Browning of subcutaneous adipose tissue in wild-caught rodents of Siberia

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Abstract. The physiological role of uncoupling protein 1 in white fat depot is not yet fully understood. Analysis of adipose tissues of animals from natural populations may be an informative addition to the laboratory experiment in the study of this problem. The paper presents the first case of the detection of uncoupling protein 1 in inguinal white fat depot of short-tailed field voles (Microtus agrestis) and Korean field mice (Apodemus peninsulae). The animals were caught in West Sayan in the summer of 2015. Their inguinal fat was of non-typical dark-pink color with the brown colored inclusions, increased content of total protein in comparison with these parameters of laboratory mice. Immunoreactive UCP1 was identified in this white fat pad of about two-thirds of the animals. The interscapular brown fat weight, morphology, biochemical properties were typical. We assumed the inguinal fat with uncoupling protein 1 to be responsible for heating the low limb muscles and triggering their high activity at low summer night temperatures in West Sayan.

1. Introduction
Evolutionary emergence and development of animal homeothermy run parallel with the development of adipose tissues [1,2]. In the conditions of pronounced fluctuations in food supply white adipose tissue functions as the energy depot for the process of intensive metabolism. Brown adipose tissue protects animals from hypothermia by thermogenesis through uncoupling electron transport from ATP synthesis within mitochondria [3]. Accordingly, there are two types of fat cells - white and brown adipocytes. At present there is no doubt that the adipose tissue includes the third type of cells called beige or bright adipocytes [4, 5]. The shape, size, protein and lipid content of these cells represent intermediate characteristics between the values of morphological and biochemical parameters of typical white and brown adipocytes [6]. Their common feature with the brown adipocytes is the presence of mitochondrial uncoupling protein 1 (UCP1), which has never been detected in white adipocytes. At the same time, the origin and location of these UCP1-containing cells markedly differ from those of brown adipocytes [4]. In small laboratory mammals they are diffusely scattered in the subcutaneous and visceral white fat, while brown adipocytes have never been observed in these depots. The latter often form isolated pads of predominantly globular type [3]. In fact, the presence of UCP1 indicates the potential involvement of these cells in thermogenesis [5-7]. Therefore, both brown and beige cells are often included into a group of thermogenic adipocytes [8]. For this reason, the increase of their number or the content of UCP1 along with browning of the fat depots either in conditions of cold temperatures or beta-adrenergic stimulation is usually regarded as a manifestation of thermogenic activity of white fat [5,7].
Virtually all the information on the UCP1-containing cells in white fat depots was obtained in the experiments on laboratory animals. Until now it is not studied whether these cells are present in the white fat depots of free-living small mammals. Therefore, we cannot assume with certainty that the browning of white adipose tissue is a common biological phenomenon.

The aim of our research was to identify the uncoupling protein 1 in white fat depots of rodents from natural populations. The inguinal adipose tissue samples were isolated directly in the field conditions. The obtained results are the first to demonstrate the presence of UCP1-containing cells in subcutaneous white adipose tissue of short-tailed field voles (*Microtus agrestis*) and Korean field mice (*Apodemus peninsulae*).

2. Material and methods

2.1. Animals

Eight male short-tailed field voles (*Microtus agrestis*) and eight mature male Korean field mice (*Apodemus peninsulae*) were caught in Usinsk basin and adjacent slopes of Western Sayan (52°32′20″N, 93°17′68″ E), at an altitude of about 730 m a.s.l., in July 2019. To cause instant death of animals we have used a spring-loaded trap bars. Interscapular brown adipose tissue (IBAT) and inguinal white adipose tissue (IWAT) were removed carefully and homogenized in 10 mM Tris-HCl buffer with 1 mM EDTA, pH 7.2. A tissue-buffer ratio was 30-50 mg of IBAT and 60-100 mg of IWAT per 0.5 ml. The period from the moment of death to the isolation of adipose tissues did not exceed 5:00. The homogenates were kept and transported to the laboratory in liquid nitrogen.

