemented. Dexamethasone had in both fat culture types a pronounced lipolytic effect (in the presence of insulin) throughout the whole culture period. Interestingly, dexamethasone induced lipolysis was further elevated by the presence of T_3 in BAT as well as in WAT cultures. In order to examine more closely the effects of dexamethasone and T_3 on lipolysis of brown and white adipocytes, we measured the glycerol release induced by 48 hours treatment with different concentrations of dexamethasone and T_3 (Fig. 4). At 1 nM, dexamethasone already had a pronounced lipolytic effect on both WAT and BAT cultures. T_3 on the other hand, strongly increased lipolysis in WAT cultures in a dose dependent matter, but had little effect on BAT cultures even at high concentrations.

Cell respiration

Using a Clark type electrode we could measure respiration rates i.e. metabolic activity of cultured BAT and WAT cells. In Table 2 the mean respiration rates of BAT and WAT cultures grown with different hormonal supplements are summarized. In both culture types respiration rates were lowest when adipocyte differentiation was inhibited by the presence of dexamethasone, i.e. in phenotype III. In the presence of insulin, when differentiated adipocytes were present, unstimulated as well as maximum (uncoupled) respiration was always higher. As we had estimated the number of differentiated adipocytes in the different

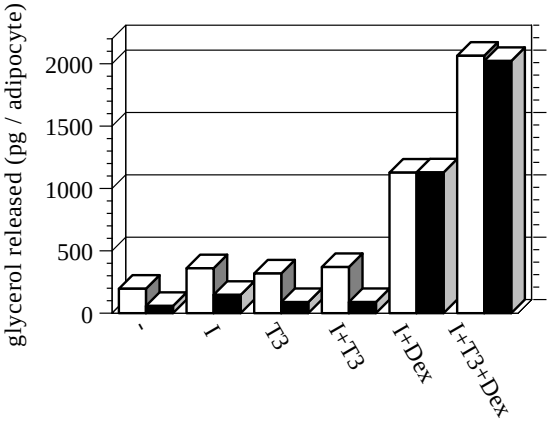

Fig. 3. Lipolysis of adipocytes from WAT (open bars) and BAT cultures (filled bars). Preadipocytes were cultured from day 3 in medium containing 7% FCS and indicated hormonal supplements (see text for abbreviations). Glycerol content of the culture medium was measured at day 8 of culture and the glycerol released per adipocyte was calculated from the mean values listed in Table 1.

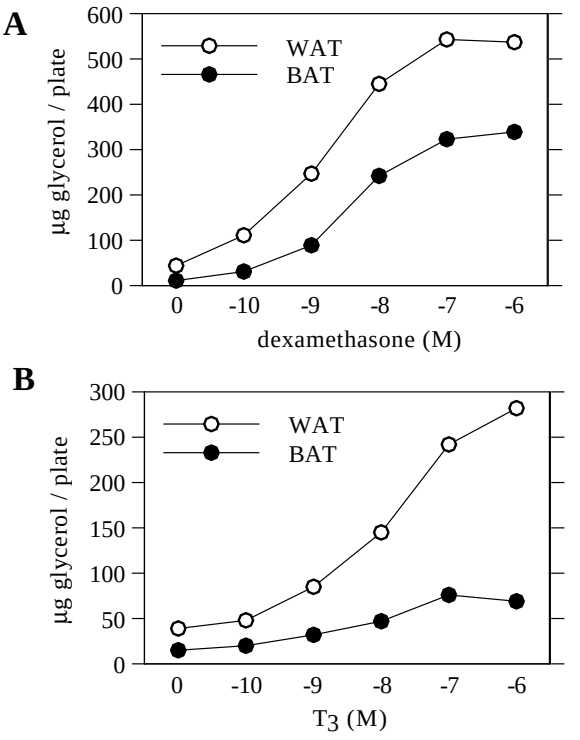

Fig. 4. Dose response curve of lipolysis of WAT and BAT cultures treated with dexamethasone or T_3 for 48 hours. Preadipocytes were cultured from day 3 in 3 cm Petri dishes in medium containing 7% FCS and 17 nM insulin. At day 6 dexamethasone (A) or T_3 (B) were added and glycerol content in the medium was measured 48 hours later (day 8). Glycerol content before the addition was subtracted from the values.

