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Interscapular brown adipose tissue recruitment is hindered by a temperature environment of 33°C: uncoupling protein-1 underexpression is not associated with obesity development in rats

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Abstract: Brown adipose tissue (BAT) generates heat due to unique thermogenic UC-mitochondria, an event known as nonshivering thermogenesis. Cold, adrenergic agents, hormones, etc., activate nonshivering thermogenesis, resulting in lipid mobilization, an increase in the mitochondria and mitochondrial cristae, and increased uncoupling protein-1 (UCP1) expression and its incorporation into mitochondrial cristae. BAT precursor cells mature and contribute to BAT growth in a process known as BAT recruitment. For the first time, we herein report the effect of a thermoneutral environment of 33°C on interscapular BAT (IBAT) in rats delivered and raised at 33°C. The control animals were housed at 20°C. Thermoneutral IBAT was atrophic (73 mg vs. 191 mg) but with more adipocyte precursor cells; euthermia (37.6°C) was maintained without nonshivering thermogenesis. Although IBAT was inactive, the thermoneutral animals did not develop obesity, and on the contrary, the thermoneutral environment of 33°C hindered the rats' growth, weight (65 gm vs. 139 gm), volume (67 gm vs.136 gm) and length (12 cm vs. 16 cm). The thermoneutral brown adipocytes were smaller (7234 μm^3 vs. 9198 μm^3) with more lipids (4919 μm^3 vs. 4507 μm^3) and a smaller mitochondrial cristae area (52504 μm^2 vs. 61288 μm^2/adipocyte). Lipoprotein lipase mRNA expression was 11% (vs. 58% in control) and UCP1 mRNA expression was 34% (vs. 93% control). UCP1 immunoelectron microscopic study detected 160 UCP1-gold particles (vs. 700 in control) per UC-mitochondrion; thermoneutral brown adipocytes had 9-fold fewer UCP1-gold particles ($0.34x10^6$ vs. $2.99x10^6$ UCP1-gold particles), and thermoneutral UC-mitochondria developed specific intramitochondrial tubular inclusions.

Key words: brown adipose tissue; lipoprotein lipase; uncoupling protein; heat stress; mitochondria

INTRODUCTION

Brown adipose tissue (BAT) and its variant convertible adipose tissue (CAT, also referred to as beige or bright adipose tissue) generates heat and helps rewarm hibernating animals. BAT contributes to maintaining body temperature during cold exposure in mammals and, as recent data suggest, BAT may be involved in controlling obesity by burning excess calories [1-2]. Fatty acids imported from the circulation or from brown adipocyte lipid stores are oxidized inside the uncoupled brown adipocyte mitochondria (UC-mitochondria) to produce heat. The heat production is dependent on, firstly, fatty acids as thermogenic substrates, primarily provided by lipoprotein lipase (LPL) and, secondly, on thermogenesis being sustained by uncoupling protein-1 (UCP1). LPL and UCP1 are transcribed on the brown adipocyte genome and synthesized in the cytoplasm. LPL is transferred to the surface of endothelial cells where it hydrolyzes triglycerides and liberates fatty acids that are imported inside brown adipocytes and used as fuel for heat production. UCP1 is transferred and incorporated into the inner mitochondrial membranes. There, UCP1 allows the translocation of protons from the intermembranous space to the mitochondrial matrix and generates

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heat instead of ATP, a process known as nonshivering thermogenesis [3-5].

At delivery, rat pups have two adipocyte populations within their IBAT: mature adipocytes filled with lipids and specific UC-mitochondria, and another population of adipocyte-precursor cells, described as endothelial cells, pericytes, preadipocytes, young adipocytes, etc. [3-4]. With delivery, as the newborn adjusts from intrauterine temperatures to an external, lower temperature milieu, their mature brown adipocytes begin nonshivering thermogenesis. This process is accompanied by the growth and maturation of adipocyte precursor cells, which are filled with lipids and UC-mitochondria to join already mature adipocytes in nonshivering thermogenesis. This BAT activation process after delivery has been described as BAT recruitment and includes an up to 3-fold increase in BAT volume and weight, as well as increased adipocyte count, mitochondrial content, mitochondrial cristae surface area, mitochondrial enzymes, including UCP1, and oxidative, lipolytic and lipogenic capacity [5-6]. Similarly, the increased expression of UCP1 and increased nonshivering thermogenesis have been postulated as a mechanism to prevent/control obesity [1-2,5,7].

Since the discovery of nonshivering thermogenesis more than 50 years ago [8], substantial data have been generated regarding the effect of cold on the structure and function of brown adipocytes [3-7]. However, with the exception of a few sporadic studies that examined some aspects of young and adult animal behavior in a thermoneutral environment or BAT response in mature animals acclimated to a thermoneutral environment, there have been no detailed analyses of BAT in animals delivered and raised in a thermoneutral environment or in an elevated temperature environment that does not activate nonshivering thermogenesis. Our comprehensive study fills that gap by analyzing BAT in rats delivered and raised in a temperature environment of 33°C during their first month of life.

The thermoneutral environment as an environment in which the basic metabolic rate maintains euthermia without additional heat production by either shivering or nonshivering thermogenesis, has a minimal and constant metabolic rate, animals are inactive, and the elimination of CO_2 is lowest, as is the core body temperature [5,9-10].

Historically, the thermoneutral environment, or thermoneutral zone (TNZ), delineated by lower critical temperature (LCT) and upper critical temperature (UCT), had been considered as the basic metabolic rate, and for rats it had been reported to be in the range of 28-34°C [9-10]. Considering the updated definition of thermoneutrality [11] where the core body temperature is maintained through dry heat loss regulation, and after measuring such dry heat loss through the skin blood flow, the TNZ for 5 adult male rat strains had been reported to be in the range of 28-32°C [12].

The TNZ is species- and strain-dependent, influenced by the animal age, gender, nutritional status, caging and temperature conditions, pregnancy, lactation, day-night circadian cycle, animal body temperature, health conditions, etc. [9-10,12-17]. It has been reported that pregnant and lactating Sprague-Dawley rats tolerated the environmental temperature of 33°C better [18]. Therefore, in our search for the temperature environment around the UCT of TNZ where nonshivering thermogenesis and BAT are inactive, we analyzed the effect of a temperature environment of 33°C on the postnatal development of IBAT.