The males of outbred mice ICR from Vector breeding center (Novosibirsk, Russia) were used for comparative studies. The animals were housed at 23±2 ºC with free access to water and standard chow pellets (BioPro, Russia). 14-week-old mice were killed by decapitation. The adipose tissue homogenates were prepared as described above. All the investigations were conducted in accordance with the European Convention for the protection of vertebrate animals (Strasbourg, 18 March 1986) and its updating.

2.2. Biochemical analysis

The protein content of tissue homogenates was determined by the modified Lowry method [9] with bovine serum albumin as standard. 80 μg of IWAT total protein or 30 μg of IBAT total protein were separated by electrophoresis on 12% SDS-polyacrylamide gel. Protein was transferred to nitrocellulose membrane (pore size 0.2 μm, Sigma Aldrich, USA) by a semidry method in 25 mM Tris-glycine buffer (pH 8.5) with 20% ethanol and 0.1% sodium dodecyl sulfate at 3 W for 30 min [9]. The membranes were blocked for one hour in 5% bovine serum albumin with Tris-buffered saline-0.5% Tween 20 (BSA/TBS-T) and then incubated overnight at 4°C with rabbit polyclonal antibodies to synthetic UCP1 peptide (Anti-UCP1(U6382) Sigma Aldrich, USA). These primary antibodies were detected after a 2-hour incubation of membranes with the goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Sigma Aldrich, USA). The primary and secondary antibodies were diluted 1:1000 in BSA/TBS-T. UCP1 bands were quantified using the GelAnalyzer 2010a software. The intensity of UCP1 bands was normalized to the content of loaded protein and expressed in arbitrary units per μg protein (AU/μg).

2.3. Data analysis

All the data are presented as group mean values ± SE. The statistical analysis was performed with Statistica 6.0 (StatSoft, Inc., 2003). Differences among the groups of animals were determined by one-way ANOVA followed by post hoc least significant difference (LSD test). The differences between the parameters of IBAT and IWAT within the same group of animals were estimated using Student’s t-test. P<0.05 was considered to be statistically significant.
3. Results and discussion

Interscapular brown adipose tissue was easily identified in all the animals. A layer of white fat over brown fat pads was observed only in the laboratory mice but not in wild-caught animals. There were no differences in wet mass of IBAT, the total protein contents and relative intensities of UCP1 band of IBAT samples among the studied groups of animals (P>0.05, table 1, figure 1). The inguinal and intra-abdominal white fat depots of wild-caught rodents were greatly reduced in comparison with the laboratory mice (P<0.05, table 1) and were of a dark pink color (figure 1).

|                      | Laboratory mice | *Apodemus peninsulae* | *Microtus agrestis* |
|----------------------|-----------------|-----------------------|---------------------|
|                      | *ICR*           | *peninsulae*          | *agrestis*          |
| Body mass, g         | 42.80±1.04 (10) | 27.57±3.3* (8)       | 39.08±4.57b (8)    |
| IBAT, mg             | 112.60±7.99 (10)| 104.40±10.08 (8)     | 124.40±20.28 (8)   |
| IBAT total protein, μg/mg | 64.06±5.71 (10)| 65.67±8.11 (8)       | 56.67±7.84 (8)     |
| IBAT UCP1, AU/μg of loaded protein | 89.28±9.21 (5) | 153.71±39.04 (5)     | 94.58±11.49 (5)    |
| IWAT, mg             | 320.40±31.84 (10)| 66.83±12.22* (8)     | 83.67±15.34* (8)   |
| IWAT total protein, μg/mg | 12.23±1.89* (10)| 56.00±6.55* (8)      | 28.01±4.65*ab (8)  |
| IWAT UCP1, AU/μg of loaded protein | 18.68±3.04* (4) | 21.27±2.88* (6)      | 29.45±5.86* (5)    |

Table 1. Thermogenic properties of interscapular brown adipose tissue (IBAT) and inguinal white adipose tissue (IWAT) of wild-caught and laboratory rodents.