Table 2. Effect of culture medium supplements on cell respiration

	Respiration non stimulated (nmol O/min per 10^6 cells)		Respiration +isoproterenol (nmol O/min per 10^6 cells)		Respiration +FCCP (nmol O/min per 10^6 cells)	
	WAT	BAT	WAT	BAT	WAT	BAT
None	9.4±0.9	9.1±1.0	12.3±1.2	10.8±1.3	19.1±4.3	14.6±2.0
I	19.2±4.6	25.1±6.1	23.2±4.8	30.7±6.9*	28.3±4.4	41.2±5.9*
T3	10.7±1.2	23.6±4.2*,†	13.7±1.7	30.6±5.2*,†	17.0±1.9	29.4±5.5*
Dex	2.7±1.5*	3.5±1.1*	3.7±2.4	4.9±1.9	6.9±2.7	7.0±2.8
I+T3	28.6±6.5*	52.7±6.6*,†	35.4±7.2*	72.5±10.7*,†	42.3±6.9*	85.4±11.2*,†
I+Dex	15.4±4.2	13.4±3.8	18.3±3.5	17.0±3.8	31.1±4.4	22.5±4.5
T3+Dex	5.3±1.5	3.9±1.1	8.9±2.3	4.2±1.3*	11.3±2.6	6.2±2.5†
I+T3+Dex	19.1±5.6	18.2±5.1	22.5±4.7	27.0±5.4	27.6±3.7	28.4±6.0

Respiration rates of dispersed cells from WAT and BAT cultures with different hormonal supplements. All values are means ± s.e.m. from at least 4 different cell cultures at day 9 of culture.

*Value significantly different (P<0.05) from non-treated control.

†Value of BAT culture significantly different (P<0.05) from corresponding WAT culture.

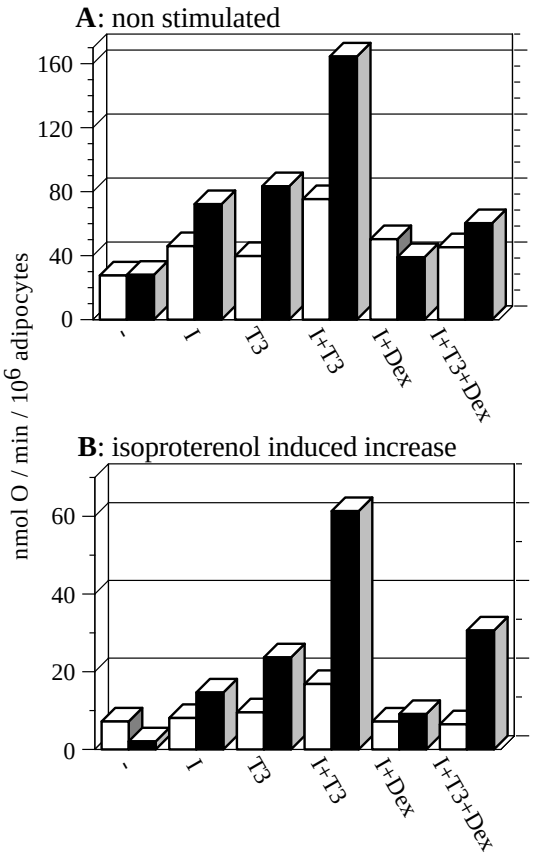

Fig. 5. Respiration rates of adipocytes from WAT (open bars) and BAT cultures (filled bars). Preadipocytes were cultured from day 3 in medium containing 7% FCS and indicated hormonal supplements (see text for abbreviations). Respiration was measured at day 9 and adipocyte specific respiration rates were calculated from the values listed in Tables 1 and 2.

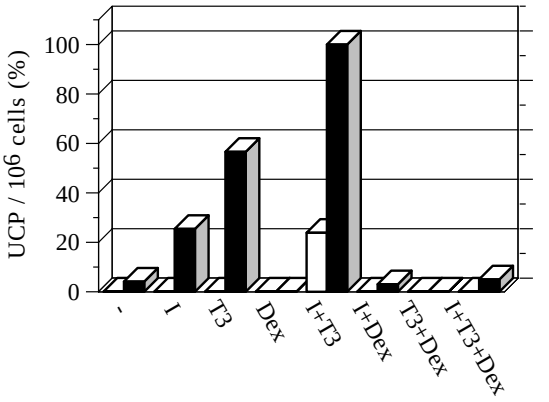

Fig. 6. Western blot analysis of UCP expression in total cell homogenates from WAT (open bars) and BAT (filled bars) cultures at day 9. Preadipocytes were cultured from day 3 in medium containing 7% FCS and indicated hormonal supplements (see text for abbreviations). A 20 μg sample of total cell protein was separated by SDS-PAGE, blotted onto Hybond C membrane and probed with anti-UCP immune serum using an ECL detection system. Signals were quantified by densitometrical scanning. Maximum UCP expression was set at 100% and relative UCP expression in 10^6 cells was calculated. Means of three independently performed cell cultures are shown.