Our results show that newborn rats delivered and raised for one month in a temperature environment of 33°C had low basic metabolism and halted animal growth, size, weight and volume increase. To maintain euthermia and to prevent hyperthermia, the rats did not recruit IBAT that remained disproportionally small, atrophic and contained fewer brown adipocytes while maintaining more adipocyte precursor cells and underdeveloped adipocytes. Within atrophic IBAT, the mature brown adipocytes remained smaller and contained more lipids compared to the control. LPL mRNA and UCP1 mRNA expression were drastically decreased. Within brown adipocytes, UC-mitochondria and their cristae were well developed but contained a drastically reduced amount of UCP1 protein. Without recruitment, intramitochondrial tubular inclusion within UC-mitochondria became more frequent, perhaps caused by the inadequate amount of UCP1 protein, or as a reflection of an imbalance among mitochondrial proteins and/or substrates. Although UCP1 was significantly underexpressed at 33°C, the rats did not develop obesity; on the contrary, the thermogenic animals remained smaller and leaner than the animals raised at 20°C.

MATERIALS AND METHODS

Animals

The care and use of animals were approved by the Animal Welfare Committee of the Faculty of Medicine, University of Zagreb. The review of literature [18], but also our pilot studies, showed that pregnant Sprague-Dawley rats and their delivered pups had good tolerance to a thermoneutral environment of 33°C. Therefore, the experimental rats were delivered and raised at this temperature, while the control group of animals was delivered and raised at a room temperature of 20°C, as had been already reported before by us and other authors [19-21].

The rats were housed at a 12-h light-dark cycle and had unlimited access to standard laboratory chow diet and tap water. On the 17th day of pregnancy, six pregnant Sprague-Dawley rats were placed in a temperature environment of 33°C, and six pregnant animals were kept at room temperature (20°C). All pregnant animals were caged individually. The fewest number of pups delivered was 9, and so all litters were reduced to 9 pups per litter. After delivery, unless killed earlier, the young pups spent the first 20 days lactating and an additional 10 days feeding on their own. The experimental and control groups of animals were killed simultaneously at 7, 14 and 30 days of age. Six young animals from each age group were killed: 3 for morphology/electron microscopy studies and 3 for mRNA essays. At 30 days of age, 10 randomly selected animals from each group (experimental and control) were measured in detail as described in Table 1.

Electron microscopy (EM)

Three animals per age group in deep anesthesia were perfused transcardially, and the IBAT was fixed for EM analysis as previously described in detail [22]. We used Ringer's solution warmed to 37°C and then a freshly prepared fixative (0.1 M phosphate buffer containing 2% glutaraldehyde and 1% paraformaldehyde at pH 7.4). After perfusion, IBAT adipose tissue was dissected, sliced into small samples and immersed in the fixative. Half of the IBAT sample was randomly selected to be stored in the fixative for 2 h and then processed for immunoelectron microscopy (IEM) (see below). The other half of the IBAT sample was maintained in the fixative for 1-2 days, rinsed in saline solution and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C. After dehydration in cold acetone and propylene oxide, the IBAT samples were embedded in Epon 812 and sectioned to 70 nm on a Reinchard Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate and examined under a JEOL 100 S electron microscope.

UCP1 immunoelectron microscopy

IBAT for immunoelectron microscopic analysis of UCP1 was prepared as previously described in detail [20]. Perfused and freshly fixed IBAT was stored in the fixative for 2 h and then small samples of IBAT were washed in 0.1 M phosphate buffer and embedded in a mixture of 20% polyvinylpyrrolidone in 2 M sucrose. The samples were frozen in liquid nitrogen 2 h later and cut on a LKB Cryo Nova cryo-ultramicrotome.

Antibodies against rat UCP1 were prepared as described previously [23]. Preparation of colloidal gold markers measuring 10 nm in diameter and preparation of protein A-gold complexes were carried out as per the method of Slot and Geuze [24]. The frozen tissue, in 70-nm-thick sections, was transferred to Formvar-covered carbon-coated copper grids and processed for staining. After washing the samples in a phosphate buffer containing 0.02 M glycine, the sections were treated with the diluted specific antibody for 30 min. After washing in a phosphate buffer containing 0.1% bovine serum albumin, the sections were treated with protein A-gold for 30 min. Thin sections were negatively contrasted with 4% uranyl acetate for 5 min, embedded in methyl cellulose and analyzed by EM. To demonstrate the specificity of the labeling, control labeling was performed by omitting the incubation step with the primary antibody, or by blocking the primary antibody with purified UCP1 in the incubation solution as previously described [20].

Morphometry

The diameter of adipose cells was determined at the optical microscopic level using eyepieces with an inbuilt calibrator and a square net. Four IBAT samples were randomly selected from each animal. One-μm-thick

sections were cut from each block and stained with toluidine blue. From these samples, sections were randomly selected for further study. To simplify the measurement of brown adipocytes' diameter, we considered that the adipocytes had a spherical shape. From this data, 100 of the largest diameters were used to calculate the mean adipocyte diameter; from the data, 10% of the largest cells were identified as the largest adipocytes.

Following the procedures described by Weibel et al. [25], we measured the adipocytes' cytoplasm occupied by lipids and/or mitochondria. The average mitochondrial volume and mitochondrial cristae area were determined as previously described in detail [26-27]. Volumetric densities of lipid droplets and mitochondria refer to the adipocyte cytoplasm excluding nuclei that occupied approximately 10% of the cell volume. All data were subjected to statistical analysis using a Student's t test to assess the differences between the control animals and thermoneutral animals.

Determination of LPL mRNA and UCP1 mRNA

The amount of LPL mRNA and UCP1 mRNA was determined as previously described [28]. Three animals per age group (7-, 14- and 30-day-old rats) were killed by cervical dislocation. The IBAT was excised and homogenized in guanidine extraction buffer. Total RNA was isolated according to Jakobsson et al. [29]. For the slot blots, an amount of the RNA preparation corresponding to 4 μg RNA was dissolved in 300 μL 10 x saline-sodium citrate buffer (SSC)/18% formaldehyde with water to yield a total of 400 μl. After a 15-min incubation at 65°C, the solution was applied to a Zeta-Probe filter in a Minifold Slot-Blot apparatus, washed with 400 μl 10 x SCC and dried at room temperature. After prehybridization with salmon sperm DNA (Sigma) and a poly A/poly C mixture, the filter paper was hybridized with cDNA probes and nick translated with a Bethesda Research Laboratories kit. The UCP1 probe utilized was previously characterized by Jacobsson et al. [29], and the LPL probe was that characterized by Kirchgessner et al. [30]. The filter papers were washed, dried and exposed to Kodak X-OMAT AR film at -80°C, and the amount of darkening was evaluated with an LKB laser densitometer.

