Change in Ucp1 mRNA Expression Following Long-Term Cold Exposure under Normal or High-Fat Diet Regimes in the Cold-Intolerant Mammal, Suncus murinus

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Abstract: The house musk shrew (Suncus murinus), or suncus, is a unique experimental mammal that is cold intolerant. However, even basic knowledge of brown adipose tissue (BAT), which is important for non-shivering thermogenesis (NST), is minimal. Therefore, we exposed suncus for 18 days to mild cold temperatures (8–14°C) and/or a high-fat diet, which are factors that increase NST, and measured two mRNAs that are critical for NST in BAT, uncoupling protein 1 (Ucp1) and type II 5'-deiodinase (D2). Neither mild cold exposure nor a high-fat diet alone induced up-regulation of the mRNAs. However, combinations of cold exposure and high-fat diet significantly increased both mRNAs. Therefore, cold intolerance in suncus may be partly caused by dietary components.

Key words: brown adipose tissue, cold intolerance, uncoupling protein 1

Brown adipose tissue (BAT) clearly differs from white adipose tissue (WAT) and is the most important tissue for non-shivering thermogenesis (NST) in mammals. BAT plays a role in adaptation to cold environments, diet-induced thermogenesis, and arousal from hypothermia in hibernation and daily torpor (reviewed in [4, 12]). In addition, BAT is involved in regulating total energy homeostasis and body weight. For example, some strains of mice and rats with genetic obesity mutations decrease the thermogenic activity in BAT by suppressing the sympathetic nervous system via direct noradrenergic innervation of BAT (reviewed in [12]). Brown adipocyte is found in adult humans, and thermogenesis in BAT is related to obesity and diabetes [6, 10, 11, 13, 26, 33]. Therefore, by consuming extra calories, the up-regulation of thermogenic activity in BAT is important for combating obesity and diabetes in humans.

The house musk shrew (Suncus murinus), or suncus, is a unique experimental animal belonging to the Insectivora [17]. We recently discovered that suncus are cold intolerant compared to C57BL/6J mice [22], and one reason for this may be the low thermogenic activity of uncoupling protein 1 (UCP1), which is one of the most important proteins for non-shivering thermogenesis in BAT [23]. Interestingly, suncus resemble Ucp1-ablated mice [8], not only in their cold intolerance, but also in the histology of BAT and their
resistance to obesity. Our laboratory has maintained several strains of suncus established from different countries for over 20 years, and over 300 individuals are bred every several years. However, old suncus with abundant gut fat have not been observed to date, whereas C57BL/6J mice typically accumulate fat with age. These observations in both animals have been confirmed by other researchers [30]. Moreover, in a pilot study, when adult male suncus were fed a cafeteria diet of high-calorie palatable foods (candy, potato chips, and chocolate) for about 1 month, their body weight did not change. It is of interest to understand this combination of low thermogenic activity and resistance to fat accumulation, and studies of this discrepancy may provide information for understanding human obesity. However, knowledge of NST in suncus is minimal; for example, it is unknown why suncus are intolerant to cold and what mechanism regulates \( \text{UCP1} \) in this species.

To examine potential reasons for cold intolerance and to investigate the regulating mechanisms of \( \text{UCP1} \) mRNA in suncus, we exposed suncus to mildly cold conditions and/or a high-fat diet. We then compared mRNA levels using Northern blot analysis because cold exposure and a high-calorie (carbohydrate and fat) diet are factors that bolster NST by activating \( \text{UCP1} \) mRNA expression which in turn activates the sympathetic nervous system via noradrenergic innervation of BAT [9, 14, 16, 20, 24, 25, 27, 31, 32]. In addition, we measured changes in mRNA of type II 5’-deiodinase (\( \text{D2} \)) in BAT, which is a critical enzyme that supplies local triiodothyronine (\( \text{T3} \)) catalyzed from thyroxine (\( \text{T4} \)) and rapidly increases upon cold exposure through the activation of the sympathetic nervous system via noradrenergic innervation of BAT [7, 9, 14, 16], because local \( \text{T3} \) production is an effective factor for increasing \( \text{UCP1} \) in BAT [2, 3].