culture conditions as well as the total cell number (Table 1), we could thus calculate the respiration per adipocyte, by subtraction of respiration of non differentiated cells (mean value of 3.9 nmol O/min per 10^6 cells). Fig. 5 shows the non stimulated respiration and the isoproterenol induced increase in respiration of adipocytes from BAT and WAT cultures. It should be noted that even in WAT cultures the respiration of mature adipocytes was over 10 times higher than that of non differentiated cells (46 nmol O/min per 10^6 adipocytes versus 3.9 nmol O/min per 10^6 cells) and there is an excellent correlation between respiration and adipogenic index. An increased respiratory capacity can thus be considered as an additional marker of terminal differentiation for white adipocytes. In both culture types non stimulated respiration as well as β-adrenergic induced increase in respiration was highest when cells were grown with insulin + T_3. However, in this case adipocytes of BAT cultures had much higher respiration rates than those of WAT cultures. This is also reflected in cytochrome *c* oxidase activity, which was 84 nmol O/min per 10^6 total cells in WAT cultures compared to 147 nmol O/min per 10^6 total cells in BAT cultures (both treated with insulin + T_3). A further difference between WAT and BAT cultures was the increased respiration of poorly differentiated BAT adipocytes due to T_3 alone, without addition of insulin. The β-adrenergic stimulation of adipocyte respiration, i.e. thermogenesis was two and three times higher in BAT cultures than in WAT cultures supplemented with T_3 and insulin + T_3, respectively. Fully differentiated brown adipocytes cultured in standard medium (insulin + T_3) increased their respiratory capacity by 40 times as compared to non differentiated cells. The maximum, uncoupled respiration of 260 nmol O/min per 10^6 adipocytes was reached by brown adipocytes treated with insulin + T_3. This is very similar to respiration rates found by Nedergaard et al. (1977), who evaluated respiration of freshly isolated adipocytes from Syrian hamster brown fat. They report noradrenaline stimulated respiration rates of about 230 nmol O/min per 10^6 freshly isolated adipocytes.

UCP expression

As the uncoupling protein UCP represents a qualitative marker for brown adipocytes, we analyzed the UCP expression in the cell cultures by western blot analysis and immuno-fluorescent assay. Fig. 6 shows the level of UCP expression in total cell homogenates (20 μg protein of WAT and BAT cultures). In BAT cultures UCP expression was only significant in cells treated with insulin, T_3, and insulin + T_3 with highest levels always in insulin + T_3 treated cultures. This pattern of UCP expression is very much like the one of respiration rates (Fig. 5). Taken together with the adipogenic index this means that in brown adipocytes it is possible to dissociate to a certain extent terminal differentiation from thermogenic function. As UCP expression is restricted to brown adipocytes, we did not expect a UCP signal in WAT cultures. However, a weak signal was apparent in the insulin + T_3 treated cells which could indicate the presence of brown adipocytes in this cultures. As western blot analysis does not permit us to discriminate between changes in UCP expression per cell and the number of UCP expressing cells, we performed an immuno-fluorescence microscopy study to address this problem. Fig. 7 shows typical examples of immuno-fluorescent detection of lipopro-

