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Opposite regulation of uncoupling protein 1 and uncoupling protein 3 in vivo in brown adipose tissue of cold-exposed rats

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Abstract Earlier we reported a 14-fold increase of glycogen in the brown adipose tissue (BAT) in rats when the animals were placed back from cold to neutral temperature. To elucidate the mechanism, here we compared the level of glucose transporter 4 (GLUT4) protein, uncoupling protein (UCP) 1 and UCP3 mRNA and protein expressions in the BAT under the same conditions. We found that the increased GLUT4 level in cold was maintained during the reacclimation. After 1 week cold exposure the mRNA and protein content of UCP1 increased parallel, while the protein level of UCP3 decreased, contrary to its own mRNA level.

Key words: Uncoupling protein; Brown adipose tissue; Glucose transporter 4

1. Introduction

Uncoupling proteins belong to the large group of solute carriers, which are located in the inner mitochondrial membrane. They are supposed to dissipate energy by transporting H^+ ions from the outer side of the mitochondrial membrane into the matrix, bypassing the ATPase. This function causes an uncoupling of respiration from ATP generation, resulting in heat production. Uncoupling protein (UCP) 1 was discovered first as mitochondrial integral membrane protein induced by cold exposure in brown adipose tissue (BAT) of rats [1]. It has also been demonstrated that the expression of UCP1 is restricted to BAT, in the tissue playing a role in the thermogenesis mostly in rodents [2,3]. It was a milestone in the research on UCP1 when the other homologues, UCP2 [4] and UCP3 [5,6], were discovered. Both of these latter discovered homologues are present in BAT, but they also show a broad expression pattern [7]. UCP2 is expressed in liver, muscle and mostly in white adipose tissue. UCP3 is located mostly in skeletal muscle [8] and both of them can be found in rodents and humans [5,9]. The recently discovered UCP4 is expressed only in nervous tissue [10].

The uncoupling action of UCP1 is essential for the thermogenesis in BAT [2]. Genetic deletion of UCP1 in mice causes cold sensitivity but not obesity [11]. This result revealed that the function of UCP1 could not be substituted by the other UCPs [2,11]. On the other hand, UCP2 may be involved in the body weight regulation, since mutations of this protein correlate with obesity [4,9]. UCP3 is supposed to play a regulatory role in the respiration of muscle [8].

UCPs are subjected to a complex regulation. Cold exposure strongly induces UCP1 mRNA and protein expression [12]. The adrenergic system [13–15] and the thyroid [15,16] hormones play the most significant role in the mediation of this effect. A slight induction in the UCP2 and UCP3 mRNA expression in BAT upon cold exposure has also been demonstrated [12]. There are, as yet, few results on UCP3 protein expression, e.g. [17].

It was a widely held view that for the H^+ transport activity UCPs the fatty acids are the cofactors. This view was recently challenged [18], and others demonstrated that ubiquinone [19] and retinoic acid [20] are more potent activators of UCPs, therefore, they were suggested to be the natural cofactors. Cofactor availability as a possible regulatory factor has also been discussed. Finally, the activity of UCPs is subjected to an allosteric regulation. Intracellular free fatty acids and nucleotides, such as ATP/ADP, GTP/GDP, were shown to regulate the H^+ transport activity [21].

These studies shed light on the events in BAT followed by cold adaptation, however, little information is available on the molecular events during the reacclimation to normal temperature, when the heat production and metabolic rate in BAT decrease. We reported earlier a strong accumulation of glycogen in the BAT when the rats were transferred from cold to normal temperature [22]. This was a consequence of an increased synthesis of glycogen by the glycogen synthase and its decreased breakdown by the phosphorylase enzyme [22]. To study the mechanism further, we investigated here the expression of glucose transporter 4 (GLUT4) under the same conditions. Since glycogen synthesis is an energy consuming process, we also addressed the question, what happens to the expression of UCP1 and UCP3, which have a strong effect on the energy status of BAT. Therefore, we followed the expression of these UCPs during cold exposure and reacclimation spanning the glycogen accumulation period.