RESULTS

Animal growth and IBAT growth were retarded in the thermoneutral environment. Pregnant rats were housed in a temperature environment of 33°C from the 17th day of pregnancy, and their pups were delivered and raised at 33°C. The control group of animals was raised at 20°C. All animals had free access to food and water, the consumption of which was similar in both groups during the first 3 weeks. However, after 3 weeks, the consumption of water appeared to be higher in the animals caged at 33°C, although that could also have been the result of greater water evaporation at 33°C. At 20 days old, the pups were weaned, and the mother rats were removed from the cages.

We checked on our animals twice daily, then during feeding and bedding changes and during the conduction of the experiments. We found that during the first 7-10 days of life, the animals in both groups behaved similarly. However, after 3 weeks of age, the pups raised at 33°C became less active in their cages, appeared to have slower growth rates, and their fur was shorter and thinner when compared to the animals raised at 20°C. At one month of age, both groups were euthermic, but the 33°C-animals weighed approximately half as much as the control group, their body volume was half and their length was only ¾ of the measurements of the control group (Table 1). After transcardial perfusion during which blood was washed from the tissue, IBAT appeared brown macroscopically in both groups, but the IBAT in the 33°C-animals was significantly smaller, had an atrophic-like appearance and its wet weight was 2.5-fold lower than the control animals (Table 1).

Basic BAT morphology is preserved in animals raised at 33°C

Animals delivered and raised at 33°C had IBAT that was comparable to the structure and morphometry of the IBAT of the control animals (Fig. 1, Table 2). The IBAT of both groups was dominated by typical multilocular brown adipocytes, the cytoplasm of which contained a (peri)centrally located nucleus, multilocular lipid droplets and a cytoplasm crowded by mitochondria (Fig. 1). Well-developed networks of capillaries and neural axons ran between the adipocytes. The intercellular junctions of brown adipocytes, as well as the distribution of the intercellular matrix,

Table 1. The effect of temperature environment on 30-day-old rats delivered and raised at 33°C, as compared to rats delivered and raised at 20°C (control).

30-day-old rats						
	Rectal body core temperature (°C)[a]	**IBAT wet weight (mg)**	**mg IBAT/g body weight**	**Body weight (g)**	**Body volume (mL)[b]**	**Body length excluding tail (cm)**
Control (20°C) (n-10)	37.3±0.3	191±21	1.73	139.4±6.9	136±4.2	16±0.3
Temperature of 33°C-rats (n=10)	37.6±0.4	73±9	1.11	65.5±7.5	65.5±7.5	12.3±0.6

[a]Rectal digital thermometer was inserted up to 20 mm beyond the anal sphincter and the stable core temperature was recorded;
[b]Body volume was measured by immersing the killed animals into a graduated cylinder.
The mean (±SD) represents the measurements of ten randomly selected animals from each group.

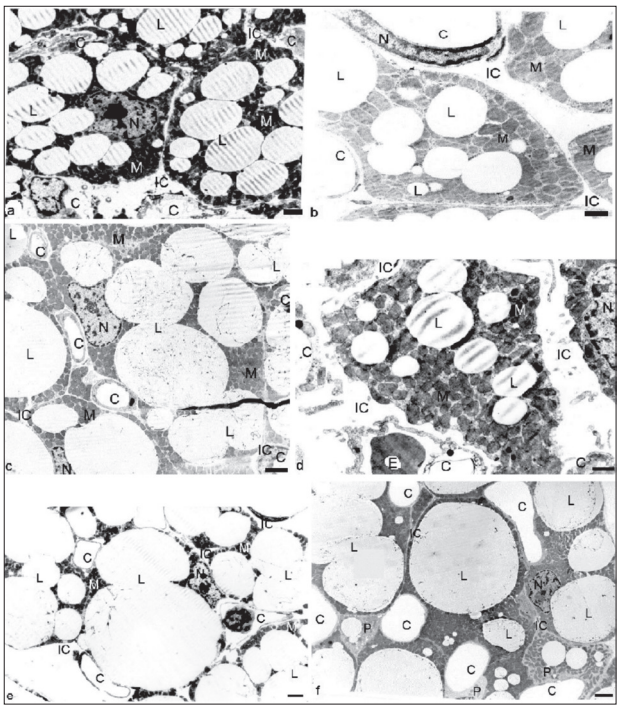


Fig. 1. Control IBAT at 20°C: (**a**) 7-day-, (**b**) 14-day-, (**c**) 30-day-old-rats and IBAT of rats delivered and raised at 33°C: (**d**) 7-day-, (**e**) 14-day-, (**f**) 30-day-old rats. At 7 and 14 days of age, the IBAT of both groups had a similar appearance, but in IBAT at 30 days of age, the adipocytes of rats raised at 33°C had more lipids (L). Preadipocytes (P) with less developed mitochondria (M) were more frequent. Capillaries (C), erythrocyte (E), intercellular matrix (IC), nucleus (N). The bars are 2 μm.

were similar in both groups. Both groups of animals had adipocyte precursor cells (pericytes and preadipocytes) and occasional macrophages and basophils near endothelial cells.

The size of the adipocytes increased with time in both groups (Table 2). The maximal adipocyte diameter grew with time and was approximately 33 μm at 1 month of age in both groups (Fig. 1, Table 2). In rats raised at 33°C, the average diameter was 24 μm at 30 days, i.e., slightly smaller than in the control animals, which resulted in a smaller adipocyte volume, so that at 1 month, their brown adipocyte volume was 7234 μm^3 compared to 9198 μm^3 in the control group (Fig. 1, Table 2).

