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

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Abstract. A new cellular model for the study of brown adipocyte development and differentiation in vitro is presented. Preadipocytes isolated from brown adipose tissue (BAT) of the djungarian dwarf hamster *Phodopus sungorus* are able to proliferate and differentiate in vitro into true brown adipocytes able to express the BAT marker protein the uncoupling protein (UCP). Whereas basal UCP expression is very low, its mRNA levels as well as the UCP detected by immunoblotting are highly increased by β-adrenergic stimulation. The novel, atypical β-adrenergic compound D7114 (ICI Pharmaceuticals, Macclesfield, Cheshire, England) was found to increase the number of adipocytes as well as UCP mRNA and UCP content of mitochondria, indicating the involvement of an atypical or β3 receptor. Insulin was found to play an important role in brown adipocyte differentiation and mitochondrial development, whereas T_3 seemed to be implicated more directly in UCP expression. In a defined, serum-free medium a synergistic stimulatory action of insulin and T_3 on UCP expression was found, which seems to involve a pathway different from that of β-adrenergic UCP stimulation.

Two types of adipose tissue with quite different functions are known to exist in mammals: firstly the white adipose tissue whose main function is energy storage, secondly the so-called brown adipose tissue (BAT)[1], which functions as a heat dissipating organ in small and newborn mammals. This function is due to the uncoupling protein (UCP), uniquely expressed in BAT, which is located in the inner mitochondrial membrane (for review see 24). UCP functions as a proton translocator and can thus short circuit the coupling between the respiratory chain and ATP production, resulting in elevated respiration rates and dissipation of the energy as heat (28). Contrary to white fat, the BAT is highly innervated and vascularized and brown adipocytes have very high respiratory capacities, i.e., a high content of mitochondria. Brown fat is considered to play an important function in energy balance, by dissipating excess energy intake as heat in certain animal models (38). It is also known that many types of obesity in animals are associated with a defective BAT function (17). Despite their quite contrary physiological function, it is not yet clear if brown and white adipocytes should be considered as two distinctly different cell types or if they just represent two different differentiation or maturation states of the same cell type. According to our present knowledge, every adipocyte that expresses UCP is considered a brown adipocyte, whereas an adipocyte not expressing UCP is not automatically a white adipocyte. In small mammals, like rodents, BAT preserves throughout the animal life its thermogenic ability, i.e., the ability to express UCP. But in larger mammals, like bovine and ovine species, it was shown that fat depots of a true brown nature are present at birth, which very rapidly lose their "brown" nature and become typical white depots which will never express UCP anymore (9). We don't know yet if this means that brown adipocytes become white adipocytes or if new white adipocytes are recruited which replace brown adipocytes. This leads to two crucial questions. (*a*) Do brown and white adipocytes originate from different precursor cells? (*b*) How is the gene expression of UCP, the only true marker for brown adipocytes, regulated? Numerous in vivo studies have shown that acute UCP function as well as UCP gene expression are stimulated by Noradrenaline and numerous β-adrenergic agents (for reviews see 28, 35, 37). Studies on the cellular level, however, had been unsuccessful for many years. Only recently the group of B. Cannon found expression of UCP in primary cultures of preadipocytes, isolated from mouse BAT (31). Since then, several reports from the same group (16, 32) and others (20, 27) showed that UCP expression of mouse brown adipocytes differentiated in vitro can be stimulated by various β-adrenergic agonists.

In this study we introduce a new animal model for the study of brown adipocyte development and differentiation,

1. *Abbreviations used in this paper*: BAT, brown adipocyte tissue; COX, cytochrome-c-oxidase; LPL, lipoprotein lipase; UCP, uncoupling protein.

the siberian (or djungarian) dwarf hamster *Phodopus sungorus*. Several considerations prompted us to use this species. *Phodopus sungorus*, a small hamster of ~40 g body weight shows extreme adaptive abilities of the cold resistance by a high capacity of nonshivering thermogenesis which is located mainly in brown adipose tissue (15). This animal possesses very large amounts of BAT, which can make up to 5% of its body weight (29). It has further been shown that the respiratory capacity of BAT as well as UCP activity and mRNA levels in BAT of *Phodopus sungorus* can be highly stimulated by cold exposure (29, 43). It seems thus to be an ideal animal model for the study of brown adipocyte development and UCP expression in vitro. Furthermore, this species possesses large quantities of white adipose tissue, which will allow comparative studies on white and brown adipocyte differentiation. Using this new cell model we demonstrate the stimulation of UCP expression by an atypical β-agonist and the dependence of brown adipocyte thermogenic function on insulin and T_3.