2. Materials and methods

2.1. Rat treatment protocol and tissue sampling

Male Wistar rats weighing 200–250 g were used. The animals were housed in individual cages, fed commercial laboratory chow ad libitum and submitted to a 12/12 h light/dark period (from 8:00 a.m. to 8:00 p.m.) both in cold and at neutral temperature. During the experiment the food consumption and weight gain of the rats were moni-

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Abbreviations: UCP1, 2, 3, uncoupling protein 1, 2, 3; BAT, brown adipose tissue; GLUT4, glucose transporter 4

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tored. At termination of the experiments, they were sacrificed by decapitation between 8:00 and 9:00 a.m. A group of cold-exposed animals was replaced for 24 h to neutral temperature. Throughout the text, cold means +5°C, while neutral temperature means 24–25°C, which is near to thermoneutrality. After decapitation the blood was collected into tubes containing minimal necessary heparin. Interscapular BAT was quickly removed, put on an ice-cold glass plate where the white and brown tissues were separated within 1 min, while an other member of the staff clamp froze a part of gastrocnemius muscle by using tongs pre-cooled in liquid nitrogen. The trimmed BAT was also clamp frozen and stored under liquid nitrogen until analyses.

2.2. Immunoblotting analyses (Western blot)

The BAT samples were extracted with chloroform/methanol (2:1) after grounding and then were washed three times with the same mixture. The samples were dried under vacuum, dissolved in 1 ml distilled water and agitated by ultrasonication. 750 μl of the 1.0 ml sample was mixed with 3.0 ml acetone, frozen at −70°C and its protein content was precipitated by centrifugation. The dried protein was resolved at 2 μg/μl concentration in sample buffer (0.0625 M Tris–HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8). The protein components were separated on 12% acrylamide gel and blotted onto nitrocellulose membrane, which was followed by immunoblotting with appropriate antibodies. From the residual 250 μl sample the protein concentration was determined with BCA reagent (Sigma, Budapest, Hungary), in order to equal adjust the protein concentrations.

The rabbit antibody for UCP1 was a kind gift from Barbara Cannon (Stockholm University, Stockholm, Sweden), which was tested not to cross-react with UCP3 (not shown). The anti-rabbit peroxidase-conjugated second antibody was purchased from Sigma (Budapest, Hungary). The blots were developed with ECL plus (Amersham Pharmacia, UK).

The guinea pig antibody for UCP3 was purchased from Linco Research, Inc. (St. Charles, USA). This antibody is specific for UCP3, however, it reacts only with the long form of UCP3 (UCP3 L). The anti-guinea pig peroxidase-conjugated second antibody was bought from Rockland (Gilbertsvill, USA). The blots were developed as in case of UCP1. The intensity of the lanes was quantified by UTHCSA Image (IT Version 1.27).

2.3. Production of antibodies against human frataxin

A cDNA fragment encoding human frataxin was amplified from a human brain cDNA library using AAAAGATCTATGATAGCAGCGGCAGGAGGA as forward and AAACTCGAAGAGAGTCGATGGATAAGTG reverse primers. The product of the reaction was cloned into pGEX 4T-1 plasmid resulting in a fusion between glutathione-*S*-transferase (GST) and frataxin. The fusion protein was expressed in BL21 *Escherichia coli* cells and isolated using glutathione Sepharose as suggested by the supplier (Pharmacia). The isolated GST–frataxin fusion protein was used as antigen to generate antibodies in rabbits.

2.4. Northern blot analyses

Total RNA from rat interscapular BAT was purified by the method of Chomczynsky et al. [23], separated in a 1.2% agarose gel containing formaldehyde and transferred to Hybond-N membranes. The membranes were prehybridized at 42°C for 1 h in 10 ml/membrane of prehybridizing solution (1 M NaCl, 50% Denhardt's, 1% SDS, 50% formamide, and 100 μg/ml salmon sperm DNA). RNA membranes were hybridized overnight in the same solution with the addition of probes that were labeled by random priming with [α-^{32}P]dCTP to a specific radioactivity of approximately 6×10^8 dpm/μg DNA, then washed in a solution of 2×SSC/0.1% SDS at 63°C for 1 h and in 0.1×SSC/0.1% SDS at 40°C for 1 h. The DNA probes both for UCP1 and UCP3 were kind gifts from Barbara Cannon (Stockholm University, Stockholm, Sweden). The blots were detected by PhosphorImager and the data were analyzed by OptiQuant software.