As the size of brown adipocytes increased, the lipid amounts within the adipocytes also increased (Fig. 1, Table 2). The stored lipids in the 33°C brown adipocytes at 30 days of age occupied 2/3 of the brown adipocyte volume, while in the control adipocytes the lipids occupied approximately 1/2 of the brown adipocyte volume. Although the 33°C adipocytes had a slightly smaller diameter, their larger stored lipid volume resulted in a larger volume of lipids per brown adipocyte compared to the control brown adipocytes (4919 μm^3 vs. 4507 μm^3) (Fig. 1f, Table 2). Such an increase in the lipid volume in slightly smaller 33°C brown adipocytes resulted in a smaller volume of the brown adipocyte cytoplasm (Table 2).

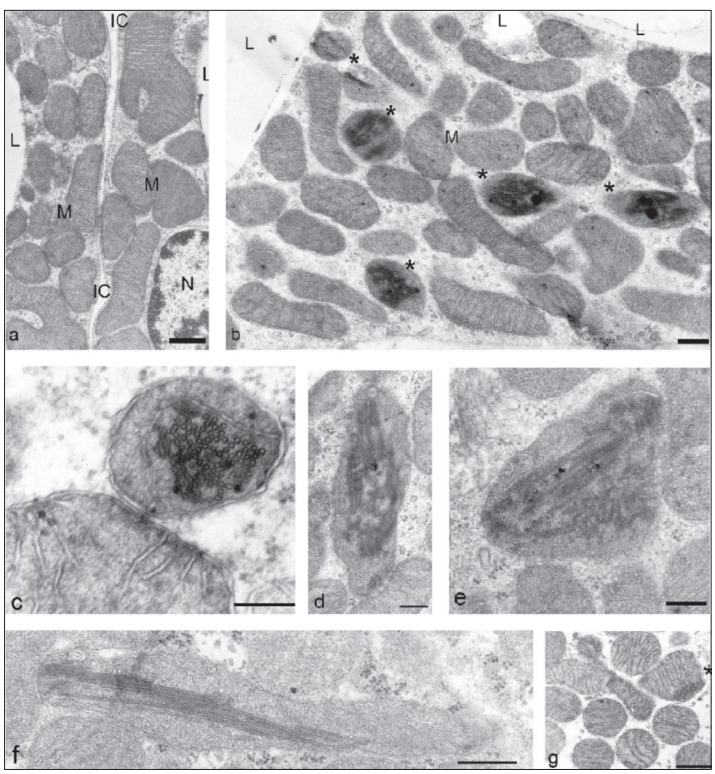
Adipocyte precursor cells are more prevalent in IBAT of animals raised at 33°C

In animals raised at 33°C, the IBAT had small, pale cells with no lipids or a very small amount of lipids near capillaries. EM identified those cells as adipocyte precursor cells with less mitochondria and less mitochondrial cristae: there were 2-3 adipocyte precursor cells per EM section, while the control IBAT had less than one adipocyte precursor cell per EM section (Fig. 1c and f, Table 2), because these cells were recruited and grew into mature brown adipocytes, thereby contributing significantly to the growth and thermogenic potential of control IBAT.

Table 2. The effect of environmental temperature on the morphological parameters (±SD) in brown adipocytes of rats delivered and raised at 33°C, as compared to animals delivered and raised at 20°C (control).

	Age					
	7-day-old		14-day-old		30-day-old	
	20°C (n-3)	33°C (n-3)	20°C (n-3)	33°C (n-3)	20°C (n-3)	33°C (n-3)
Mean maximal diameter of adipocytes (μm)	27±8	25±6	26±8	29±7	33±5	33+4
Brown adipocytes diameter (μm)	21±8	20±11	23±5	22±6	26±3	24±5
Brown adipocytes volume (μm^3)[a]	4846	4186	6367	5572	9198	7234
Adipocyte volume occupied by lipids (%)	34±15	35 ± 16	37±16	36±19	49±14	68±8***
Volume of lipids per adipocyte (μm^3) [a]	1647	1465	2355	2005	4507	4921
Adipocyte cytoplasm volume without nucleus (μm^3) [a]	2897	2448	3610	3210	4221	2083
Preadipocytes per EM section	<1	<1	<1	<1	<1	2-3
Adipocyte average mitochondrial volume (μm^3) [b]	0.38±0.1	0.34±0.4	0.37±0.5	0.40±0.4	0.43±0.1	0.47±0.1
Volumetric density of mitochondria per cytoplasm (%) [bc]	45±9	40±12	51±7	46±4	44±3	60±2***
Volumetric density of mitochondria per adipocyte (μm^3/adipocyte) [b]	1296	979	1841	1477	1857	1249
Mitochondrial cristae per mitochondrion ($\mu m^2/\mu m^3$) [b]	27±4	31±6	29±7	33±8	33±7	42±3##
Mitochondrial cristae per cytoplasm ($\mu m^2/\mu m^3$) [bc]	12±3	12±4	15±5	15±7	14±3	25±2***
Mitochondrial cristae per adipocyte (μm^2/adipocyte) [d]	34992	30366	53399	48727	61288	52504
Mitochondria with inclusions per EM adipocyte section (%) [e]	<<1	<<1	<<1	<<1	<<1	1.3±6.9

[a]Recalculated from brown adipocyte diameters and postulated that the adipocyte is a sphere.
[b]Four randomly selected IBAT samples from each animal, and one hundred EM images per a sample were analyzed at magnifications of x8000 and x20 000. Multipurpose test system M168 (for mitochondrial volume; volumetric density of mitochondria per cytoplasm) and M42 (for mitochondrial cristae per mitochondrion) were used for EM morphometric estimation (see reference 26).
[c]Per μm^3 of adipocyte cytoplasm excluding the nucleus and lipid droplets (nucleus occupies 10% of the cytoplasm).
[d]Recalculated data from the above measurement.
[e]See Fig. 1.
***P<0.01, ##P<0.02;
Data are the mean±SD from three animals in each group.