Materials and Methods

Isolation of Preadipocytes

Isolation and culture of brown preadipocytes was adapted from the protocol used in reference 32. Animals aged between 4 and 6 wk were anaesthetized with chloroform and killed by cardiac puncture. The following depots of BAT were excised: axillary, interscapular, dorsal-cervical, and parts of subscapular depot. Tissue was cut in small pieces and digested at 37°C for 30–40 min in a buffer containing 100 mM Hepes (pH 7.4), 123 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 5 mM glucose, 1.5% (wt/vol) BSA, and 1 mg/ml collagenase (10 ml/g tissue). Digestion mixture was vortexed every 5 min. All this as well as following procedures were performed under sterile conditions. After digestion, mixture was filtered over a 250-μm nylon filter and left at room temperature for 5–10 min, until a discrete fat layer on top had formed. This layer, which contains mature adipocytes, was discarded and the lower phase centrifuged at 700 *g* for 10 min at room temperature. The pellet containing the stromal-vascular fraction was washed with 20–40 ml cell culture medium (preheated to 37°C) and repelleted. This pellet was resuspended in 20–40 ml culture medium and cells counted in a Malassez counting chamber.

Cell Culture

Cells were diluted to a concentration of 15,000 cells/ml and inoculated in petri dishes (10 cm diameter) at 10 ml medium/dish (i.e., ~2,000 cells/cm^2. Cells were grown at 37°C in air with 5% CO_2 content and 100% relative humidity. Standard cell culture medium consisted of 50% MEM (Gibco/BRL, Cergy Pontoise, France) and 50% F12 Ham's F12 medium (Gibco/BRL) supplemented with Na HCO_3 (1.2 g/l), biotine (4 mg/l), Ca-panthotenate (2 mg/l), glutamine (5 mM), glucose (4.5 g/l) and Hepes (pH 7.4, 15 mM), penicillin G (6.25 mg/l), and streptomycin (5 mg/l). Until appearance of differentiated adipocytes the medium was supplemented with 10% FCS. After one day, medium was aspirated, cells were washed with 5 ml/dish PBS 137 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and 10 ml new medium was added. Unless otherwise indicated, cell medium was changed at day 3 and supplemented with T_3 (1 nM) and insulin (20 nM). Cells were harvested at days indicated after rinsing with 5 ml PBS by scraping into either mitochondria isolation buffer or RNA isolation buffer, according to analysis performed later on.

Cell Counting

Cells were counted directly in the dish using a microscopy field of 0.5 mm^2. At least six randomly chosen fields were counted in at least two separately treated dishes. Cell numbers thus obtained corresponded very well to those obtained by counting of trypsinized cells in a Malassez counting chamber. Mature adipocytes were distinguished from preadipocytes by the presence of visible lipid droplets.

Isolation of Mitochondria

Routinely, cells from two petri dishes were pooled, suspended in 1–2 ml isolation buffer containing 250 mM sucrose, 10 mM TRIS-HCl, 1 mM EDTA (pH 7.0) and homogenized using a glass/teflon Potter-Elvehjem homogenizer. An aliquot of this homogenate was frozen at −20°C for subsequent DNA, protein and cytochrome c oxidase measurement. From the rest, mitochondria were isolated by differential centrifugation as described in 10.

Protein Content and Cytochrome-c-oxidase (COX) Activity Measurement

Protein concentration in cell homogenate was measured by the method of Bradford. COX activity was measured spectrophotometrically (42).

DNA Measurement

For measurement of DNA content, cell homogenates were sonicated two times for 10 s. DNA content was measured fluorometrically in a DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Immunoblotting

Western blotting of mitochondria was performed as described before (10) after separation of 15–40 μg protein by SDS-PAGE (12% acrylamide) and electrotransfer onto a nitrocellulose membrane. Antibodies used were anti rat UCP raised in sheep. Anti-sheep JGS (Sigma Chemical Co., St. Louis, MO) linked to peroxidase were used as second antibody.

RNA Isolation

Total RNA was isolated as described in (21) using a guanidin/HCl extraction buffer. Routinely cells from two petri dishes were pooled and scraped directly into 1 ml extraction buffer. Homogenization was performed by repeated aspiration with a syringe fitted with a Luer needle (0.5 × 16 mm). The yield of total RNA at day 10 of culture was usually between 30 and 100 μg/dish.

Northern Blotting

RNA electrophoresis and Northern blotting was performed as described before (36). Blots were hybridized with a ^{32}P-labeled pUCP 36 insert, containing the whole cDNA for rat UCP mRNA (7), with a pBR 325 ST41 containing the total mouse mitochondrial genome (6), or a pGEM-2 containing a mouse lipoprotein lipase (LPL) cDNA insert (23).

Results

Growth and Differentiation of Brown Preadipocytes

Phodopus sungorus used for cell culture aged between 4 and 6 wk had a body weight between 15 and 30 g. Average amount of BAT dissected was 0.7 g per animal and the average yield of isolated preadipocytes was 1.1 × 10^6/g BAT, i.e., 0.77 × 10^6 per animal. Proliferation and differentiation of preadipocytes was obtained in a medium supplemented with insulin (20 nM), T_3 (1 nM) and 10% FCS. Preadipocytes that were routinely inoculated at a density of 15–20/mm^2 reached confluency around day 5 or 6 of culture at a density of ~350 cells/mm^2. Differentiation started around confluency, i.e., around day 5 of culture. Cells always differentiated in distinct colonies, surrounded by nondifferentiated cells. This can be seen in Fig. 1 which shows a microphotograph of cells at different days of culture. Differentiated cells can be easily distinguished from fibroblast-like preadipocytes by their rounded shape and the presence of intracellular lipid droplets. At day 8 to 10 of culture, routinely, between 45 and 60% of the cells showed morphological characteristics of mature adipocytes.