3. Results

Investigating the mechanism of our earlier finding [22], that the glycogen content in BAT decreased during cold exposure,

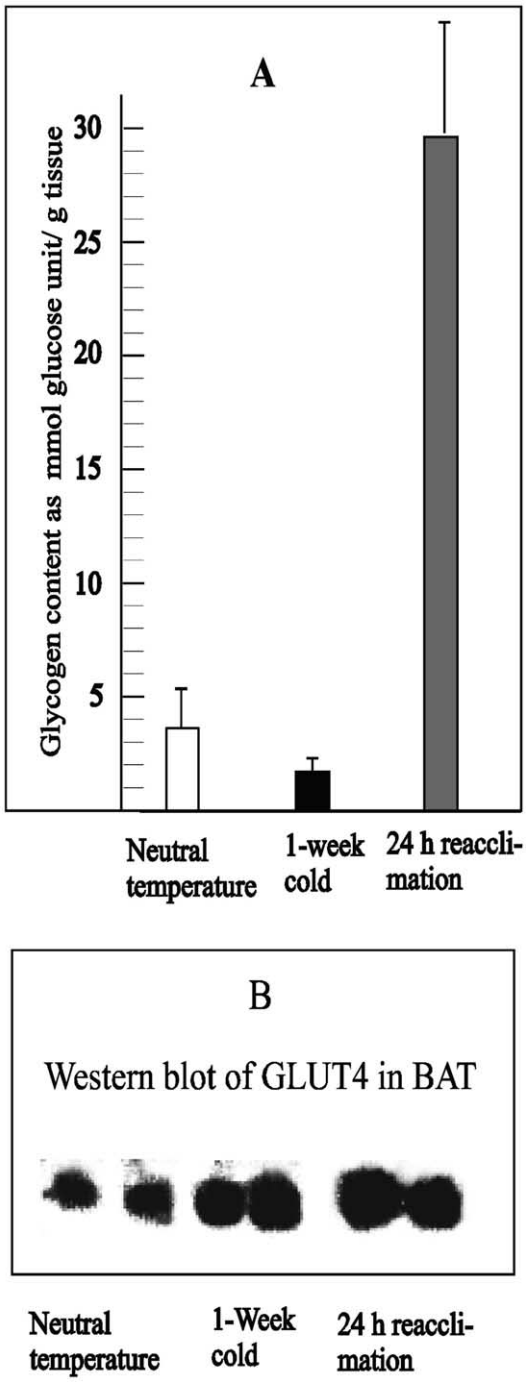

Fig. 1. A: Glycogen accumulation in BAT of rats after 1 week cold exposure and after 24 h reacclimation at neutral temperature in BAT of rats. B: Expression of GLUT4 protein under the same conditions as in A.

then accumulated after 24 h reacclimation to normal temperature, here we compared the glycogen levels (Fig. 1A) and GLUT4 protein expression (Fig. 1B) under the same conditions. GLUT4 protein expression was markedly enhanced in cold and this high level was maintained during the reacclimation period, supporting the possibility of a facilitated glucose uptake to supply the glycogen synthesis. Similarly, in the rat muscle an enhanced expression of GLUT4 mRNA was reported on the effect of cold [24].

To reveal the energy status of BAT in the cold we inves-

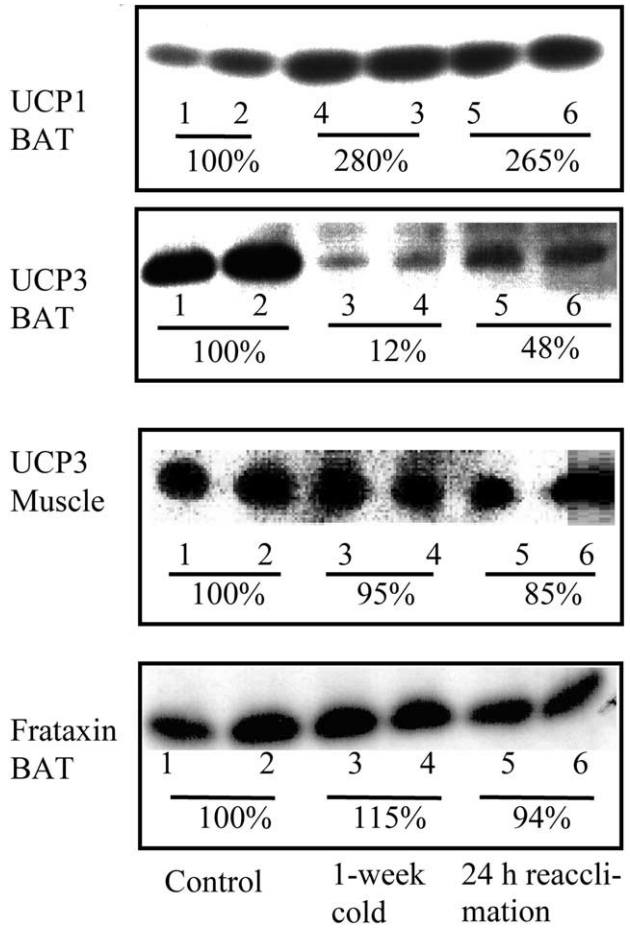

Fig. 2. Western blots of UCP1 and UCP3 proteins on the effect of 1 week cold exposure and after 24 h reacclimation at neutral temperature in BAT and muscle of rats. Each lane represents an individual animal. Frataxin served as control to demonstrate that equal amounts of proteins were loaded.

