When mammals are exposed to cold or exercise, heat production occurs because of the change of nutrient catabolism. The respiratory quotient (RQ) indicates the changes of catabolism because the oxidation of fat, carbohydrates, and protein has characteristic RQ values [1–3]. The RQ of animals fed a mixed diet at room temperature is about 0.8 [4, 5], and the combustion of pure carbohydrates and pure lipids results in RQs of 1.0 and 0.7, respectively [6]. It was reported that when animals became acclimated to cold, the RQ value decreased to about 0.7, and the thermogenesis occurred accompanying fat combustion [7]. Brown adipose tissue that contains a large amount of mitochondria and lipid droplets may be a major metabolic site of the thermogenesis in small animals such as rats [8].

Genetically obese Zucker (fa/fa) rats have been shown to have several metabolic defects involving the apparent inability to burn off calories as heat [9, 10]. This thermogenic defect is shown early in life as a reduction in body temperature, in cold tolerance [11, 12], and in metabolic rate [13]. Although some authors claim that lean and obese Zucker rats are equally able to endure cold stress [14–16], most claim that the obese Zucker rats are less resistant to cold [17, 18]. Some researchers have indicated that obese Zucker rats exposed to a cold envi-

### Ion Transport and Morphological Changes of Mitochondria in Brown Adipocytes of Warm- and Cold-Acclimatized Obese Zucker Rats

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**Abstract:** Brown adipose tissue plays the dominant role in response to cold acclimatization through its capacity to produce heat. To demonstrate the cellular function for thermogenesis induced by cold acclimation in the brown adipose tissue of obese Zucker rats, we examined the changes for the area as well as the Na, K, Cl, and Ca concentrations in the mitochondria of brown adipocytes after the warm (25°C, WG) and the cold acclimations (10°C, CG). Moreover, the respiratory quotients (RQs) of these rats were measured. After the acclimations, the RQ in the CG was decreased and the oxygen consumption increased. A morphometric analysis of electron micrographs of brown adipocytes from the two groups of rats showed a marked increase in the area of the mitochondria in the CG. An electron probe X-ray microanalysis showed an increase in the Ca concentration and decreases in the Na and K concentrations in the matrix of the mitochondria of the cells in the CG. These results suggest that the reduction in the RQ of obese Zucker rats acclimated to cold is the consequence of the metabolism of a large quantity of lipid in the brown adipocytes. Our data also indicate that the observed change in the mitochondrial area and the increase for Ca in the mitochondria were associated with the cold-induced thermogenesis in brown adipocytes of obese Zucker rats. [Japanese Journal of Physiology, 51, 531–537, 2001]

**Key words:** obese Zucker rats, cold acclimation, brown adipocytes, mitochondria, X-ray microanalysis.

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environment have lower norepinephrine-stimulated non-shivering thermogenesis than lean Zucker rats and that the rate of lipolysis and the ability to use free fatty acids are more suppressed than those of the lean Zucker rats. Moreover, the binding of purine nucleotides to the uncoupler protein (UCP) in the mitochondria of obese Zucker rats is weaker than that of the lean Zucker rats [19]. However, these defects of brown adipose tissue metabolism in obese rats can be improved by acclimation to mild cold [17].

To investigate the contribution of cold acclimation to the development of mitochondrial thermogenic function, we measured the RQ and the oxygen consumption and the changes in the area and the elemental concentrations of mitochondria in interscapular brown adipocytes of warm- and cold-acclimated obese Zucker rats.

**MATERIALS AND METHODS**

**Animal care.** Sixteen obese male Zucker rats were used according to the Guiding Principles in the Care and Use of Animals approved by the Council of the Physiological Society of Japan. They were seven weeks old at the start of the experiment and were divided into a warm-acclimated group (WG, n=8) and a mildly cold-acclimated group (CG, n=8). The rats were housed in cages at 25°C for the WG and at 10°C for the CG under lights for 12 h a day. The animals were fed Purina laboratory chow and water ad libitum for 10 weeks.