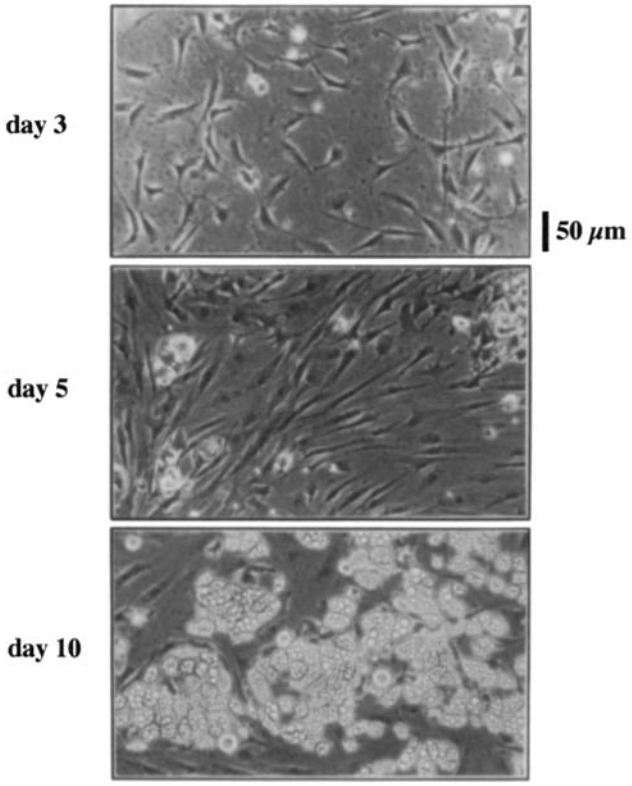

Figure 1. Microphotograph of preadipocytes isolated from brown adipose tissue in primary cell culture at different days of culture. Cells were inoculated at a density of 15 cells/mm^2 and grown in a medium supplemented with 10% FCS, 1nM T_3, and 17 nM insulin.

Mitochondrial development was followed by measuring COX activity, a mitochondrial marker enzyme. In Fig. 2 it can be seen that COX activity is almost nondetectable until day five, after which it increases linearly. This means that mitochondrial proliferation follows closely cell differentiation rather than cell growth. For a single cell this represents an almost 20-fold increase in COX activity, i.e., mitochondrial content between day 5 and 10. The mRNA for the mitochondrial uncoupling protein UCP, which is unique for brown adipocytes, appears very late during cell differentiation, and basal UCP expression was very low and not detectable by either Western or Northern blotting before day 7 of culture (data not shown). Even at day 10, in most cultures the expression level was very low. This shows that the UCP expression observed in these cells is not due to a survival of already differentiated adipocyte, but rather a new synthesis of UCP in adipocytes differentiated in vitro. Even in the standard medium supplemented with 10% FCS, this basal UCP expression required the presence of insulin/T_3. When these two hormones were omitted, cells grew normally and reached confluency at day 5, but no following lipid accumulation could be observed (data not shown). When insulin/T_3 was subsequently added at day 7 of culture, UCP mRNA could be detected only 48 h later (Fig. 3), when lipid accumulation was visible. Mitochondrial transcription products and LPL mRNA, on the other hand, were not changed by this treatment, indicating cell differentiation had already taken place, even without visible lipid accumulation.

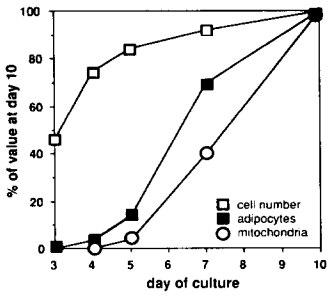

Figure 2. Time course of cell growth, appearance of mature adipocytes, and mitochondrial activity in cell culture. Total cell number and number of mature adipocytes were obtained by counting several microscopic fields. Cytochrome c oxidase activity was used as a measure of mitochondrial activity. All values are per petri dish with the value at day 10 of culture normalized to 100%.