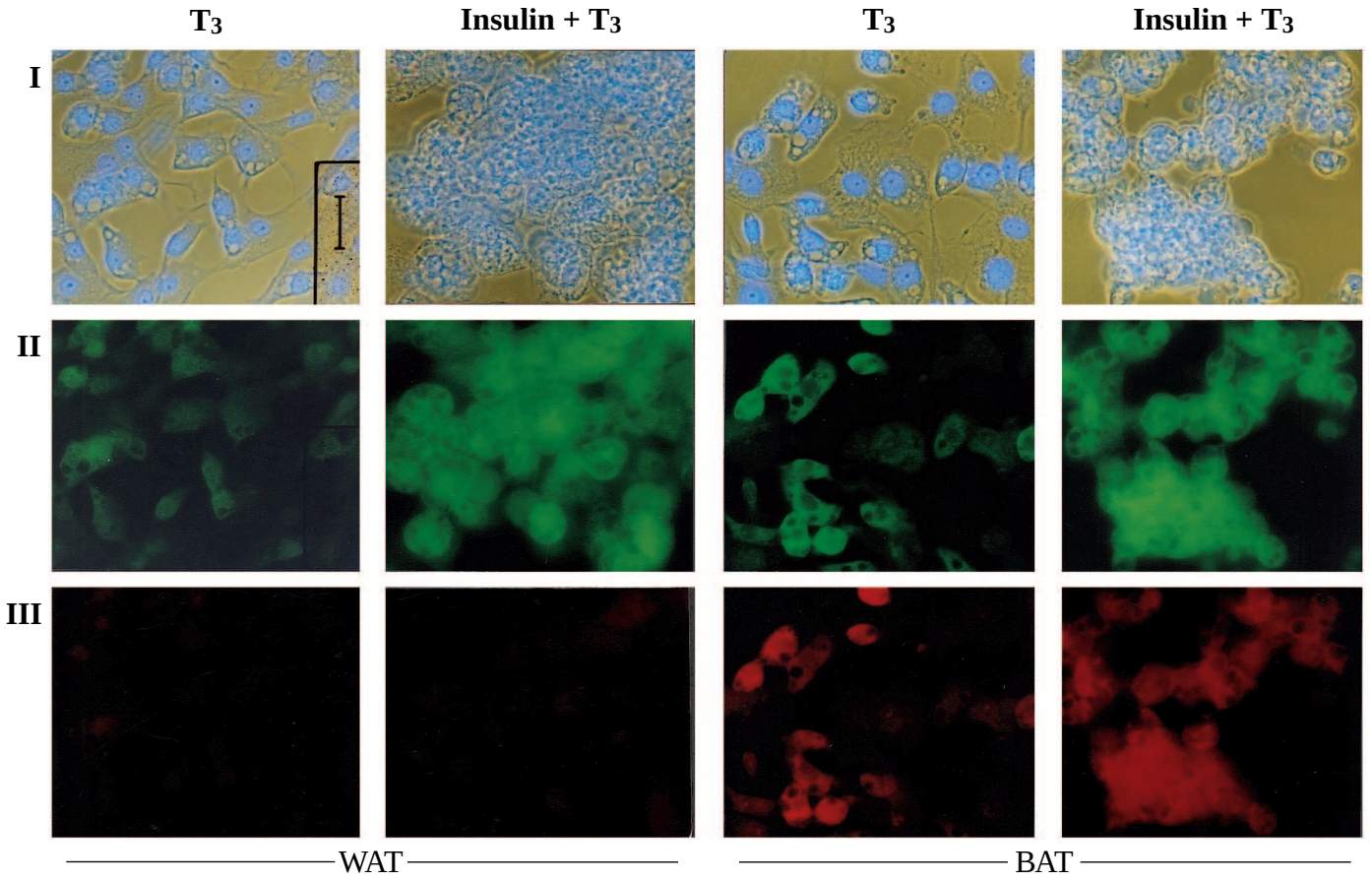

Fig. 7. Microphotograph of cultured stromal-vascular cells isolated from white adipose tissue (WAT) and brown adipose tissue (BAT). Preadipocytes were cultured from day 3 in medium containing 7% FCS, supplemented with T_3 (1 nM) alone or insulin (17 nM) + T_3 (1 nM). I: phase contrast image with DAPI stain of nucleus (blue), II: immuno-fluorescent imaging of LPL (FITC, green) III: immuno-fluorescent imaging of UCP (rhodamine, red). I, II, and III are from the same microscopic field. Bar, 40 μm.

tein lipase (LPL) and UCP in WAT and BAT cultures treated only with T_3 or insulin + T_3. LPL was used as a marker for adipocyte differentiation and we found an excellent correlation between cells which we considered as adipocytes by visual criteria (i.e. lipid inclusions) and LPL positive cells. UCP-expression was always restricted to LPL-positive cells. It was found in about 90% of adipocytes from BAT cultures and in 10-13% of adipocytes from WAT cultures (Fig. 8) under standard culture conditions (insulin + T_3). This means that the low expression of UCP found by western blot analysis in WAT cultures was due to the existence of a certain percentage of brown adipocytes within WAT cultures and not to a low 'leaky' expression of UCP in white adipocytes. The immuno-fluorescent image (Fig. 7) also confirms the expression of UCP in some cells of BAT cultures treated only with T_3 although these cells do not resemble fully differentiated adipocytes. Using immuno-fluorescence microscopy it is now possible to discriminate clearly between white and brown adipocytes.

DISCUSSION

Due to the quite opposite physiological functions of brown fat and white fat in mammalian energy metabolism, the comparative study of white and brown adipocyte development and metabolism is important for the elucidation of cellular components of energy metabolism. For small mammals like the Siberian hamster, brown fat is a powerful tool for survival in the cold due to its thermogenic function. White fat is very important as an energy store and its mass and metabolic activity is known to be highly regulated in this species. In our investigation we therefore focused on several function-related parameters that are known to be important in vivo conditions and for the differentiation state of cultured cells.

Terminal differentiation

Dexamethasone completely inhibited white and brown adipocyte differentiation without having an effect on total cell number, i.e. proliferation (Table 1). This is somewhat surprising, as it is known to be implicated in terminal adipocyte differentiation (reviewed by Ailhaud et al., 1992; Cornelius et al., 1994). But as our culture medium contained 7% FCS, possible interactions with serum factors cannot be excluded. Furthermore the effect of dexamethasone was shown to be different depending on the time of its addition (Xu and Björntorp, 1990) and in our cultures it was added before confluence. Anyway, the complete inhibition of adipocyte differentiation by dexamethasone provides us with excellent control groups. Our culture conditions thus permit us to distinguish between 3 distinct differentiation types (non differentiated cells, poorly differen-