The mitochondrial cristae surface area is smaller in adipocytes of rats raised at 33°C

The average mitochondrial volume expanded with adipocyte growth in both groups (Fig 2a and b). At 30 days, the average mitochondrial volume was approximately 20% bigger than the 7-day-old adipocyte mitochondria. Compared to the control at 30 days of age, the brown adipocytes in rats raised at 33°C had

Fig. 2. Thirty-day-old rats had a brown adipocyte cytoplasm crowded by UC-mitochondria (M). Control rats delivered and raised at 20°C (**a**), rats delivered and raised at 33°C (**b**). Intramitochondrial tubular inclusions (asterisk) were frequently present in adipocytes of rats delivered and raised at 33°C (**b**-**g**). The inclusions were assemblies of cylindrical tubules. Each tubule had an osmophilic wall, clear lumen, was 50 Å wide (**c**), up to 2 μm long (**d**-**f**) and controlled the shape of the mitochondria (**b**-**g**). Intercellular matrix (IC), lipid droplets (L), nucleus (N). The bars are 0.25 μm.

mitochondria with a slightly larger volume (0.47 μm^3 vs. 0.43 μm^3) (Figs. 2a-b, 3a-b, Table 2). The larger mitochondria packed in a smaller cytoplasmic volume of the 33°C brown adipocytes (likely "compressed" by an increased lipid volume) resulted in a relatively larger cytoplasmic volumetric density of mitochondria (60%) compared to control brown adipocytes (44%). However, the adipocytes raised at 33°C had a smaller cytoplasmic volume. This resulted in a smaller mitochondrial mass per adipocyte during all three measuring points compared to the control group (Table 2).

Although the mitochondrial cristae area per mitochondrion appeared to be slightly larger in the brown adipocytes of rats raised at 33°C at 7 and 14 days of age, the final measurement of the mitochondrial cristae area per 1 μm^3 of cytoplasm remained stable (12-15 μm^2/μm^3) (Table 2) during the first 2 weeks of life in both groups. At 30 days of age, the control brown adipocytes had a mitochondrial cristae area per cytoplasm of 14 μm^2/μm^3. However, the 30-day-old brown adipocytes in rats raised at 33°C had a 30% larger mitochondrial cristae area per 1 μm^3 of mitochondrion, and a 60% larger mitochondrial cristae area per 1 μm^3 of cytoplasm (Figs. 2a-b, 3a-b). The total mitochondrial cristae area per adipocyte increased with age in both groups, and more so in the control group. At 7 and 14 days of age, the brown adipocytes of rats raised at 33°C had 10% less cristae area than the control group, while at 30 days of age, the adipocytes in the 33°C rats had 20% less mitochondrial cristae per adipocyte than the control group (52504 μm^2/adipocyte vs. 61288 μm^2/adipocyte) (Table 2).

Intramitochondrial tubular inclusions are more frequent in adipocytes raised at 33°C

Intramitochondrial tubular inclusions were exceptionally rare findings in the brown adipocytes of the 7- and 14-day-old rats, but in rats raised at 33°C, 1-7% of the mitochondria per adipocyte electron microscope section had tubular inclusions (Fig. 2b-g, Table 2). The inclusions were comprised of uniform tubules. On transversal sections, each tubule appeared as a well-rounded cylinder with a dark peripheral osmophilic wall and a clear central lumen (Fig. 2g-c). On longitudinal sections, the tubules had straight shapes with a dark osmophilic wall and a clear central lumen (Fig. 2g-c). Each tubule had a diameter of about 50 Å, while the length of the tubule could span the entire mitochondrial length (i.e., up to 2 μm), and appeared to determine the shape of the mitochondria (Fig. 2f). Some intramitochondrial tubular inclusions were comprised of only a few tightly packed tubules, but most of the inclusions were assembled from several dozen tubules, reaching up to 100 plus tubules per inclusion (Fig. 2c). Most inclusions were present inside mitochondria with very few or no intramitochondrial cristae (Fig. 2), although a few mitochondria with well-developed cristae also had inclusions (Fig. 2g).

LPL mRNA expression is significantly depressed in the IBAT of rats raised at 33°C

Table 3 shows that the expression of LPL mRNA declined with animal age in both the control and animals raised at 33°C. At 14 days of age, the control IBAT expressed 78% and at 30 days of age, only 58% of LPL mRNA was expressed compared to LPL mRNA expressed in the 7-day-old control. In rats raised at 33°C, IBAT at 7 days of age expressed only 68% of the LPL mRNA, while at 14 and 30 days, the rates of LPL mRNA were 44% and 11%, respectively, compared with LPL mRNA expression in the 7-day-old control IBAT. By the time the animals were 1 month old, LPL mRNA expression was about 1/6 of the LPL mRNA expressed in the 7-day-old pups raised at 33°C (Table 3).

UCP1 mRNA expression is lower in brown adipocytes of rats raised at 33°C

Table 3 shows the effect of the temperature environment of 33°C on the expression of UCP1 mRNA at 7, 14 and 30 days of age. During the first month of life, the UCP1 mRNA expression in the control group was the highest at 14 days (12% higher than at 7 days of life), while at 30 days, the UCP1 mRNA level decreased to 93% of that in the 7-day-old pups.

Compared to the control animals, the brown adipocytes of the rats raised at 33°C expressed significantly less UCP1 mRNA. At 7 days of age, the thermoneutral adipocytes expressed only 2/3 of the UCP1 mRNA, at 14 days of age 1/2 and at 1 month of age 1/3 as compared to UCP1 mRNA expression

Table 3. The effect of temperature environment on the expression of UCP1 mRNA and LPL mRNA in the interscapular BAT of rats delivered and raised at 33°C as compared to rats delivered and raised at 20°C (control).

	Age					
	7-day-old		14-day-old		30-day-old	
	20°C (n-3)	33°C (n-3)	20°C (n-3)	33°C (n-3)	20°C (n-3)	33°C (n-3)
UCP1 mRNA (%)	100±17	64±12	112±23	52±16	93±11	34±9
LPL mRNA (%)	100±20	68±21	78±19	44±26	58±15	11±9

Interscapular BAT of the killed animals was dissected, total RNA was extracted and UCP1 mRNA and LPL mRNA levels were quantified by the Slot-Blot technique, as described in the Materials and Methods. UCP1 mRNA and LPL mRNA data were normalized and presented relative to the corresponding mean levels of the 7 day-old-pups housed at 20°C, with mean values set to 100 %.
Data are the mean±SD from three animals in each group.

in the 7-day-old control pups. The brown adipocytes of rats raised at 33°C had the highest expression of the UCP1 mRNA at 7 days of age and by the time the animals were one month old, UCP1 mRNA expression was nearly halved (table 3).