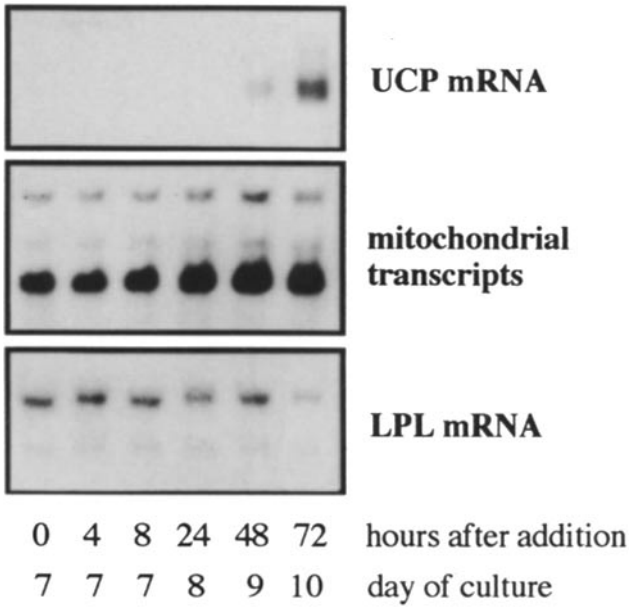

Figure 3. Northern analysis of brown adipocytes. Cells were grown in a medium containing 10% FCS until day 7. Under these conditions confluency had been reached at day 5. At day 7 1nM T_3 and 17 nM insulin were added into the medium and cells harvested immediately (0) or 4, 8, 24, 48, and 72 h later. 10 μg of total RNA were analyzed per lane. The same blot was consecutively hybridized with rat UCP cDNA, a plasmid containing mouse total mitochondrial genome, and a rat LPL cDNA insert.

β-Adrenergic Stimulation

UCP expression in brown adipocytes was highly increased by the addition of β-adrenergic agonists like Noradrenaline, Isoproterenol (a β1/β2 agonist), and D7114, a newly developed atypical β-adrenergic agonist (18, 19). Addition of 1 μM of either compound into the medium 24 h before harvest of cells, led to a similar increase in UCP mRNA (Fig. 4). None of these treatments caused a significant increase in mitochondrial activity. Neither COX activity (data not shown) nor mitochondrial RNA amount was significantly changed by adrenergic stimulation (Fig. 4).

The induction of UCP synthesis by D7114 in mature adipocytes was a very fast event, already 2 h after addition a significant increase in UCP mRNA could be observed, which reached a maximum after 8 h and stayed at this level

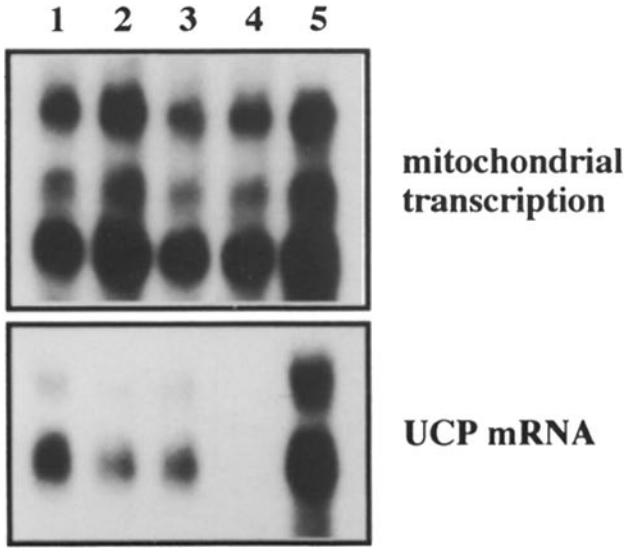

Figure 4. Northern analysis of brown adipocytes harvested at day 10 of culture after 24 h treatment with different β-adrenergic agonists. Each lane corresponds to 10 μg total RNA. (Lane *1*) noradrenaline (1 μM); (lane *2*) isoproterenol (1 μM); (lane *3*) D7114 (1 μM); (lane *4*) control; (lane *5*) RNA from brown adipose tissue of Phodopus sungorus subjected to 0°C for 12 h. The same blot was consecutively hybridized with a plasmid containing the whole mouse mitochondrial DNA (*top*) and a rat UCP cDNA probe (*bottom*).

for at least 48 h (Fig. 5, *top*). The appearance of UCP in the mitochondrial fraction (as determined by performing immunoblotting) was clearly visible after 8 h and increased still until 48 h after addition of D7114 (Fig. 5, *bottom*).

Further experiments were performed in order to examine more closely the effect of D7114 on brown adipocyte differentiation and UCP expression. The effect of different concentrations of D7114 present for only 1 h at day 3 or continuously from day 3 on were compared (Fig. 6). When cells were treated during exponential growth phase for only 1 h, the number of mature adipocytes detectable at day 10 was increased by 60% at concentrations of 10^{-7}–10^{-6} M, whereas no significant effect on UCP mRNA levels was observed. Chronical treatment with the same D7114 concentrations,

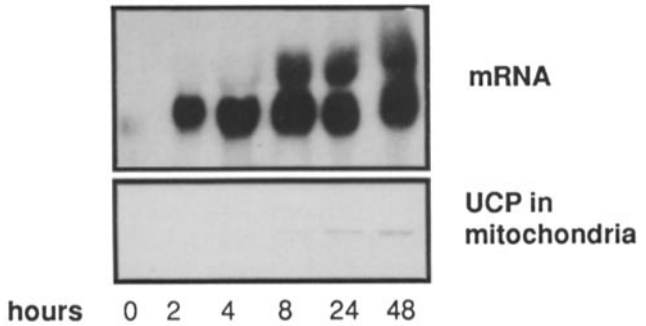

Figure 5. Time course of UCP expression in brown adipocytes treated with D7114. At day 9 of culture 1 μM D7114 was added into the medium and cells were harvested immediately (0 h), 4, 8, 24, and 48 h later. (*top*) Northern blot of 10 μg total RNA per lane hybridized with UCP cDNA. (*bottom*) Western blot of 40 μg mitochondrial protein hybridized with serum raised against rat UCP.