**Respiratory quotient.** The rats were tested in a 6-l cylindrical plastic chamber through which air was drawn at 1,500 ml/min. O₂ and CO₂ contents of the expired air were measured with an O₂ analyzer and a CO₂ analyzer (Oxygen and Carbon Dioxide Analyzer, Osaka Microsystem Inc., Osaka, Japan). Water was absorbed by silica gel and CO₂ with soda lime (Wako, Osaka, Japan). The chamber was maintained at 25±1°C for WG and at 10±1°C for CG.

The RQs of the animals of the two groups were tested at 25±1°C before acclimation. After an acclimation period of 3, 6, or 10 weeks, the RQs of the animals were tested at 25±1°C for WG or 10±1°C for CG. Every RQ measurement was performed from 1 to 15 min. The values are expressed as means±SD. The statistical significance of values was assessed by a Student’s t-test for the comparison between warm- and cold-acclimated rats. The multiple comparison technique (variant analysis: ANOVA) was performed for comparison of the data of different acclimation periods. p<0.05 was considered to be statistically significant.

After measuring the RQ, the rats were anesthetized with pentobarbital sodium, and small pieces of interscapular brown adipose tissue were removed.

**X-ray microanalysis and conventional electron microscopy.** For X-ray microanalysis, small pieces of brown adipose tissue were rapidly frozen in liquid nitrogen by pressing them against a copper block that had been precooled in liquid nitrogen. Cryosections (70 nm thick) were cut at −150°C and mounted on gold grids. The sections were freeze-dried at −120°C overnight on a freezing dryer, then carbon coated. The elemental concentrations were determined by X-ray microanalysis and data processing [20]. The freeze-dried sections were observed by using a Hitachi H-7100 electron microscope (Hitachi Corp., Tokyo, Japan) with an energy dispersive X-ray detector (Kevex Corp., Burlingame, CA, USA) and a microanalyzer system (Horiba Corp., Kyoto, Japan). Frozen hydrated sections of brown adipose tissues were also mounted on grids, then immediately set in cooling specimen holders attached to an electron microscope for estimating the dry mass fraction. The elemental concentrations (mmol/kg dry weight) of mitochondria of brown adipocytes were originally determined by using the peak/continuum ratio of the X-ray spectra from the freeze-dried sections and converted to the values (mmol/kg wet weight) by the calculation using the dry mass fraction [21]. The dry mass fractions of mitochondria matrix were estimated to be 31±3.7 and 35±4.5% in brown adipocytes of WG and CG, respectively (mean±SD, n=6). For conventional electron microscopy, small pieces of brown adipose tissue were fixed successively in glutaraldehyde and osmium, dehydrated in ethanol, and embedded in araldite. Block staining of specimens was done by using uranyl acetate. The specimens were cut 1 μm thick and examined under a Hitachi-H7100 electron microscope at an accelerating voltage of 100 kV.

**Morphometric analysis.** The areas of mitochondria of brown adipocytes in conventional electron micrographs were measured by using a computer system (Power Mac 9600: NIH Image Ver 1.61) equipped with a scanner (GT 9500, Epson, Tokyo).

**Statistical analysis.** The values are expressed as means±SD. The statistical significance of values was assessed by a Student’s t-test for the comparison between warm- and cold-acclimated rats. The multiple comparison technique (variant analysis: ANOVA) was performed for comparison of the data of different acclimation periods. p<0.05 was considered to be statistically significant.
RESULTS

Body weight
Before the temperature acclimation, the body weights of the WG and the CG were not significantly different (284±20 and 299±31 g, respectively). The body weights of the two groups were not significantly different after 3 weeks of acclimation (452±43 for the WG and 426±27 g for the CG) or after 6 weeks of acclimation (524±52 for the WG and 504±26 g for the CG). After 10 weeks of acclimation, the body weights in the WG and the CG were significantly different (643±74 and 576±36 g, respectively). In variant analyses, the values of different acclimation periods for both groups were significant (F=69.04 in the WG and F=125.07 in the CG).