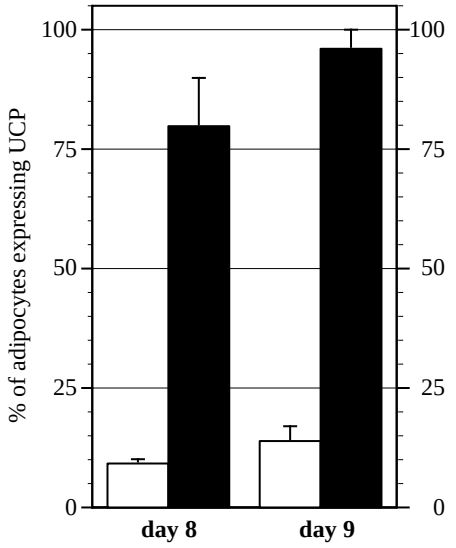

Fig. 8. UCP expressing adipocytes in cultures from WAT (open bars) and BAT (filled bars) at day 8 and 9. Preadipocytes were cultured from day 3 in medium containing 7% FCS, supplemented with insulin (17 nM) and T_3 (1 nM). UCP expression was limited to LPL expressing cells which were considered adipocytes. Shown are the percentages of adipocytes (+ s.e.m.) positive for UCP from multiple wells of one cell culture.

tiated adipocytes and fully differentiated adipocytes), both by morphological and by metabolic criteria. Non differentiated cells showed no phenotypic characteristics of adipocytes (rounded shape, lipid accumulation) had low respiration rates (approximately 4 nmol O/min per 10^6 cells) and no lipolytic activity. These cells were indistinguishable in WAT and BAT cultures. Poorly differentiated adipocytes were rounded, displayed few lipid inclusions, showed basal lipolysis, and had severalfold increased respiration rates (e.g. 27 nmol O/min per 10^6 adipocytes in non treated WAT cultures). Finally, fully differentiated adipocytes were characterized by large triglyceride accumulations (i.e. larger size), basal lipolytic activities and further increased respiration rates (e.g. 45 nmol O/min per 10^6 adipocytes in non treated WAT cultures).

Differentiation of many cell types is accompanied by increased protein synthesis and triglyceride accumulation is generally considered a marker for terminal adipocyte differentiation (Ailhaud et al., 1992; Cornelius et al., 1994). Applying these two criteria, it is obvious that insulin was the prerequisite for terminal differentiation of both white and brown adipocytes, because it significantly increased protein content and adipocyte size (as an index for lipid accumulation) in both WAT and BAT cultures (Table 1). Furthermore it increased the number of cells differentiating into adipocytes (Table 1) resulting in a 7-fold increase in the adipogenic index of WAT cultures (Fig. 2). For WAT cultures insulin was even the only hormonal factor necessary for terminal differentiation. It cannot be excluded that this effect of insulin was mediated by IGF-1 receptors rather than insulin receptors as the concentration used (17 nM) is rather supra physiological and therefore likely to act via IGF-1 receptors (reviewed by Cornelius et al., 1994).

The hormonal regulation of brown adipocyte terminal differentiation proved to be more complex. Adipocyte size (i.e. lipid accumulation) was significantly increased by about 65% when T_3 was present in addition to insulin (Table 1). A similar picture evolves if we consider cell respiration as a marker for terminal differentiation, because T_3 further increased basal brown adipocyte respiration to 165 nmol O/min per 10^6 adipocytes as compared to 72 nmol O/min per 10^6 adipocytes with insulin alone (Fig. 5).

Lipid metabolism

The main function of adipose tissue lies in its capacity for lipid storage and lipid mobilization and both white and brown adipocytes are equipped with the necessary 'machinery' for lipogenesis and lipolysis. The hormonal regulation, however of lipid storage and lipid mobilization can be quite different in white and brown fat. Lipoprotein lipase activity for example is decreased in white fat upon adrenergic stimulation, but is increased in brown fat (Carneheim et al., 1988). The hormonal regulation of lipogenic and lipolytic capacity in developing white and brown adipocytes is thus of special interest. Here we used adipocyte size as an index of triglyceride accumulation and glycerol release into the medium as an index of the lipolytic activity. Lipid accumulation has already extensively been discussed above, but it is obvious that in our culture system white adipocytes had a higher lipogenic capacity as well as basal lipolytic activity than brown adipocytes (Table 1, Fig. 3). The lipolytic capacity, however, was the same in both adipocyte types because it was stimulated to exactly the same extent by dexamethasone (Fig. 3) and in the same dose dependent manner (Fig. 4). The lipolytic action of T_3 on the other hand was very different on white and brown adipocytes. Treated with 1 μM T_3, WAT cultures released 4 times more glycerol within 48 hours than did BAT cultures (Fig. 4). Taken together, these results imply higher triglyceride turnover rates in white adipocytes and a more stringent regulation of lipolysis in brown adipocytes.