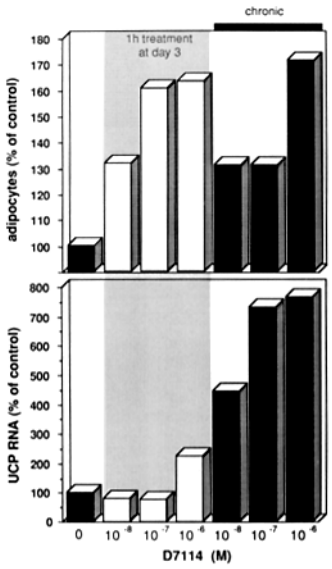

Figure 6. Effect of D7114 on brown preadipocyte differentiation and UCP expression. Preadipocytes at preconfluent state (*day 3*) were treated with different concentrations of D7114 either for 1 h (*light bars*) or chronically (*dark bars*) until day 10 when they were harvested. The number of adipocytes was evaluated before harvest by counting cells containing visible lipid inclusions in a microscopical field. UCP mRNA levels were obtained by densitometrical scanning of Northern blot hybridized with UCP cDNA. All values are expressed in % with control cells normalized to 100%.

however, resulted in seven- to eightfold increases in UCP mRNA level. This indicates a dual role of D7114 in brown adipocyte development. Firstly, it acts on the level of preadipocytes, where it promotes differentiation; secondly, it acts upon mature brown adipocytes by stimulation of UCP expression.

Stimulation of Mitochondrial Activity and UCP Expression in a Serum-free, Defined Medium

Growth and differentiation of Phodopus sungorus preadipocytes required the presence of FCS. When standard culture medium was replaced by serum-free medium during exponential growth phase or at confluency, within several days most cells died. Differentiated adipocytes, however, could be maintained in serum-free medium for several days. We observed that after removal of serum, nondifferentiated cells had a tendency to detach from the dish, leaving the mature adipocytes (Fig. 7), resulting in a loss of ~50% of proteins and 40% of DNA content, i.e., cells after 24 h (Table I). In the presence of insulin no decrease in protein content was observed and only 10% of cells were lost (Table I). As can be seen in Fig. 7, adipocytes in the absence of insulin were smaller in diameter and had fewer lipid inclusions than cells supplied with insulin. Mitochondrial activity (i.e., cytochrome c oxidase activity) was increased by insulin treatment on the average by 60% after 24 h (Table I). This means a net increase in COX activity per adipocyte. COX activity was maintained in cells not supplied with insulin, although cell number decreased. This results from the preferential loss of preadipocytes, in which COX activity is almost negligible. UCP mRNA levels decreased by ~70% within 24 h in a serum-free medium whether or not insulin was present, which could be prevented by addition of 1 nM T_3 (Table I). This shows clearly that basal UCP gene expression in these brown adipocytes is independent on β-adrenergic stimulation, but requires the presence of T_3.

Surprisingly, the addition of insulin plus T_3 resulted in a significant increase of UCP mRNA (Table I, Fig. 8). However, contrary to β-adrenergic stimulation of UCP mRNA expression, this was a rather slow process. The first significant increase could be observed only after 24 h and after