We used adult male suncus (body mass; 80–120 g) of an outbred KAT strain established from a wild suncus population in Nepal-Katmandu [18]. Suncus were bred and maintained through a partnership with the Laboratory of Animal Management and Resources, Graduate School of Bio-Agricultural Science, Nagoya University, Japan, and the Research Institute of Environmental Medicine, Nagoya University. This experiment was performed under the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. For this research, we used two types of chow: commercial trout pellets (Nippon Formula Feed Manufacturing, Yokohama, Japan) under standard breeding and housing conditions for the low-fat diet, and Marshall Premium Ferret Diet (Marshall Pet Products, Wolcott, NY) for the high-fat diet. The nutritional compositions are shown in Table 1. In addition, we prepared two condition rooms, a standard room (25–27°C, 30–60% humidity, 12L:12D light cycle [photoperiod: 08:00–20:00]) as the warm-acclimation room and a room with no heaters in winter (range: 8.5–14.6°C [>12°C during the daytime], no regulation of humidity, and natural daylight) as the cold-acclimation room.

We used 3 to 4-month-old suncus for the experiments and divided them into four groups: 1) warm-acclimated with low-fat diet (WALF, \( n=3 \)); 2) warm-acclimated with high-fat diet (WAHF, \( n=3 \)); 3) cold-acclimated with low-fat diet (CALF, \( n=3 \)); and 4) cold-acclimated with high-fat diet (CAHF, \( n=4 \)). The time of each treatment is shown in Fig. 1. The cold exposure was performed from 7 to 18 December 2002 in Nagoya, Japan (35.1° N latitude).

Following the treatments, suncus were sacrificed by cardiac exsanguination under ether anesthesia, and blood sugar levels were measured using a Medisafe Reader GR-101 (Terumo, Tokyo, Japan). BAT was immediately dissected from the interscapular regions, washed in 1× PBS, minced into small pieces, frozen in liquid nitrogen, and kept at −80°C until use. Total RNA was extracted using the acid guanidium thiocyanate–phenol–chloroform extraction (AGPC) method [5]. The total RNA of each BAT (15 \( \mu \text{g} \)) was fractionated on 0.8% agarose gels and transferred onto GeneScreen Plus Membrane (PerkinElmer Life Sciences, Wellesley, MA, USA). The same cDNA fragment that was previously used as the probe for \( \text{UCP1} \) [23] was also used in this study. As the probe for \( \text{D2}, \text{D2} \) cDNA fragment A [21] inserted into pGEM-T Easy vector I (Promega) was digested with \( \text{EcoRI} \) in a multiple cloning region and recovered using TaKaRa RECOCHIP. These fragments were labeled with \( \text{[32P]-dCTP} \) (PerkinElmer Life Sciences) using the Random Primer DNA Labeling Kit Ver. 2 (TaKaRa). The membrane was prehybridized in 5× SSCP containing 1% SDS, 10× Denhardt’s solution, 50% formamide, and 50 mg of fish sperm DNA (Roche Diagnostics, Mannheim, Germany) at 42°C over
Ucp1 EXPRESSION IN SUNCUS WITH COLD AND DIET

The membrane was then labeled with the radiolabeled probe (10^6 cpm/mL) in prehybridization buffer at 42°C overnight. After washing (twice in 2 × SSC for 5 min; once in 2 × SSC containing 1% SDS at 65°C for 20 min; twice in 0.1 × SSC for 5 min), the membrane was exposed to a Molecular Imaging Screen-CS (Bio-Rad Laboratories, Hercules, CA) and analyzed using a Molecular Image System (GS-363; Bio-Rad). The hybridized membranes were rehybridized with a rat 18S ribosomal RNA (rRNA) radioisotope probe.

Throughout the experiment, we observed no severe problems, such as immobilization or death, and all cold-exposed animals were resistant to cold, which contrasted with our previous data of exposure of suncus to 6–8°C [22]. A cold environment of >8.5°C was therefore not considered to represent severe conditions for suncus. We conclude that to evaluate the physiological changes in cold-exposed suncus without heavy stress, temperature conditions should be no lower than 9°C.

Our experiment adequately confirmed physiological differences between suncus that consumed a low-fat diet and those on a high-fat diet, even though the cold condition was mild. At the start of cold exposure, suncus were somewhat inactive and were seldom observed outside of the nest box until about 5 days into the experiment. However, after 1 week, they often left the nest box, and motor activity resembled that of suncus housed in the warm room. The woodchip bedding was not very soiled in the first week, but became dirtier after 1 week, suggesting increased motor activity. This implies that adaptation to cold in suncus takes about 1 week. However, on the last day of cold exposure, individuals in the CAHF treatment appeared to be more active than those in the CALF treatment, indicating that NST in CALF may have been lower than in CAHF. Compared to our previous experiment [22], the cold exposure conditions were mild. Nevertheless, we observed a tendency for decreasing body weight with cold exposure, although it was not significant (Fig. 2). Blood glucose levels also did not differ significantly, although they tended to decrease on the high-fat diet (Fig. 3).