The amount of UCP1 protein is significantly reduced in thermoneutral UC-mitochondria

The qualitative and quantitative analysis of UCP1 protein by immunoelectron microscopy in 30-day-old IBAT is detailed in Table 4 and in Fig. 3a-b. The distribution of anti-UCP1 antibody protein A-gold complex particles (UCP1-gold particles) over the mitochondria follows the shape of the mitochondrial cristae (Fig. 3a-b). The intermitochondrial membrane space and the mitochondrial matrix were not substantially labeled by the UCP1-gold particles; when they were labeled by UCP1-gold particles, they were labeled near the cristae, indicating that such labeling very likely represented the incorporation of UCP1 protein into the cristae, or UCP1 protein *en route* to cristae incorporation. Occasional UCP1-gold particle labeling was present throughout the cytoplasm of the brown adipocytes, particularly near the mitochondria, likely representing the UCP1 already synthesized *en route* to the mitochondria. The endothelial cell cytoplasm and their C mitochondria did not display any significant labeling by UCP1-gold particles.

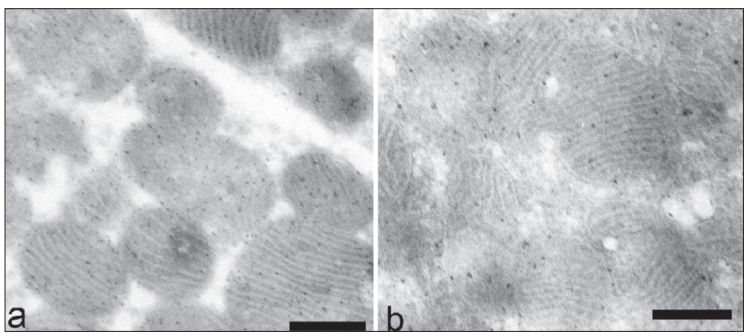


Fig. 3. IEM of UCP1 distribution in rats of 30-day-old control brown adipocytes (**a**) and brown adipocytes of rats raised at 33°C (**b**). Well-developed mitochondria with numerous straight cristae were labeled by UCP1-antibody protein A-gold complex particles (UCP1-gold particles), over the mitochondrial cristae with a few UCP1-gold particles, over the mitochondrial matrix, the intermembranous space and in the cytoplasm. Compared to control mitochondria, thermoneutral mitochondria had 5-fold fewer UCP1-gold particles per one μm^3 of mitochondrion (Table 4). The bars are 0.15 μm.

In the control brown adipocytes, 85% of the UCP1-gold particles were distributed over the mitochondrial cristae, 11% over the intramembranous space and the mitochondrial matrix, and 4% over the outer mitochondrial membrane. With a 33-μm^2 mitochondrial cristae area per 1 μm^3 of mitochondria (Table 2), there were approximately 1400 UCP1-gold particles in the cristae per μm^3 of mitochondria. With the UCP1-gold particles over the mitochondrial intramembranous space, the mitochondrial matrix and over the outer mitochondrial membrane, it would appear that 1 μm^3 of mitochondria had about 1600 UCP1-gold particles (Table 4). Combining these data with an average control brown adipocyte mitochondrion volume of 0.43 μm^3 (Table 2), we established that one control UC-mitochondrion had about 700 UCP1-gold particles or, expressed through a control adipocyte, there were approximately $3x10^6$ UCP1-gold particles in the 30-day-old rats.

The mitochondria in the brown adipocytes of rats raised at 33°C exhibited a similar UCP1-gold particle labeling pattern as the control mitochondria (Fig. 3a-b), with the most UCP1-gold particle labeling (72%) observed over the mitochondrial cristae, 23%

over the intermembranous space and the mitochondrial matrix, and 5% over the outer mitochondrial membrane (Table 4). However, the total number of UCP1-gold particles per mitochondrion was drastically reduced (Fig. 3b). There were only 5 UCP1-gold particles per μm^2 of mitochondrial cristae, 1.6 UCP1-gold particles per μm^2 of the intramembranous space and the mitochondrial matrix, and 0.3 UCP1-gold particles per μm^2 in the outer mitochondrial membrane, totaling 6.8 UCP1-gold particles per μm^2 of mitochondria. With 42 μm^2 of mitochondrial cristae area per 1 μm^3 of mitochondrion (Table 2), there were about 200 UCP1-gold particles in the cristae per 1 μm^3 of brown adipocyte mitochondria in rats raised at 33°C. Adding to this the number of UCP1-gold particles present over the mitochondrial intermembrane space and the mitochondrial matrix and the outer mitochondrial membrane, it appears that 1 μm^3 of mitochondria contained about 270 UCP1-gold particles (Table 4). As the average thermoneutral brown adipocyte mitochondrion had a volume of 0.60 μm^3 (Table 2), there were approximately 160 UCP1-gold particles per mitochondrion. Thus, there were about 0.34×10^6 UCP1-gold particles expressed per brown adipocyte in the 30-day-old rats raised at 33°C, which was 9-fold fewer than in control brown adipocytes.

DISCUSSION

Control rat pups and pups delivered at 33°C were similar in appearance, lactation and behavior for 7-10 days post-delivery. In spite of being raised at 33°C from the time of delivery, the experimental group of pups behaved as poikilotherms and huddled with the mother in their nests until about 10 days of life when they began to leave the nests more often. After weaning at 20 days of age, the pups raised at 33°C moved in the cage less and spent more time out of the nest and away from each other, similarly as reported previously [9-10,13-17,31], and at thirty days of age the rats were moving very little in the cages; they mostly lay down and appeared to be less interested in food. Their fur was sparse with short hair, and their bodies were significantly smaller, lighter and shorter than those of the control animals (Table 1).

Our animals raised at 33°C maintained normothermia, preventing hyperthermia by minimizing muscle movements and maintaining low basal metabolism, which caused the global, retarded growth. It has been well documented that animals raised in a similar temperature environment have the lowest basal metabolism and maintain euthermia without an additional heat source, i.e., without shivering or nonshivering thermogenesis [9-10,12,18,31-32]. The findings that the IBAT of the animals raised at 33°C was atrophic, 2.5-fold lighter than the IBAT of control rats and more growth-arrested than the rest of the animals' body (1.1 mg vs. 1.7 mg IBAT per 1 g of animal body weight) are similar to others [9,32]. In comparison, fully recruited IBAT in cold-acclimated rats had up to about 3.0 mg of IBAT per 1 g of animal body weight [33].