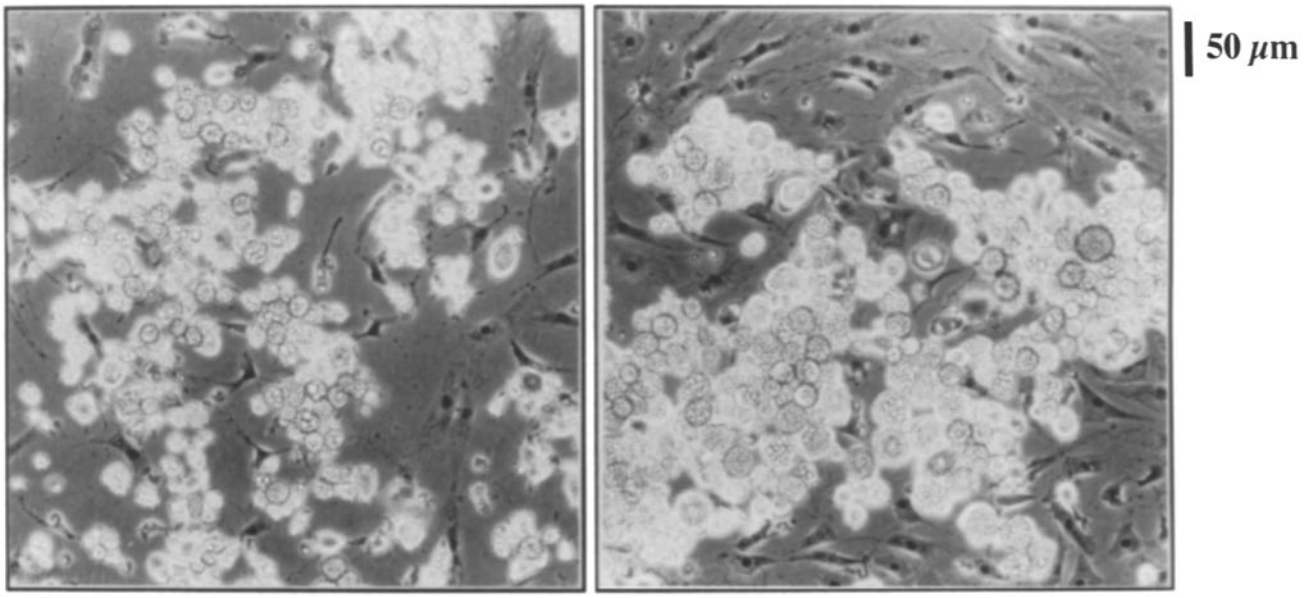

Figure 7. Microphotograph of brown adipocytes kept for 24 h in a defined, serum free medium. Preadipocytes were grown until day 9 in the standard medium containing 10% FCS, 1 nM T_3, and 17 nM insulin. At day 9 adipocytes were rinsed twice with PBS and medium replaced by serum-free standard medium (*A*) or standard medium containing 17 nM insulin (*B*).

3 d there was still an increase in UCP mRNA levels (Fig. 8). In parallel, an increase in mitochondrial transcription products and LPL mRNA could be observed. In the presence of 10% FCS, no stimulating effect of insulin plus T_3 could be found which might indicate an inhibitory factor present in serum preventing the induction of UCP expression. The stimulation of UCP by insulin/T_3 in a serum-free medium could be prevented by addition of actinomycin D, a transcription inhibitor (data not shown).

Discussion

Brown Adipocyte Growth and Development

For small mammals like rodents, the heat production in BAT plays a very important role in maintainance of homeothermy under severe cold stress. For a species like the Siberian hamster, which in its natural environment is frequently exposed to very low temperatures in winter, this is essential for survival. For physiologists, the Siberian hamster has been proved to be a very useful animal model to study extreme adaptive responses to environmental factors like cold or photoperiod. The high abundance of brown adipose tissue is just one of several intriguing features of this animal. Although BAT thermogenesis of this species has been very well studied in vivo, so far there have been no studies about the cellular aspects of the adaptive mechanisms responsible for this.

The Siberian dwarf hamster proved to be a very good model for the study of brown adipocyte development in primary cell culture with a high yield of brown preadipocytes per animal. Compared to reported yields from mouse BAT (32), that means 10–15 times more brown preadipocytes from one hamster than from one mouse. Furthermore, a good differentiation into brown adipocytes could be obtained, which display the most important features of brown adipocytes in vivo, i.e., high mitochondrial activity and inducible presence of the uncoupling protein UCP in its mitochondria. In Fig. 2 the time courses of cell growth, differentiation, and mitochondrial development are summarized. Cell differentiation starts around confluency, i.e., growth arrest of preadipocytes, which is a typically observed in primary cultures of brown as well as white adipocytes (2). It should be noticed that mitochondrial development follows closely the appearance of mature adipocytes. This means a

Table I. Hormonal Requirements in a Serum-free Medium

Addition	Protein (per dish)	DNA (per dish)	COX activity (per dish)	UCP mRNA
–	53 ± 15 (3)	62 ± 12 (3)	92 ± 7 (3)	27 ± 8 (3)
Insulin	100 ± 5 (3)	86 ± 11 (3)	161 ± 15 (3)	30 ± 14 (5)
T_3	52 ± 10 (3)	60 ± 8 (3)	111 ± 24 (3)	117 ± 47 (4)
Insulin + T_3	103 ± 12 (5)	89 ± 19 (3)	160 ± 18 (5)	325 ± 103 (8)

24-h treatment of brown adipocytes in a defined, serum-free medium supplemented with different hormones. Effect on total protein content (per dish), mitochondrial activity (COX activity per dish), cell number (DNA content per dish), and UCP mRNA levels. Given are values in % ±SD of control values at beginning of treatment, which were set at 100%. Numbers in brackets indicate the number of cell cultures. Cell cultures were performed independently at different times. UCP mRNA levels were quantified by densitometrical scanning of Northern blots where the same quantity of total RNA was analyzed.