Oxygen consumption and RQ
Before temperature acclimation, the oxygen consumption was not significantly different in the WG and the CG (6.40±0.6 and 7.31±1.1 ml/min, respectively). After the acclimation of 3 weeks, the oxygen consumption of the CG was significantly greater than that of the WG. The oxygen consumption was also significantly different between the two groups after 6 and 10 weeks of acclimation. In variant analyses, the values of different acclimation periods for both groups were significant (F=23.73 in the WG and F=45.77 in the CG).

When the oxygen consumption was expressed in terms of oxygen consumption per body mass^{0.67} per minute, the differences between the two groups were also significant after 3, 6, and 10 weeks of acclimation. Oxygen consumption per body mass^{0.67} per minute of the CG was increased from 0.163±0.019 ml/g/min before acclimation to 0.203±0.016 ml/g/min after 3 weeks of acclimation, and thereafter up to 0.215±0.023 ml/g/min after 10 weeks. The oxygen consumption in the WG decreased slightly from 0.149±0.016 ml/g/min before acclimation to 0.135±0.018 ml/g/min after 10 weeks of acclimation (Fig. 1). In variant analyses, the values of different acclimation periods for the CG were significant, and the values for the WG were not significant (F=11.39 in the CG, p<0.05).

Fig. 1. Changes in the O_2 consumption/body mass^{0.67} min during acclimation. Data are expressed as means±SD (n=8), * mean is different (p<0.05) from that of the warm group (WG). In variant analyses, the values of different acclimation periods for the cold group (CG) were significant, and the values for the WG were not significant (F=11.39 in the CG, p<0.05).

Before acclimation, the RQs of the WG and the CG were not significantly different (0.92±0.03 and 0.90±0.03, respectively). After an acclimation of 3 weeks, the RQs of the WG and the CG were significantly different (0.84±0.03 and 0.71±0.01, respectively). The RQs were also significantly different between the two groups after 6 weeks and 10 weeks of acclimation. The RQ of the CG decreased from 0 weeks to 3 weeks and was then stable from 6 weeks (0.73±0.04) to 10 weeks (0.73±0.01) of acclimation (Fig. 2). In variant analyses, the values of different acclimation periods for both groups were significant (F=19.98 in the WG and F=104.86 in the CG).

In variant analyses, the values of different acclimation periods for the CG were significant, and the values for the WG were not significant (F=11.39 in the CG, p<0.05).

Fig. 2. Changes in the respiratory quotient during acclimation. n=8. Data are expressed as means±SD (n=8), and * mean is different (p<0.05) from that of the warm group. In variant analysis, the values of different acclimation periods for both groups were significant (F=19.98 in the WG and F=104.86 in the CG, p<0.05).

We investigated the RQs of warm- and cold-acclimated obese Zucker rat and confirmed earlier reports that exposure to cold markedly reduced the RQ of obese Zucker rats. We also confirmed that these reductions in RQ are the consequence of the utilization of a larger proportion of lipid combustion in the cold [22–24].

The level of O_2 consumption in the CG was also found to be higher than in the WG in this study, and the RQ and O_2 consumption measurements suggest that cold acclimation activates the mitochondria respiratory function and lipid catabolism in the brown adipocytes of obese Zucker rats.
Morphometric measurement of mitochondria of brown adipocytes

Figure 3 shows conventional electron microscopic images of the WG and the CG brown adipocytes from obese Zucker rats. We observed nucleus, mitochondria, and a few large lipid droplets in the WG brown adipocytes, whereas many lipid droplets were observed in the cytoplasm, and most of the rest of the cytoplasm was occupied by mitochondria in the CG brown adipocytes. The mitochondria of the brown adipocytes were small and electron-dense, and few cristae were identified in the WG. However, mitochondria swelling and the development of cristae (arrows) were observed in the brown adipocytes of the CG, and these mitochondria structures were comparable to those observed in brown adipocytes of normal rats in disuse or in cold exposure [25, 26]. The mitochondria area in brown adipocytes from the CG was significantly larger than the same area in brown adipocytes from the WG (Fig. 4).