tigated also the changes of UCPs, which are supposed to undermine the energy status of the cell, that is, the synthetic processes. As expected, the amount of UCP1 protein increased upon the effect of cold exposure, however this increased amount was maintained after 24 h reacclimation (Fig. 2), allowing an intensive glycogen synthesis to go on at the same time. Surprisingly, UCP3 protein in BAT exerted an opposite pattern, since its level markedly decreased upon the effect of cold but started to increase after the reacclimation (Fig. 2). At the same time, UCP3 protein did not show significant changes in the skeletal muscle under the above conditions (Fig. 2). Equal amounts of proteins were loaded as judged by the measurement (see Section 2) and the frataxin control.

For the explanation of the contrasting changes of UCP1 and UCP3 proteins in BAT, further experiments were necessary. Detecting the expression of mRNA of the two genes, we found that both mRNA levels showed an approximately equal (280–220%) increase upon the effect of cold and started to return to normal during reacclimation increase (Fig. 3). The bottom panel demonstrates that equal amounts of total RNA were loaded. Thus, comparing Figs. 2 and 3, there is a controversy in changes of protein versus mRNA levels of UCP3.

For the detailed analysis of the decrease of UCP3 protein in cold, we followed its changes within the 1 week cold period (Fig. 4). The protein level decreased already by the sixth hour and then started to increase until the end of the period.

4. Discussion

During cold exposure the glucose utilization in BAT of rats increased [25], in accordance with our finding showing a decreased glycogen content (Fig. 1A). The intensive glucose utilization was accompanied with an increased expression of mRNA and protein of the insulin sensitive GLUT4 isoform of glucose transporters [25]. While a part of our experiment (Fig. 1B) confirms the mentioned report, we also demonstrated that the increased GLUT4 protein level persists after 24 h reacclimation from cold to neutral temperature (Fig. 1B). Since the insulin level during the reacclimation was about the same as in normal temperature [22], it seems probably that the higher level of GLUT4 protein was in active form, supplying substrate for the glycogen accumulation (Fig. 1A).

To elucidate further the mechanism of glycogen accumulation we also investigated the effect of cold on UCPs, which supposedly have substantial impact on cell metabolism, such as on synthetic and/or catabolic processes. On the effect of cold UCP1 protein expression, being an essential protein in

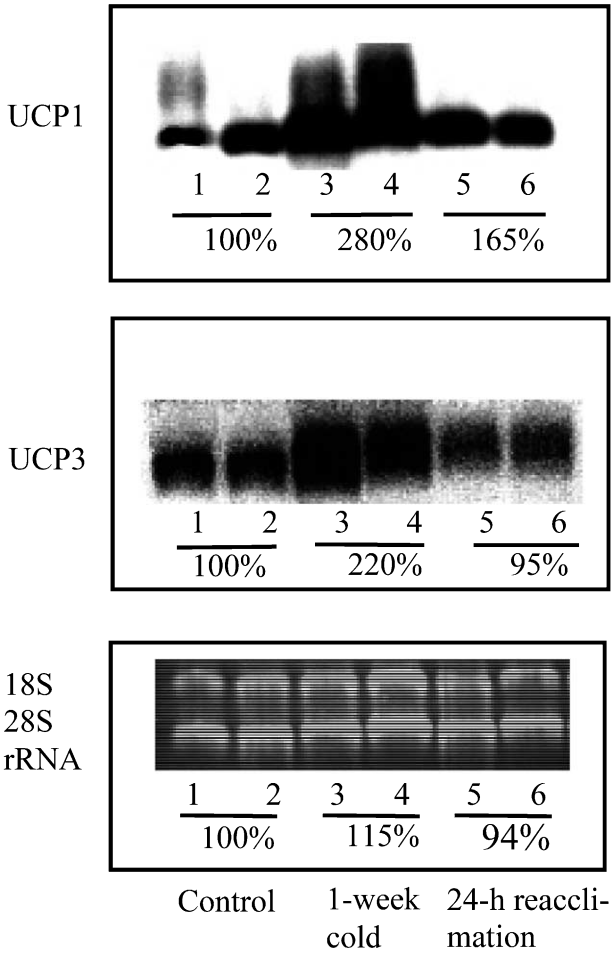

Fig. 3. Northern blots of UCP1 and UCP3 mRNA on the effect of 1 week cold exposure and of 24 h reacclimation to neutral temperature in the BAT of rats. The ribosome RNAs demonstrate that equal amounts of RNA were loaded. Each lane represents an individual animal.