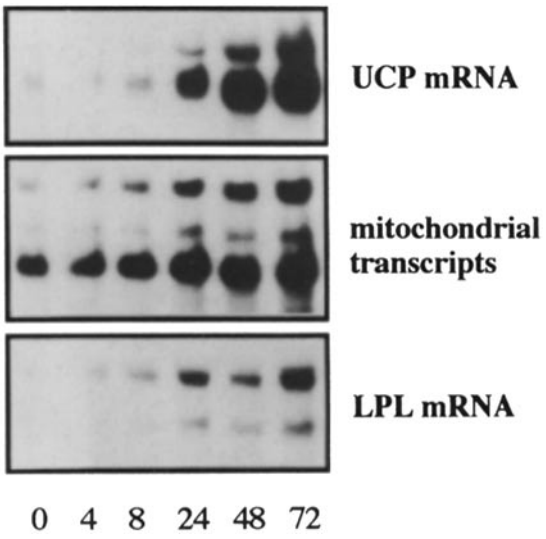

Figure 8. Northern analysis of adipocytes kept for different times in a defined, serum-free medium. Preadipocytes were grown until day 9 in the standard medium containing 10% FCS, 1 nM T_3, and 17 nM insulin. At day 9 adipocytes were rinsed twice with PBS and medium replaced by serum-free standard medium supplemented with 1 nM T_3 and 17 nM insulin. Cells were harvested immediately (hour 0), 4, 8, 24, 48, and 72 h later. 10 μg total RNA was analyzed per lane. The same blot was consecutively hybridized with rat UCP cDNA, a plasmid containing mouse total mitochondrial genome, and a rat LPL cDNA insert.

20-fold increase of COX activity per cell between confluency, i.e., start of differentiation and full maturation. The terminal differentiation of brown adipocytes required the addition of insulin and T_3 even in the presence of 10% FCS. No lipid accumulation could be observed even 2 d after confluency when insulin and T_3 were omitted in the medium. The subsequent addition induced very rapidly lipid accumulation. LPL mRNA appearance, as an early marker for differentiation, was not dependent on insulin/T_3 addition (Fig. 3) and could be observed from confluency on (25). This conforms to observations in different lines of white adipocytes (reviewed in 1). It is also known from white preadipose cells lines that growth hormone (which is present in FCS), insulin and T_3 are required for obtaining a fatty phenotype (2). LPL mRNA is furthermore increased by insulin (Fig. 8) and decreased by β-adrenergic agonists (25) in brown adipocytes of Phodopus, i.e., exactly as in white adipocytes (30). In the animal, however, LPL activity in BAT is increased by cold exposure (8, 26) which is normally considered to be mediated by β-adrenergic stimulation (8). It thus seems that brown adipocytes differentiated in vitro have the same basic requirements and display the same pattern of differentiation as white adipocytes. The only difference lies in the presence of a basal UCP expression in brown adipocytes in culture conditions allowing terminal differentiation. This indicates that preadipocytes isolated from brown fat are already determined to become brown adipocytes.

UCP as a Late Differentiation Marker of Brown Adipocytes

In primary cell culture, UCP expression is restricted to the terminal differentiation state of Phodopus brown adipocytes. LPL as an early differentiation marker appears already at day five of culture, i.e., around confluency and concomitant growth arrest. UCP expression, however, can first be observed when adipocytes show lipid accumulation (around day 7), which coincides with a large increase in mitochondrial activity. Even with β-agonists present from the beginning of culture, no UCP expression could be observed at an earlier state. This indicates firstly that UCP is only produced by mature adipocytes and secondly that a certain mitochondrial equipment of the cell is necessary for UCP appearance. When terminal differentiation (i.e., lipid accumulation) was prevented by omitting insulin/T_3, also no basal UCP mRNA could be observed, although confluency was well reached (Fig. 3). Upon subsequent addition of insulin/T_3, UCP mRNA was found only 48 h later, when lipid accumulation was visible. This again confirms UCP as a marker of terminal differentiation in Phodopus brown adipocytes.

This is somewhat in contrast to findings by Rehnmark et al. (32), who found highest UCP mRNA expression in mouse brown adipocytes around confluency and thereafter a rapid decline in the ability to stimulate UCP expression. Phodopus cultured brown adipocytes retain their ability to express high UCP levels over a long period. Species specific differences might explain this discrepancy. But both cell models have in common that no UCP can be found in brown preadipocytes and that its expression seems to be coupled to a certain differentiation state.

Role of Atypical β-Adrenergic Stimulation in Brown Adipocyte Development and Differentiation

The ability to stimulate UCP expression in mouse brown adipocytes differentiated in primary culture by β-adrenergic agonists like noradrenaline or isoproterenol has recently been reported by different groups (27, 32). This is also true for Siberian hamster adipocytes (Fig. 4). However, in our cell model we could demonstrate a stimulation of UCP expression not due to stimulation of either β1 or β2 receptors. We tested the novel, atypical β agonist D7114, which has no β1 or β2 adrenergic action and is considered as a β3 agonist (18, 19). This compound seems to play a dual role in brown adipocyte development. It can increase the number of mature adipocytes as well as UCP gene expression (Fig. 6). Apparently, these are two independent effects, the first acting on preadipocytes, the second on adipocytes. It has been proposed for several years that thermogenic action of brown fat is mediated by atypical or so called β3 receptors present in brown fat, which are not inhibited by classical beta antagonists (3, 4). Recently, atypical β receptors from human adipose tissue (12) and rat brown fat (34) were cloned and sequenced. It remains to be established if they represent the same pharmacological characteristics and if the action of atypical beta agonists like D7114 is mediated by these receptors. In mouse brown adipocytes differentiated in vitro, it has been shown that type II 5′ deiodinase activity, required for the conversion of T_4 into T_3, is increased by noradrenaline, which is apparently acting via the classical β-adrenergic pathway (20). This suggests a dual action of noradrenaline in vivo, firstly, supplying the brown fat with T_3, which is required for basic UCP gene expression, and secondly, directly modulating the UCP gene expression by acting on a different receptor type.