Thermogenesis

Thermogenesis in brown adipocytes is due to the uncoupling protein UCP whose expression is unique to the mitochondria of brown adipocytes (Cannon et al., 1982; Ricquier et al., 1992). UCP is therefore classically considered the qualitative marker protein for brown adipocytes as compared to white adipocytes and often used as an index for thermogenic capacity. However, from a physiological point of view, the important difference between the two adipose types is the high respiratory i.e. heat producing capacity of brown adipocytes compared to the very low respiratory capacity of white adipocytes. Brown fat thermogenesis is under the control of the sympathetic nervous system, acting through β-adrenergic receptors (Seydoux and Girardier, 1978; Bukowiecki et al., 1981). In order to investigate more directly the thermogenesis of cultured adipocytes, we measured UCP expression as well as unstimulated respiration and the stimulation of respiration by a β-adrenergic agonist (isoproterenol). This method has been used to evaluate the thermogenesis of brown fat cells isolated from the brown fat of animals adapted to different temperatures (Bukowiecki et al., 1980; Locke et al., 1982). To our knowledge, respiration measurements have only once been applied to cultured preadipocytes from WAT and BAT (Néchad et al., 1983); however, those respiration rates were very low compared to our data and no stimulation by β-adrenergic agonist was found.

Our results show clearly that development of increased thermogenic capacities of brown adipocytes depends largely on the hormonal environment during differentiation. T_3 proved to be very important as assessed by respiration measurements as well as UCP detection (Figs 5, 6, and 7). T_3 was able to increase thermogenesis as well as UCP expression alone and synergistically with insulin. This corroborates and extends previous studies which have shown the importance of T_3 for UCP expression in vivo (Silva and Bianco, 1990; Reiter et al., 1990) and in vitro (Rehnmark et al., 1990; Klaus et al., 1991b; Guerra et al., 1994). In cultured WAT preadipocytes a small effect of T_3 on thermogenesis was observed (Fig. 5B). But if we take into account that 10 to 15% of adipocytes in WAT cultures are really brown adipocytes (Fig. 8) and subtract the respective respiration rates, the β-adrenergic induced increase in respiration of white adipocytes was very similar in all experimental groups (between 6.5 and 8.5 nmol O/min per 10^6 adipocytes) and thus 7 to 8 times lower than the value for brown adipocytes treated with isulin + T_3 (61 nmol O/min per 10^6 adipocytes). This increase in respiration is surprisingly similar to the norepinephrine induced stimulation of respiration of brown adipocytes isolated from warm adapted guinea pigs (Locke et al., 1982; Rafael et al., 1986). Adipocytes isolated from cold adapted guinea pigs and thermoneutrally kept rats were reported to show up to 10-fold increased respiration rates upon stimulation by β-adrenergic agonists (Locke et al., 1982, Bukowiecki et al., 1980, Rafael et al., 1986). It will be interesting to use our culture system in order to investigate if the presence of β-adenergic agonists will improve the thermogenic capacity of brown adipocytes differentiated in vitro. Overall, a very good correlation of adipocyte thermogenesis (Fig. 5B) with UCP expression (Fig. 6) indicates that UCP is functional in brown adipocytes differentiated in vitro. Relatively high unstimulated respiration rates in our study (up to 164 nmol O/min per 10^6 adipocytes) might indicate that respiration of these cells is, in culture, already partially uncoupled in the absence of adrenergic stimulation.

Although in the inguinal fat pad that we used for isolation of preadipocytes normally no UCP (protein or mRNA) could be detected (data not shown), we could detect UCP in total cell homogenates of WAT cultures treated with insulin + T_3 (Fig. 6). Our immuno-fluorescent results prove that this was not due to an expression leakage but that UCP expression was confined to discrete sub-populations of adipocytes. This corroborates results obtained in vivo, indicating the presence of UCP expressing fat cells i.e. brown adipocytes, in typical white fat deposits (Lonçar, 1991; Cousin et al., 1992). It will be interesting to investigate if β-adrenergic stimulation during adipocyte development might increase the number of UCP expressing cells, i.e. recruit new brown adipocytes in WAT cultures. This is now possible using immuno-fluorescent detection of UCP expression.

Considering the very low metabolic activity of white fat in vivo, the respiration rates of cultured white adipocytes seem to be rather high. This is partly due to the presence of brown adipocytes as shown in Fig. 8. However, it is also possible that mitochondria are more abundant during early development of white adipocytes and reduced later on. Therefore it will be interesting to investigate the thermogenic capacity of WAT and BAT cultures during long term cultures.

Conclusions

Using a comparative approach we could clearly demonstrate a different determination of brown preadipocytes as compared to white preadipocytes. The major difference between the two preadipocyte types lies in their reaction to T_3, which evokes different metabolic responses in the two preadipocyte types. The presence of T_3 is necessary for the development of maximal thermogenic capacity of brown adipocytes but does not increase the thermogenic capacity of white adipocytes. It remains to be established if this is due to different characteristics of T_3 binding to nuclear receptors or post receptor binding events. Our results also demonstrate for the first time in brown adipocytes a dissociation of the thermogenic function from the lipid storage function. The hormonal regulation of the latter appears to be very similar in white and brown adipocytes, whereas the ability to develop thermogenic functions seems to be intrinsic to brown preadipocytes.