Figure 4 shows the difference in the mRNA expression levels of Ucp1 and D2. Levels of both mRNAs did not differ between Walf and CALF, whereas the levels were significantly higher in CAHF than in Walf and CALF. In addition, levels of both mRNAs were significantly correlated (Fig. 5, r^2=0.676, P<0.001). In cold-exposed mice and rats, Ucp1 and D2 mRNA in BAT is increased by a shared mechanism, activation of the sympathetic nervous system via direct noradrenergic stimulation [7, 15]. In addition, T3 supplied from D2 synergistically induces mRNA expression of Ucp1 in BAT [29]. Perhaps these mechanisms of direct and/or indirect regulation of Ucp1 are common in suncus. However, we found that mRNA was increased by cold exposure only in the high-fat diet treatment (CAHF). A high-calorie diet is a factor that activates Ucp1 mRNA expression [9, 14, 16, 24, 25, 30, 31]. Even when the period of high-fat diet was shorter, Ucp1 mRNA did not increase in the high-fat diet condition alone (WAHF) in contrast to the high-fat diet condition with cold. In addition, D2 mRNA, which is increased by a mechanism common to Ucp1 [7, 16], did not increase significantly in WAHF. This may have been because the level of fat used in the high-fat group was relatively

Table 1. Components of commercial trout pellets (low-fat diet) and Marshall Premium Ferret Diet (high-fat diet)

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<th>crude protein (more than)</th>
<th>crude fat (more than)</th>
<th>crude fiber (less than)</th>
<th>crude ash (less than)</th>
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<tr>
<td>low-fat</td>
<td>45.0%</td>
<td>3.5%</td>
<td>3.0%</td>
<td>13.0%</td>
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<tr>
<td>high-fat</td>
<td>38.0%</td>
<td>18.0%</td>
<td>3.5%</td>
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Fig. 1. Time periods of warm and cold conditions and low- and high-fat diets for each experimental group of suncus. The lines on the left and right indicate start and end dates, respectively. Numbers in the boxes represent the number of days in each experimental condition.

3 h. The membrane was then labeled with the radiolabeled probe (10^6 cpm/mL) in prehybridization buffer at 42°C overnight. After washing (twice in 2 × SSC for 5 min; once in 2 × SSC containing 1% SDS at 65°C for 20 min; twice in 0.1 × SSC for 5 min), the membrane was exposed to a Molecular Imaging Screen-CS (Bio-Rad Laboratories, Hercules, CA) and analyzed using a Molecular Image System (GS-363; Bio-Rad). The hybridized membranes were rehybridized with a rat 18S ribosomal RNA (rRNA) radioisotope probe.

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low, weakening the effect of increasing mRNA by a high-fat diet in the warm condition, or because the an-
imals did not like the high-fat chow (ferret food), i.e.,
diet-induced thermogenesis via direct noradrenergic
stimulation of BAT was not induced by overfeeding [1, 19].
For example, both mRNAs increased more in WAHF than in WALF and CALF, although the differ-
ence was not significant. In addition, the experimental
period may have been too long. von Praun et al. [28]
reported that in hamsters, the level of Ucp1 mRNA
decreased to about 200% after 7 days of cold exposure,
even after its expression had increased to about 400%
after 2 days of cold exposure. In our previous study,
most immobile and dead suncus were observed until 1
week after the initial cold exposure [22]. Therefore, to
elucidate the relationship between cold intolerance and
Ucp1 regulation in BAT, increased mRNA levels in-
duced by cold exposure should be investigated over a
shorter time, i.e., 1 h to several days. It was unclear
why Ucp1 mRNA did not increase on a high-fat diet
alone, but it is evident that thermogenic activity in
suncus is affected by diet components, which may be a
**Fig. 5.** Significant positive correlation between expression levels of *Ucp1* and *D2* in suncus (*r²=0.676, *P*<0.001).

reason for the cold intolerance of this species.

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**References**