Sample sizes are shown in parentheses. The data are mean ± SE. Different superscripts indicate significant differences: *The wild-caught rodents versus laboratory mice; **A. peninsulae versus M. agrestis (one-way ANOVA LSD test, p<0.05); * IWAT versus IBAT within the same group of animals (Student’s t-test, p<0.05).

The values for total protein in IWAT samples of short-tailed voles and Korean field mice were 2-5 times higher than those for the laboratory mice (P<0.05, Table 1). UCP1 band was revealed in these fat samples in six of the eight of Korean field mice and in five of the eight of short-tailed field voles (figure 1).

Figure 1. Adipose tissues of wild-caught small rodents *On the left* Morphology of adipose tissues of *Microtus agrestis* Adipose tissues are shown by arrows. IBAT – interscapular brown adipose tissue; IWAT – inguinal white adipose tissue and GWAT – gonadal white adipose tissue. *On the right* Western blot analysis of uncoupling protein 1 in adipose tissues. A stands for representative western blots of inguinal fat of *M. agrestis* (1), *A. peninsulae* (2) and western blots of interscapular brown fat of *M. agrestis* (3), *A. peninsulae* (4) and laboratory mice (5). B stands for representative western blots of inguinal fat of *M. agrestis* (1 and 2), *A. peninsulae* (3 and 4) and laboratory mice (5).
These results unambiguously indicate the presence of UCP1 containing adipocytes in IWAT in the major part of wild-caught animals. UCP1 was also detected in IWAT of about 40% of the laboratory mice despite the lack of the morphological features of browning of their fat depots. The average values of relative UCP1 band intensity were calculated except the samples without this band. The values of relative intensity UCP1 band in IWAT were not different between animal groups. They were 3-8 times lower than the corresponding values in the brown fat samples.

At present, there are only three papers on the identification of UCP1 in tissues different from the typical brown fat in small mammals from natural populations. One is devoted to a protoendothermic mammal the Lesser hedgehog tenrec (Echinops telfairi), a member of the phylogenetically ancient afrotherian fauna [10]. Two symmetrical globular brown fat-like structures were revealed in the proximity of reproductive organs. Morphological and functional parameters of this brown fat-like tissue, the content of UCP1 and beta 3-adrenergic receptor were similar to those of mice interscapular brown fat. This atypically located thermogenic fat of ancient mammals is considered to be responsible for the increase of the female body temperature to a level that is favorable for the developing fetus. In our study the darker color, the increased content of total protein and the presence of UCP1 in IWAT indicated the browning process; at the same time these parameters were significantly lower than those in IBAT.

Two other articles present data on the increased UCP1 expression in the visceral white fat of great roundleaf bat (Hipposideros armiger) [11] and pikas (Ochotona curzoniae) [12] under the influence of regular cold exposures in the laboratory. However, it should be pointed out that capturing, transporting and stressful laboratory conditions significantly affect the neuroendocrine status, in particular the sympathetic tone of wild animals [13]. It is known that the reaction of adipose tissue on stimuli may be qualitatively different in natural environment compared to the laboratory conditions. Generally speaking, these results demonstrate only the inducible nature of UCP1-positive cells. We first identified UCP1 in the samples of inguinal fat from animals in their natural habitat, and trapping procedure was performed so that the stressful factor has been completely eliminated. Thus it can be assumed that the UCP1-positive cells are a natural component of the subcutaneous inguinal fat in the majority of wild small mammals in summer.