Thanks are due to Norbert Radomski from the University in Gießen (Germany) who helped us to set up the indirect immuno-fluorescent microscopy. This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to S. Klaus (Kl 613/2-1 and Kl 613/2-2) and G. Heldmaier (SFB 305).

REFERENCES

Ailhaud, G. (1990). Extracellular factors, signalling pathways and differentiation of adipose precursor cells. *Curr. Opin. Cell Biol.* **2**, 1043-1049.

Ailhaud, G., Grimaldi, P. and Négrel, R. (1992). Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr.* **12**, 207-233.

Bianco, A. C., Sheng, X. and Silva, J. E (1988). Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by a mechanism not requiring protein synthesis. *J. Biol. Chem.* **263**, 18168-18175.

Bronnikov, G., Houstek, J. and Nedergaard, J. (1992). β-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. *J. Biol. Chem.* **267**, 2006-2013.

Bukowiecki, L., Folléa, N., Paradis, A. and Collet, A. J. (1980). Stereospecific stimulation of brown adipose tissue respiration by catecholamines via β1-adrenergic receptors. *Am. J. Physiol.* **238**, E552-E563.

Bukowiecki, L., Folléa, N., Lupien, J. and Paradis, A., (1981). Metabolic relationships between lipolysis and respiration in rat brown adipocytes. *J. Biol. Chem.* **256**, 12840-12848.

Butterwirth, S. C. (1994). Molecular events in adipocyte development. *Pharmac. Ther.* **61**, 399-411.

Carneheim, C., Nedergaard, J. and Cannon, B. (1988). Cold-induced β-adrenergic recruitment of lipoprotein lipase in brown fat is due to increased transcription. *Am. J. Physiol.* **254**, E155-E161.

Casteilla, L., Champigny, O., Bouillaud, F., Robelin, J. and Ricquier, D. (1989). Sequential changes in the expression of mitochondrial protein mRNA during the development of brown adipose tissue in bovine and ovine species. *Biochem. J.* **257**, 665-671.

Casteilla, L., Nougués, J., Reyne, Y. and Ricquier, D. (1991). Differentiation of ovine brown adipocyte precursor cells in a chemically defined serum-free medium. Importance of glucocorticoids and age of animals. *Eur. J. Biochem.* **198**, 195-199.

Champigny, O., Holloway, B. R. and Ricquier, D. (1992). Regulation of UCP gene expression in brown adipocytes differentiated in primary culture: effects of a new β-adrenoceptor agonist. *Mol. Cell. Endocrinol.* **86**, 73-82.

Cannon, B., Hedin, A. and Nedergaard, J. (1982). Exclusive occurrence of thermogenin antigen in brown adipose tissue. *FEBS Lett.* **150**, 129-132.

Cornelius, P., MacDougald, O. A. and Lane, M. D. (1994). Regulation of adipocyte development. *Annu. Rev. Nutr.* **14**, 99-129.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Pénicaud, L. and Casteilla, L. (1992). Occurrence of brown adipocytes in rat white

adipose tissue: molecular and morphological characterization. *J. Cell Science* **103**, 931-942.

Darimont, C., Gaillard, D., Ailhaud, G. and Négrel, R. (1993). Terminal differentiation of mouse preadipocyte cells: adipogenic and anti mitogenic role of triiodothyronine. *Mol. Cell. Endocrinol*. **98**, 67-73.

Geloen, A. and Trayhurn, P. (1990). Regulation of uncoupling protein in brown adipose tissue by insulin. *Am. J. Physiol.* **258**, R418-R424.

Grimaldi, P., Dijan, P., Négrel, R. and Ailhaud, G. (1982). Differentiation of Ob17 preadipocytes to adipocytes: requirements of adipose conversion factor(s) for fat cell cluster formation. *EMBO J.* **1**, 687-692.

Guerra, C., Porra, A., Roncero, C., Benito, M. and Fernandez, M. (1994). Triiodothyronine induces the expression of the uncoupling protein in long term fetal rat brown adipocyte primary cultures: role of nuclear thyroid hormone receptor expression. *Endocrinol*ogy **134**, 1067-1074.

Hauner, H. (1990). Complete adipose differentiation of 3T3-L1 cells in a chemically defined medium: comparison to serum-containing culture conditions. *Endocrinol*ogy **127**, 865-872.

Klaus, S., Casteilla, L., Bouillaud, F. and Ricquier, D. (1991a). The uncoupling protein UCP: A membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. *Int. J. Biochem*. **23**, 791-801.

Klaus, S., Cassard-Doulcier, A.-M. and Ricquier, D. (1991b). Development of Phodopus sungorus brown preadipocytes in primary cell culture: effect of an atypical beta-adrenergic agonist, insulin, and triiodothyronin on differentiation, mitochondrial development, and expression of the uncoupling protein UCP. *J. Cell Biol*. **115**, 783-1790.

