Alterations of Insulin Content and Insulin Binding to Plasma Membranes in Rat Brown Adipose Tissue during Cold Exposure and Cold Acclimation

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Abstract We examined the insulin content, insulin binding to plasma membranes, and *in vitro* oxygen consumption of brown adipose tissue (BAT) from warm-acclimated (25°C), acute cold-exposed (5°C, 24 h: CE), and cold-acclimated (5°C, 4 weeks: CA) rats. Plasma insulin level was significantly lower in CE rats, but it did not differ between CA rats and warm controls. Insulin content per mg BAT weight from CE and CA rats were about three-times higher than those from controls. ¹²⁵I-insulin binding to plasma membranes of BAT from CE rats was higher and that from CA rats was lower than that from respective warm controls. Scatchard plots analysis showed that the number of insulin binding sites per mg protein of BAT plasma membranes from CE rats increased by 100%, while that from CA rats decreased by 35% without any changes of affinity constant. Basal oxygen consumption of BAT from CE rats was higher than warm controls, and suppressed by insulin. Insulin did not affect basal oxygen consumption of BAT from CA and warm control rats. Noradrenaline-induced oxygen consumption of BAT from warm controls and CE rats was suppressed by insulin. These findings suggested that insulin was involved in the regulation of BAT thermogenesis by changing insulin status in BAT (content and binding to plasma membranes) during cold exposure and cold acclimation.

Key words: brown adipose tissue, cold exposure, insulin, insulin receptor, insulin content.

Brown adipose tissue (BAT) is a major organ which carries out an enhanced nonshivering thermogenesis during cold acclimation [1] and overfeeding [2]. Its physiological function is to produce heat by burning fat, regulated mainly by sympathetic noradrenaline (NA) and several hormonal factors such as adrenaline, adrenal corticoids, thyroxine, and glucagon [1, 3–5]. These factors enhance heat production in BAT by activation of lipolysis directly or in a permissive way. In

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contrast with these lipolytic factors, insulin acts as a powerful anti-lipolytic as well as lipogenic hormone. Insulin enhances glucose uptake in BAT, and glucose serves as a thermogenic and lipogenic substrate in BAT [6, 7]. Therefore, it should be stressed that insulin would also be an essential factor to support high thermogenic capacity of BAT. BAT thermogenic capacity as well as cold tolerance in mice rendered diabetic with streptozotocin was reduced and recovered with insulin treatment. Such failure in BAT function in the diabetic animals may be mediated by atrophy of the tissue, reduced capacity for fatty acid oxidation, and decreased mitochondrial uncoupling protein, a key factor in BAT thermogenesis [8, 9]. Nedergaard and Lindberg [10] demonstrated that insulin inhibited the rate of oxygen consumption of hamster brown adipocytes stimulated by NA, whereas Howland and Bond [11] reported that insulin enhanced oxygen consumption of rat brown adipocytes. Tanti *et al.* [12] identified that insulin receptors were present in BAT and the number of insulin receptors decreased in obese mice. Previous report showed that the insulin content of BAT from cold-acclimated rats markedly increased as compared with that from warm controls [13]. These results suggested that insulin had direct and indirect (possibly through an interaction with the sympathetic NA [9]) effects on BAT function. In an attempt to clarify the role of insulin on BAT thermogenesis, we investigated in the present study changes in plasma insulin level, insulin content, insulin binding to plasma membranes, and the effect of insulin on *in vitro* oxygen consumption of BAT from acute cold-exposed and cold-acclimated rats.

MATERIALS AND METHODS

Animals. Male rats of Wistar strain, weighing about 200 g (aged 8 weeks), were used for this study. They were initially housed at $25 \pm 1^\circ\text{C}$ under artificial lighting from 7:00 to 19:00 and fed a standard commercial chow (Orient MF, Oriental Yeast Co. Ltd., Tokyo) *ad libitum*. They were divided into four groups. Two groups were kept in a warm control room ($25 \pm 1^\circ\text{C}$), and two others were transferred to a cold room ($5 \pm 1^\circ\text{C}$) in individual cages at the same time. One group in the cold was used for the experiments after 24-h cold exposure (acute cold exposure: CE), and one group in the warm (8 weeks of age at the experiments: WC 8) was used as control for CE rats. The other cold-exposed group was reared in the cold room for 4 weeks as a cold-acclimated one (CA). The last group was kept in the warm for 4 weeks (12 weeks of age at the experiments: WC12) as control for CA rats.

Determination of BAT composition. BAT composition was determined by the method of Folch *et al.* [14]. Interscapular BAT was excised, cleaned of the adhesive tissue, and minced. BAT lipid was extracted by shaking the minced BAT (ca. 100 mg) in chloroform-methanol (2:1 by volume) solution for 4 h. The extract was dried at 60°C for 1 h, and then weighed for lipid. The tissue residue was dried at 110°C for 4 h and then weighed for fat-free dry matter (FFDM).

Measurement of plasma insulin and insulin content of BAT. Animals were killed by decapitation without anesthesia, taking care not to give stress. The trunk blood samples were centrifuged to separate plasma, and stored at -20°C . Interscapular BAT was excised and minced in ten volumes of ice-cold acidified saline (pH 2.8) containing trypsin inhibitor (Antagosan, 1,000 U/ml, Behring Institute) and 1 mM EDTA, homogenized with Ultra-Disperser (LK-21, Janke and Kunkel Co., Germany) at a maximum speed (24,000 rpm) for 1 min. The homogenate was centrifuged ($12,000 \times g$) at 4°C for 30 min and then aliquots of infranant under lipid layer were filtered through glass fiber filters (Glassfiber Prefilter, Sartorius GmbH, Germany) and stored at -70°C . Immunoreactive insulin of plasma and the tissue extract was determined by radioimmunoassay using a commercial kit (Coat-A-Count Insulin, Diagnostic Products Corporation, U.S.A.).