Since in the temperature environment around UCT any heat production by IBAT nonshivering thermogenesis would cause hyperthermia (a detrimental condition to the animals), their IBAT remained atrophic, unrecruited. Such atrophic, unrecruited IBAT in addition to mature developed adipocytes, had significantly more adipocyte precursor cells and young, under-developed adipocytes. They remained unrecruited, "dormant" in 1-month-old thermoneutral IBAT, contributing to a smaller, lighter and atrophic IBAT.

Although the IBAT of rats raised at 33°C was not recruited for nonshivering thermogenesis, their mature brown adipocytes did not change into large white adipocytes as has been observed in "inactive" adipocytes of CAT [4,28,34]. The general structure, multilocular appearance, size, ultrastructure and morphometry of the IBAT of rats raised at 33°C resembled that of the control animals, where the IBAT participated in nonshivering thermogenesis. However, detailed analysis showed that by one month of age, the brown adipocytes in rats reared at 33°C had a 20% smaller volume and significantly more lipids, resulting in about 2-fold less cytoplasm per adipocyte, implying that the size of brown adipocyte and its cytoplasm volume are controlled by the stimuli present in a lower temperature environment. As the brown adipocytes of rats raised at 33°C were not recruited, one would anticipate some retardation or destruction of their UC-mitochondria [4,28,34]. However, the UC-mitochondria in brown adipocytes of rats raised at 33°C remained BAT-specific as well. Morphometric analysis demonstrated that the UC-mitochondria were

larger, with significantly more cristae per one μm^3 of mitochondrion, resulting in more mitochondrial cristae per 1 μm^3 of adipocyte cytoplasm in rats reared at 33°C (Table 2).

How brown adipocytes initiate and maintain a vast mitochondrial population and enormous mitochondrial cristae surface area is not quite clear, but we know that stimulation of noradrenergic pathway proliferates the UC-mitochondria and its cristae in animals exposed to cold and in brown adipocytes cultured *in vitro* [5,35-39]. However, mitochondriogenesis and the proliferation of UC-mitochondria also occurs before the pups are delivered and exposed to the cooler, external environment, suggesting that other, intrinsic mechanisms, likely hormonal, are involved in controlling mitochondriogenesis in brown adipocytes [1,3,5]. The animals raised at 33°C probably did not have any significant noradrenergic stimulation and therefore their extensive UC-mitochondriogenesis was probably the effects of intrinsic, hormonal stimuli.

LPL in BAT provide the fuel for nonshivering thermogenesis, and the expression of LPL mRNA and the activity of LPL enzyme in IBAT corroborate well. The expression of LPL mRNA starts early in conjunction with lactation and then declines slowly during the first few weeks following delivery, after which the expression of LPL mRNA declines slowly as there is less need for nonshivering thermogenesis [5-6,40,40-42]. In the control IBAT, the expression of LPL mRNA followed this pattern. Conversely, without the recruitment of IBAT in animals raised at 33°C, the expression of IBAT LPL mRNA expression declined sharply at the age of 1 month to only 11%.

One would expect that without LPL, fatty acid import from the capillaries would cease and the volume of lipids within the brown adipocytes would decline or remain unchanged. However, morphometric analysis revealed that thermoneutral adipocytes had more stored lipids than control adipocytes. This may be a reflection of one of two processes: (i) even small, barely detectible amounts of LPL on the endothelial lumen of BAT capillaries could generate a small but continuous import of lipids into the inactive thermoneutral adipocytes, or (ii) very low levels of LPL mRNA in 1-month-old thermoneutral adipocytes could originate not from adipocytes but from other sources (such as macrophages frequently present in BAT) [42], and the accumulated lipids within the thermoneutral brown adipocytes were thus due to a *de novo* lipid synthesis [42].

Table 4. Distribution of anti-UCP1-antibody-protein-A-gold complex particles (UCP1-gold particles)[a] in brown adipocytes of 30-day-old rats delivered and raised in a temperature environment of 33°C, as compared to animals delivered and raised at 20°C.

30-day-old rats		
	Control (20°C) (n-3)	**Temperature of 33°C (n-3)**
No. of UCP1-gold particles per μm^2 mitochondrial cristae	43.1±11.9 (85%)	4.9±6.3*** (72%)
No. of UCP1-gold particles per μm^2 mitochondrial matrix	5.8±3.9 (11%)	1.6± 3.1 (23%)
No. of UCP1-gold particles per μm^2 outer mitochondrial membrane	1.9±1.1 (4%)	0.3±0.5*** (5%)
No. of UCP1-gold particles per μm^3 mitochondria (Nx10^3/μm^3) [b]	1.41[c]-1.61[d]	0.20[c]- 0.27[d]
No. of UCP1-gold particles per adipocyte (Nx10^6) [b]	2.63[c]–2.99[d]	0.25[c] –0.34[d]

***P<0 .01
[a]See the Materials and Methods section.
[b]Recalculated data from the above measurements and the adipocyte morphometry data from Table 2.
[c]Recalculated data using the number of UCP1-gold particles per μm^2 of mitochondrial cristae.
[d]Recalculated data using the number of UCP1-gold particles per μm^2 in the mitochondrial cristae plus the number of UCP1-gold particles per μm^2 in the intermitochondrial space, mitochondrial matrix and the outer mitochondrial membrane.
Data are the mean±SD from three animals in each group.

Ricquier's team discovered that UCP was specifically expressed in brown adipose tissue [43], while Loncar [4,28] showed that UCP can also be expressed in unique white-like-adipose tissue named convertible adipose tissue (CAT), currently referred to as beige or bright adipose tissue [1-2,6]. The amount of UCP1 mRNA in brown adipocytes corroborates well with the amount of UCP1 protein, BAT thermogenic capacity [1-2,5-6,43-44], with the ultrastructural features of brown and convertible adipocytes [4,20,28,45-46], with brown adipocytes *in vitro* [35], and with BAT-related tumors [21]. We showed that the IBAT of control animals expressed UCP1 mRNA and LPL mRNA consistently during the first month of life. Although we had only 3 animals in each age group, our results corroborate well with the observation of other authors where 4 or more animals per age group were studied [5,47-48].