Klaus, S., Choy, L., Champigny, O., Cassard-Doulcier, A.-M., Ross, S., Spiegelman, B. and Ricquier, D. (1994). Characterization of the novel brown adipocyte cell line HIB 1B. Adrenergic pathways involved in regulation of uncoupling protein gene expression. *J. Cell Sci.* **107**, 313-319.

Kopecky, J., Baudysova, M., Zanotti, F., Janikova, D., Pavelka, S. and Houstek, J. (1990). Synthesis of mitochondrial uncoupling protein in brown adipocytes differentiated in cell culture. *J. Biol. Chem*. **265**, 22204-22209.

Kozak, U. C. and Kozak, L. P. (1994). Norepinephrine-dependent selection of brown adipocyte cell lines. *Endocrinology* **134**, 906-913.

Locke, R. M., Rial, E. and Nicholls, D. G. (1982). The acute regulation of mitochondrial proton-conductance in cells and mitochondria from the brown fat of cold-adapted and warm-adapted guinea pigs. *Eur. J. Biochem*. **129**, 381-387.

Lonçar, D. (1991). Convertible adipose tissue in mice. *Cell. Tiss. Res.* **266**, 149-161.

Nedergaard, J., Cannon, B. and Lindberg, O. (1977). Microcalorimetry of isolated mammalian cells. *Nature* **267**, 518-520.

Néchad, M., Kuusela, P., Carneheim, C., Björntorp, P., Nedergaard, J. and Cannon, B. (1983). Development of brown fat cells in monolayer culture. I. Morphological and biochemical distinction from white fat cells in culture. *Exp. Cell Res.* **149**, 105-118.

Néchad, M. (1986). Structure and development of brown adipose tissue. In *Brown Adipose Tissue* (ed. P. Trayhurn and D. G. Nicholls), pp. 1-30. London: E. Arnold Ltd.

Nicholls, D. G., Cunningham, S. and Rial, E. (1986). The bioenergetic mechanism of brown adipose tissue mitochondria. In *Brown Adipose Tissue*. (ed. P. Trayhurn, and D. G. Nicholls), pp. 52-85. London: E. Arnold Ltd.

Park, L. R. A., Mount, D. B. and Himms-Hagen, J. (1989). Role of T_3 in thermogenic and trophic responses of brown adipose tissue to cold. *Am. J. Physiol.* **257**, E81-E87.

Rafael, J., Fesser, W. and Nicholls, D. G. (1986). Cold adaptation in guinea pig at level of isolated brown adipocyte. *Am. J. Physiol*. **250**, C1-C9.

Reiter, J. R., Klaus, S., Ebbinghaus, C., Heldmaier, G., Redlin, U., Ricquier, D., Vaughan, M. K. and Steinlechner, S. (1990). Inhibition of 5′ deiodination of thryroxine suppresses the cold-induced increase in brown adipose tissue mRNA for mitochondrial uncoupling protein without influencing lipoprotein lipase activity. *Endocrinol*ogy **126**, 2550-2554.

Rehnmark, S., Kopecky, J., Jacobsson, A., Néchad, M., Herron, D., Nelson, B. D., Obregon, M.-J., Nedergaard, J. and Cannon, B. (1989). Brown adipocytes differentiated in vitro can express the gene for the uncoupling protein thermogenin: effects of hypothyroidism and norepinephrine. *Exp. Cell Res.* **182**, 75-83.

Rehnmark, S., Néchad, M., Herron, D., Cannon, B. and Nedergaard, J. (1990). Alpha- and beta-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. *J. Biol. Chem*. **265**, 16464-16471.

Ricquier, D., Casteilla, L. and Bouillaud, F. (1991). Molecular studies of the uncoupling protein. *FASEB J.* **5**, 2237-2242.

Ricquier, D., Raimbault, S., Champigny, O., Miroux, B. and Bouillaud, F. (1992). The uncoupling protein is not expressed in rat liver. *FEBS Lett*. **303**, 103-106.

Rothwell, N. and Stock, M. (1986). Brown adipose tissue and diet-induced thermogenesis. In *Brown Adipose Tissue* (ed. P. Trayhurn and D. G. Nicholls), pp. 299-338. London: E. Arnold Ltd.

Seydoux, J. and Girardier, L. (1978). Control of brown fat thermogenesis by the sympathetic nervous system. *Experientia Suppl*. **32**, 153-167.

Silva, J. and Bianco, C. (1990). Role of local thyroxine 5′ deiodinase on the response of brown adipose tissue (BAT) to β-adrenergic stimulation. In *Obesity: Towards a Molecular Approach*, UCLA Symposium on Molecular and Cellular Biology, New Series (ed. G. Bray et al.), pp. 131-146. Wiley-Liss, Inc., New York.

Xu, X. and Björntorp, P. (1990). Effects of dexamethasone on multiplication and differentiation of rat adipose precursor cells. *Exp. Cell Res*. **189**, 247-252.

(Received 19 June 1995 - Accepted 25 July 1995)