The study of animals from natural populations may be interesting due to the ambiguity of the laboratory data and doubts in relation to the thermogenic functions of UCP1-positive cells in white fat depots [5,8]. In fact, the presence of UCP1 in inguinal fat of outbred laboratory mice housed at room temperature permanently seemed unexpected. It should be noted, however, UCP1 can function only along with a mitochondrial respiratory chain [7]. It is well known that the darker color of fat is due to the increased content of mitochondrial cytochromes [7]. Therefore, the browning of inguinal fat of wild rodents indicated high capacity of their mitochondrial respiratory chain and, consequently, higher thermogenic activity of UCP1 in comparison with that of laboratory rodents. Although fat samples were isolated from animals in summer the obtained data seem to be in agreement with the hypothesis of the thermogenic function [5] of these cells because the climate of mountainous regions of Central Siberia is characterized by low temperatures even in summer. In July daily temperatures were within the range of 15-28°C, with night temperatures changing from 5 to 10°C, i.e. diurnal temperature gradient ranged from 10 to 20°C. Under these summer conditions, the flexible mechanism of UCP1-dependent thermogenesis is as important as that in winter. The most important organ responsible for thermogenesis would be typical brown fat [3] which was found in all the caught rodents. However, the brown fat is located in the upper dorsal part of the body to provide heating of the open parts of the back and to maintain the temperature in the blood vessels [3]. The heat losses from the ventral body surface could have reduced due to sleeping position "of rolling up into a ball". But in summer the studied rodents are active at night, consequently UCP1-positive cells of inguinal fat could be responsible for the heating of the lower limb muscles triggering their high activity at low temperatures.

Unlike the typical brown fat, UCP1 was detected in many, but not all the samples of inguinal fat and this was true for the outbred laboratory mice and two wild rodent species. Further studies are required to investigate the mechanisms and the causes of the maintenance of this polymorphism in natural and
laboratory populations of rodents, the features of the adaptive strategies of animals without UCP1-positive adipocytes in inguinal fat. These problems are of great interest, due to the discovery of other potential thermogenic mechanisms, and new evidence for polymorphisms of the UCP1 expression in different human populations.

4. Conclusions
In conclusion, our research is the first case of detection of UCP1 in subcutaneous white fat of voles and mice of natural populations. The obtained results are consistent with the hypothesis of the thermogenic function of UCP1 positive cells in white fat. The study of adipose tissues of wildlife animals promotes understanding of their evolutionary functions.

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References
[1] Jastroch M, Oelkrug R and Keipert S 2018 J. Exp. Biology. 221 jeb169425
[2] Rosen E D and Spiegelman B M 2014 Cell 156 20-44
[3] Cannon B and Nedergaard J 2004 Physiol. Rev. 84 277-359
[4] Wu J, Cohen P and Spiegelman B M 2013 Genes Dev. 27 234-50
[5] Nedergaard J and Cannon B 2014 Cell Metab. 20 396-407
[6] Okamatsu-Ogura Y, Fukano K, Tsubota A, Uozumi A, Terao A, Kimura K and Saito M 2013 PLoS One 8 e84229
[7] Shabalina I G, Petrovich N, De Jong J, Kalinovich A, Cannon B and Nedergaard J 2013 Cell Reports 5 1196-203
[8] Medvedev L N and Elsukova E I 2015 J. Physiol. Biochem. 71 847-53
[9] Elsukova E I., Medvedev L N and Mizonova O V 2016 Bull. Exp. Biol. Med. 161 321-4
[10] Oelkrug R., Goetze N, Exner C, Lee Y, Ganjam G K, Kutschke M, Muller S, Stohr S, Tscho M H, Crichton P G, Heldmaier G, Jastroch M and Meyer C 2013 Nat. Commun. 4 2140
[11] Wang Y, Zhu T, Ke S, Fang N, Irwin D, Lei M, Zhang J, Shi H, Zhang S and Wang Z 2014 Plos One. 9 e112495
[12] Bai Z, Wuren T, Liu S, Han S, Chen L, McClain D and Ge R L 2015 Comp. Biochem. Physiol. 184 171-8
[13] Fisher F M and Maratos-Flier E 2013 Nat. Med. 630 17-8