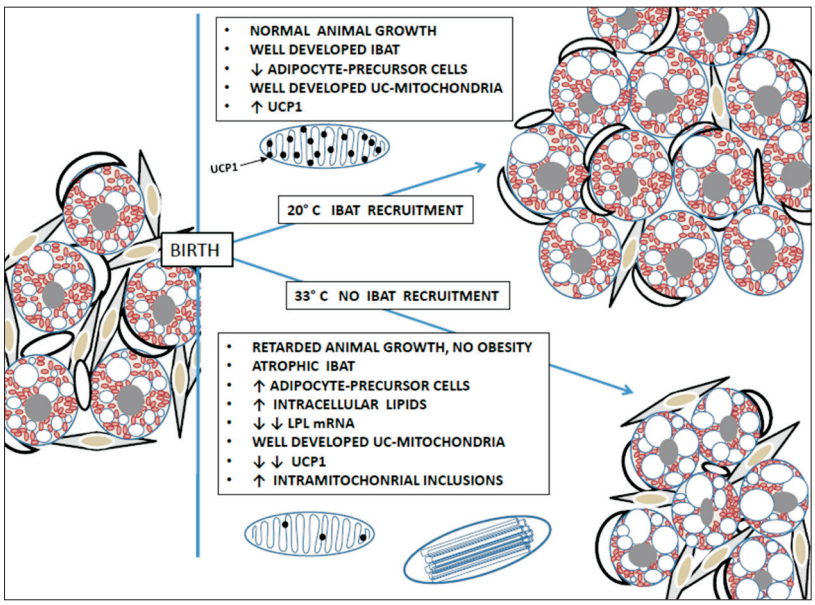


Fig. 4. Schematic illustration of the effect of a temperature of 33°C on the recruitment of IBAT in 30-day-old rats. The IBAT at delivery is composed of adipocyte-precursor cells and mature brown adipocytes filled with lipid droplets and with UC-mitochondria. At 20°C, rats activate nonshivering thermogenesis, mobilize the lipids within mature brown adipocytes, increase the mitochondria and mitochondrial cristae, and increase the expression and incorporation of UCP1 into cristae; adipocyte precursor cells are recruited, contributing to BAT growth. At 33°C, the basal metabolism maintains the animals euthermic with no IBAT recruitment. IBAT remains atrophic, with numerous adipocyte precursor cells and smaller mature adipocytes with more lipids and mitochondria with a large mitochondrial cristae area. UCP1 expression and incorporation into mitochondria is drastically reduced and its paucity could contribute to instability inside the mitochondria, leading to the development of tubular intramitochondrial inclusions. Although the UCP1 was significantly underexpressed at 33°C, the rats did not become obese.

In contrast to the control rats, the IBAT of rats raised at 33°C and without recruitment, had lower UCP1 mRNA expression, starting from the first week of life and declining further by the age of 1 month (Table 3). At this point, it is unclear why IBAT at 33°C in 1-month-olds would expresses any UCP1. Although the UCP1 gene regulation data indicate a complex interplay of numerous transcription factors in the enhancer-promoter regulatory region of the UCP1 gene [44], we need more data to explain the mechanisms controlling UCP1 mRNA expression in 1-month-old unrecruited brown adipocytes.

Good correlation between UCP1 mRNA expression and IEM quantification of the UCP1 described before [20-21,28,35,45] was confirmed, showing that UC-mitochondria at 33°C had approximately 5-fold less UCP1, and that in brown adipocytes at 33°C there was about 9-fold less UCP1 protein as compared to the control brown adipocytes. However, in spite of significant underexpression of UCP1, and without BAT recruitment, rats raised at 33°C remained small and lean suggesting that to develop obesity [49], the animals should have had higher metabolism than rats delivered and raised at 33°C.

The finding of intramitochondrial inclusions in adipocytes of 1-month-old rats raised at 33°C is significant. Such inclusions have been reported earlier by us and others [19,26, 33,34]; they were different and present around the neonatal period or at the beginning of cold exposure, i.e. during periods of intensive mitochondriogenesis, conditions that were not present in rats reared at 33°C. Instead, the UC-mitochondria in adipocytes of animals raised at 33°C already had a crowded cytoplasm and well-developed mitochondrial cristae. However, the UC-mitochondria had low amounts of UCP1 protein (Fig. 3b, Table 4).

It has been established that the composition of mitochondrial proteins and UCP1 in brown adipocytes synchronously changes during postnatal development and recruitment [3,5-6]. Induced UCP1 expression in unilocular adipocytes was shown to be followed by an increase in mitochondrial cristae and an increase in cyclooxygenase (COX) IV; the "ordinary" white adipocyte C-mitochondria also appeared as brown adipocyte UC-mitochondria [36]. These data suggest that UC-mitochondria genesis and preservation depends on the amount of UCP1 protein incorporated into the mitochondrial cristae. We are lacking data about the architecture of inner mitochondrial cristae and the required balance between the amount of UCP1 and other mitochondrial proteins. However, it is tempting to assume that a very low amount of UCP1 in the mitochondria of rats raised at 33°C, or its absence

from fully developed UC-mitochondrial cristae, creates mitochondrial cristae instability, resulting in the development of intramitochondrial tubular inclusions.

In summary (Fig. 4), rats delivered and raised at 33°C have a minimal metabolic rate. The animals remained euthermic. To prevent hyperthermia, IBAT was not recruited and nonshivering thermogenesis did not ensue. Without recruitment, the BAT remained disproportionally small and atrophic, having fewer mature brown adipocytes but more adipocyte precursor cells. Mature thermoneutral brown adipocytes were smaller but contained more lipids compared to control brown adipocytes. Considering the low level of LPL mRNA expression, the increased lipid volume in the adipocytes could be the result of absent recruitment, or the reflection of *de novo* lipid synthesis in brown adipocytes. The UC-mitochondria were well-developed. However, without the recruitment, UCP1 mRNA expression was significantly reduced and the amount of UCP1 protein in UC-mitochondria was drastically decreased. Despite significant underexpression of UCP1, the thermoneutral animals not only did not become obese due to low metabolic rate, but their growth, increase in size, weight and volume was significantly suppressed. Intramitochondrial tubular inclusion in UC-mitochondria was more frequent, perhaps because of the small amount of UCP1 protein in the mitochondrial cristae, or as a result of an imbalance between the mitochondrial proteins and/or substrates, all due to a lack of recruitment.

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