Brown Adipocyte Thermogenesis Is Regulated by Several Hormonal Factors

Cold acclimation or stimulation of BAT in vivo generally results in an increase in mitochondrial equipment (i.e., COX activity and mitochondrial protein) and a specific increase in UCP content per mitochondria. Normally these are parallel effects and both believed to be mediated by beta adrenergic stimulation. In our cell culture system we find that β-adrenergic stimulation has a positive effect on UCP expression, but little or no effect on mitochondrial activity. On the other hand, mitochondrial activity per dish was largely increased when cells were treated with insulin in a serum-free medium. UCP mRNA levels, however, were rather decreased by this treatment. This indicates that mitochondrial development and UCP expression can be independently regulated. However, a sufficient equipment with mitochondria is essential for brown fat thermogenic function. It has been shown before that in diabetic rats or mice, COX activity and mitochondrial protein content were decreased and could be at least partially restored by insulin treatment (13, 14). UCP content and proton conductance of rat brown fat are also reduced in a hypoinsulinemic state (39). Geloen and Trahurn (14) could also demonstrate a stimulating effect of insulin on UCP levels, which they showed to be partly, but not entirely mediated by the sympathetic nervous system. Insulin is furthermore important for the substrate supply of brown adipose tissue. The rate of glucose transport in brown adipocytes is highly increased by insulin (22).

T_3 seems to be more directly involved in UCP expression. It has been shown to be not only a permissive factor in the β-adrenergic stimulation of UCP expression (5), but to play an essential role for the thermogenic response of BAT (40). When the conversion of T_4 into T_3 was blocked in the Siberian hamster in vivo, the cold induced increase of UCP mRNA was largely inhibited (33). In mice brown adipocytes differentiated in vivo, maximal UCP expression was also found to require the presence of either insulin or T_3 (32). Although in our cell model T_3 alone did not change UCP mRNA levels, it prevented the decrease of UCP mRNA observed in a serum-free medium (Table 1). This underlines the importance of T_3 in UCP gene expression. However, T_3 had no effect on mitochondrial activity.

The synergistic action of insulin and T_3 leads to an increased thermogenic ability, i.e., increase in mitochondrial activity plus increased UCP expression. Their failure to induce UCP expression in a medium containing 10% FCS might indicate yet another, unknown factor involved in UCP gene expression that is inactivated by some serum factor, e.g., by binding to albumin. This demonstrates for the first time the ability to stimulate UCP expression in rodent brown adipocytes via a pathway different from β-adrenergic stimulation. Although this stimulation is due to a transcriptional process, as it can be blocked by actinomycin D, it is a much slower process than β-adrenergic stimulation of UCP expression. It might be possible that this reflects an indirect action on UCP gene, which requires the induction of other gene products which then act on the UCP gene. Anyway, it seems clear that this type of UCP induction follows a pathway different from UCP induction by β-adrenergic stimulation. It has previously been shown that in brown adipocytes from newborn lambs differentiated in vitro in a defined medium, dexamethasone was sufficient to induce a high UCP expression, which could not further be stimulated by β-agonists (11).

All these results imply that there are several humoral factors involved in the general regulation of overall brown adipocyte thermogenic activity, as well as in specific UCP expression. From our studies of *Phodopus sungorus* brown adipocytes, the following model evolves. Insulin plays an essential role in adipocyte differentiation and supply of mitochondria as the essential organelle for thermogenic activity. But it does not seem to act directly on UCP gene expression, which is on the other hand dependent on T_3. Furthermore, there are one or several as yet unidentified factors involved, which might have inhibitory functions. β-adrenergic stimulation via an untypical (β3) receptor leads to rapid, acute increases in UCP gene expression, i.e., serving as a modulator. In parallel noradrenaline, the physiological β-agonist, also stimulates brown adipocyte T_3 production via classical β-adrenoceptors, thus, also acting indirectly on UCP gene expression by supplying T_3 which is essential for permitting UCP expression.

We are presently performing studies to elucidate the regulatory elements on UCP gene promoter. For this we transfect different cell types with plasmid constructions containing various parts of the UCP promoter region coupled to a reporter gene. We hope this will help to identify the factors responsible for the unique expression of UCP in BAT and contribute to solve the question about the nature and interconvertibility of brown and white adipocytes.

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