Mitochondria elemental concentrations of brown adipocytes

The energy dispersive X-ray spectra of mitochondria of brown adipocytes from the WG (A) and the CG (B) are shown in Fig. 5. The representative spectra from the mitochondria reveal high P and K peaks and peaks of Na, S, and Ca. Figure 6 shows that the Na and K concentrations in mitochondria of brown adipocytes from the CG were significantly lower than those from the WG, and the Ca concentration of brown adipocytes from the CG was significantly lower than that of the WG. The value for the WG is expressed as 100%. Ten measurements were performed for each animal. The means of eight animals were calculated. Data are expressed as mean of means±SD (n=8), and * mean is different (p<0.05) from that of the WG.
higher than that from the WG. The concentrations of Mg, P, S, and Cl were not significantly different between the two groups.

**DISCUSSION**

Our finding was that the acclimation of obese Zucker rats to cold resulted in an increase of the mitochondria area in brown adipocytes. Moreover, the Na and K concentrations decreased, and the Ca concentration increased in mitochondria in the CG determined by X-ray microanalysis, as in the cases of norepinephrine-stimulated cultured brown adipocytes, in which the cytoplasmic Ca increased [27].

**Morphological change of mitochondria and its physiological meaning.** It has been reported that the mitochondria of obese Zucker rats reveal a remarkable variety of forms and are generally smaller than those of lean Zucker rats, suggesting that dysfunction in mitochondria is a characterized feature associated with the defective brown adipose tissue thermogenesis [19]. We observed that the mitochondria, which have well developed cristae, were round and large in the brown adipocytes of the CG, and the data are consistent with the study demonstrating that the shapes of mitochondria become round and large and that the improvement of the thermogenic function occurred during the acclimation to mild cold by the obese mouse [28].

Some studies have revealed that norepinephrine (NE) activated lipolysis and rapidly increased the synthesis of UCP-1 in the mitochondria of brown adipocytes [29], and that the stimulation of NE and/or ATP induced thermogenesis and a Ca$^{2+}$ increase in the brown adipocytes [30]. Furthermore, these stimulants trigger the Ca$^{2+}$ entry via $\alpha$ receptor and cause inositol 1,4,5-trisphosphate (InsP$_3$) production, consequently releasing Ca$^{2+}$ from the intracellular Ca store [31]. Furthermore, it has demonstrated that biogenesis of thermogenic mitochondria increased a mitochondrial mass and the capacity for the synthesis of mitochondrial proteins in the brown adipocytes of rodents in cold exposure [32]. Purine nucleotide, GDP (guanosine 5'-diphosphate) bind UCP1 and inhibit proton conductance in vitro; thus a method was developed to measure GDP binding to UCP1 from mitochondria, and it has been reported that GDP binding is rapidly increased in response to acute cold exposure [33]. On the other hand, in obese mouse there was no cold-induced increase in GDP binding to the mitochondria of brown adipocytes in response to acute cold exposure; nevertheless the adaptive response for cold acclimation appears to be relatively normal [28]. Thus an increase of mitochondrial area in response to the acclimation to 10°C in this study is dependent on normal adaptation for cold exposure in brown adipocytes of Zucker rats.