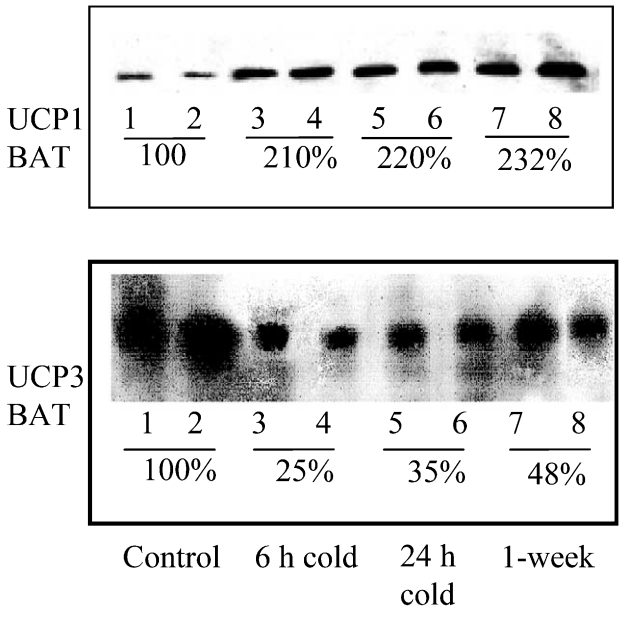

Fig. 4. Western blots of UCP1 and UCP3 proteins as a function of time during 1 week cold exposure in the BAT of rats. Each lane represents an individual animal.

non-shivering thermogenesis, expectedly increased (Fig. 2). Furthermore, the increased level was maintained during the reacclimation period, that is, during the intensive glycogen synthesis (Figs. 1A and 2). This means that despite the increased UCP1 protein level, the mitochondria were not totally in uncoupled state and were able to provide enough ATP for synthetic processes. A probable explanation is that the mere presence of UCP1 protein does not mean that the protein was fully in active form, instead it could undergo effects of regulators mentioned above [2,21]. Surprisingly, the level of an other UCP, the UCP3, decreased upon the effect of cold, and as expected started to return to normal after 24 h reacclimation in the BAT (Fig. 2). In the muscle the UCP3 protein did not change under the same conditions (Fig. 2). Others reported a three-fold increase of UCP3 protein in rodent skeletal muscle after 24 h cold exposure [17]. The explanation for the different results probably is that while in our condition the cold exposure lasted for 1 week, in their experiment [17] a 24 h cold was employed, when the shivering was prevailing. This explanation is consistent with the report that the expression of UCP3 mRNA in rat skeletal muscle increased after a short (6–24 h) but decreased after a 6 day cold exposure [24]. When the different conditions (neutral temperature, cold and reacclimation) were compared, the equal amounts of loaded proteins were ensured. Beside the careful measurement of proteins as described in Section 2, frataxin protein content was used as control in the immunoblotting.

In the further part of this work the mechanism of the opposite change of UCP1 and UCP3 proteins in BAT was to be investigated. First, the expressions of mRNA of the two genes were detected, however, both of them showed the same pattern, i.e. increased in cold and started to return normal during reacclimation (Fig. 3). Thus, the mRNA expression did not explain the difference in the protein levels of UCP1 and UCP3.

In an attempt to gain more insight into the mechanism of the reduced UCP3 protein expression in the BAT we detected its change within a 1 week cold period. The amount of expressed protein was reduced even by the sixth hour and then slowly increased (Fig. 4).

Comparing Figs. 2 and 3 we can see that the expressed mRNA and protein content of UCP1 changed in parallel. Considering the contrast between the change of UCP3 mRNA and UCP3 protein levels there are some possibilities. First, this observation is not a rare case [26], probably because the half-life times of the mRNA and its protein can be different. Second, we have to consider that two forms of UCP3, the short ($UCP3_S$) and the long ($UCP3_L$) types, exist [27]. Our radioactive probe for UCP3 mRNA recognized both forms. On the protein level, however, our antibody recognized only the UCP L. The investigations whether the ratio of the two forms changed during cold exposure are under way.

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