**The mechanism of mitochondria calcium rising in cold-acclimated adipocytes.** The Ca$^{2+}$ increase in mitochondria in the CG measured by microanalysis suggest that NE released from the sympathetic nerve stimulates the brown adipocytes to bring on the sustained intracellular Ca$^{2+}$ increase, which is thought to be an influx from extracellular space. Then the Ca$^{2+}$ causes an expression of UCP genes, followed by uptake into an intracellular Ca$^{2+}$ store, the matrix of mitochondria in the cold acclimated rats. It is reported that ATP causes the Ca$^{2+}$ release from the intracellular Ca$^{2+}$ store, and the store-operated Ca$^{2+}$ entry is inhibited simultaneously by the same transmitter in the cold-acclimated brown adipocyte [31].

Recently, studies have revealed that a time delay exists between the onsets of cytosolic and mitochondria Ca$^{2+}$ signals, and the Ca$^{2+}$ uptake into mitochondria with ACh stimulation is inhibited with ruthenium red in pancreatic acinar cells. Moreover, there exists a ruthenium red sensitive Ca$^{2+}$ uniporter and/or a gonadal steroid sensitive Na$^+$–Ca$^{2+}$ exchange on the mi-
mitochondria membrane of brain synaptosome [34, 35]. These reports could be interpreted as supporting the present microprobe data, showing that an increase of Ca and a decrease of Na in the mitochondria of brown adipocytes are induced by cold acclimation in Zucker rats.

Nonshivering thermogenesis is now known to occur primarily in brown adipose tissue. Namely, norepinephrine, the concentration of which is elevated by the cold acclimation, binds to the β-receptor on brown adipocytes, thus triggering a sequence of events leading to an increase in brown adipose tissue thermogenesis. Receptor stimulation is linked to the activation of adenylate cyclase, which produces cAMP, and this signal transduction leads to the phosphorylation and activation of an intracellular lipase [10, 36]. Free fatty acids liberated as a result of this process is transferred across the mitochondria membrane, where they are the substrate for oxidative metabolism. They also appear to uncouple this metabolic process so that the primary result is thermogenesis and not the production of ATP [10]. UCP and the enzymes of the electron transfer system in mitochondrial membrane may be induced by the increase of cytoplasmic Ca++ [37]. Increases of β oxidation of lipid droplets, water production, and H+ gradient generation by the electron transfer system in mitochondrial membrane also occur during cold exposure. It is assumed that heat is produced in the brown adipose tissues by UCP, using the driving force of the H+ gradient of the mitochondria inner membrane. The large quantity of heat produced in mitochondria of brown adipose tissues of the cold-acclimated rats is effectively conducted to the blood by the radiator function of swelling mitochondria, which have high water content [23, 38].

The ability of cold acclimation of obese Zucker rats. It is reported that the acclimation of ob/ob mice to mild cold, known to improve their cold resistance, induced normal tissue growth, mitochondrial proliferation and alterations for mitochondrial shape, and binding of purine nucleotides. An increase in the proportion of UCP in the mitochondrial inner membrane and normal mitochondrial ultrastructure are also determined [28]. The morphological changes in mitochondria observed in the present experiments may be associated with these changes of mitochondrial composition and cold tolerance.

The control mechanism for these adaptive changes is not well understood. Stimulation by norepinephrine appears to be, at least in part, responsible for tissue growth. However, norepinephrine does not seem to be responsible for the change in mitochondrial composition, such as the induction of UCP in the inner membrane [39]. The control mechanism for the adaptive changes appears to be normal in the obese Zucker rats, but the nature of the stimulus for the change in mitochondrial composition remains unknown.

There is a report that a defect in brown adipocytes mitochondria has a relationship to the defect in nonshivering thermogenesis in obese Zucker rats and thus brings on the obesity in this animal [28]. However, the results of this study suggest that the acclimation of obese Zucker rats to cold induces alteration of the mitochondrial area and a normal ion transfer of the mitochondria of cold-acclimated brown adipocytes. These changes may be associated with the improvement of thermogenic capacity of the mitochondria in cold-acclimated obese Zucker rats.

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Ion Transport in Brown Adipocytes of Rats