Plasma glucose was determined by glucose oxidase method using a commercial kit (Glucose B-Test, Wako Pure Chemical Industry Ltd., Osaka).

Preparation of plasma membrane. Plasma membrane fraction was prepared by the method of Rothwell *et al.* [15] with minor modifications. Interscapular BAT from 5 to 10 rats was rapidly dissected, finely minced in 0.25 M sucrose buffer (containing 1 mM EDTA, 1,000 U/ml Antagosan and 10 mM Tris, pH 7.4) on ice, then homogenized with Ultra-Disperser for 1 min and the homogenate was homogenized again by ten strokes of a Teflon pestle in a glass homogenizer. The final homogenate was centrifuged at $3,500 \times g$ for 5 min, followed by filtration through silk cloth to remove tissue debris. The filtrate was then centrifuged at $11,000 \times g$ for 15 min to precipitate mitochondria, and the supernatant was centrifuged at $100,000 \times g$ for 60 min. The obtained pellet was washed with Krebs-Ringer phosphate (KRP) buffer (119 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl_2 , 1.2 mM MgSO_4 , 16.3 mM phosphate buffer, pH 7.4) and centrifuged again at $100,000 \times g$ for 60 min. The final pellet was resuspended in the same buffer at a concentration of 1 mg/ml of protein. All procedures were performed at 4°C .

Protein was determined by Lowry's method with bovine serum albumin (BSA) as a standard.

Insulin binding to plasma membranes. Preliminary studies showed that specific ligand binding was proportional to membrane protein concentration over a range of 50–400 $\mu\text{g}/\text{ml}$. A membrane protein concentration of 200 $\mu\text{g}/\text{ml}$ was employed in the present binding assay. Plasma membranes (100 μg) were incubated at 25°C for 45 min in the KRP buffer containing 1% BSA, 1 mg/ml bacitracin (Sigma Chemical Co., U.S.A.) and 1.8×10^{-11} M ^{125}I -labeled insulin (^{125}I -Tyr^{A14} insulin receptor grade, Dupont/NEN Research Products) in the absence or presence of unlabeled porcine insulin (Funakoshi, Tokyo) in a final volume of 500 μl . Incubation was terminated by adding 2 ml ice-cold KRP buffer containing 0.1% BSA, followed by rapid vacuum filtration through Whatman GF/C glass fiber filters, followed by three washes with the buffer. Radioactivity of the filter was counted in a γ -scintillation counter (1282 Compugamma, LKB Wallac, Finland). Specific binding was calculated by subtracting the amount of ^{125}I -insulin not

displaced by 10^{-6} M native porcine insulin (non-specific binding) from the total count.

Measurement of in vitro oxygen consumption of BAT. *In vitro* oxygen consumption of BAT was measured as an index of thermogenic capacity of the tissue as previously described [16]. Dissected BAT was cut into about 1 mm³ blocks. These blocks were preincubated for 2 h in the KRP buffer containing 5 mM glucose and 4% defatted BSA at 37°C. Approximately 20–30 mg of the preincubated tissue blocks was incubated in the same buffer gassed with oxygen in the absence or presence of 10^{-7} M porcine insulin. Oxygen consumption of the tissue was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England), calibrated using rat liver mitochondria with addition of stoichiometric quantities of NADH. After the resting level of oxygen consumption (basal oxygen consumption) was obtained, NA (Arterenol, Hoechst) (0.01–1 µg/ml) was added to the incubation buffer. NA at a concentration of 1 µg/ml was found to give maximum response in the same *in vitro* system [16, 17].

Statistical analyses. Results are expressed as mean $\pm$ SE. Significant differences between groups were tested by Mann-Whitney test. Wilcoxon signed-ranks test was performed to evaluate the effect of insulin on *in vitro* oxygen consumption of BAT.

RESULTS

Characteristics of animals

Effects of cold exposure on rat body, BAT, and epididymal white adipose tissue (WAT) weights, and plasma glucose level are shown in Table 1. Acute cold exposure did not affect the body, BAT, and WAT weights, while it significantly raised the plasma glucose level. Cold acclimation decreased the body weight gain and WAT weight, and markedly increased BAT weight. Plasma glucose level in CA did not differ from that in WC12.

Composition of BAT

Figure 1 shows composition of BAT. FFDM level in CE was significantly higher than that in WC8 (CE: 15.8 ± 0.6 , WC8: $11.8 \pm 0.2\%$, $p < 0.01$). Lipid level in CE was lower as compared with that in WC8 (CE: 18.5 ± 2.3 , WC8: $41.3 \pm 1.7\%$, $p < 0.01$). FFDM level in CA was higher than that in WC12 (CA: 14.1 ± 0.5 , WC12: $8.4 \pm 0.1\%$, $p < 0.01$). Lipid level in CA was lower than that in WC12 (CA: 36.7 ± 2.8 , WC12: $61.2 \pm 2.8\%$, $p < 0.01$). There were significant differences between warm control groups (WC8 and WC12) in both FFDM and lipid levels ($p < 0.01$), the former being higher and the latter being lower in WC8 than WC12.

Insulin levels in plasma and BAT

Plasma insulin level was lower in CE than that in WC8 (CE: 32.1 ± 1.4 , WC8: 56.7 ± 3.9 µU/ml, $p < 0.01$). There was no difference in plasma insulin level between

Table 1. Characteristics of rats.

	Body weight (g)		BAT weight (mg)		WAT weight (g)		Plasma glucose (mg/dl)
	Initial	At experiment	Total	/100 g BW	Total	/100 g BW	
WC8	191±2.1	203±2.6	125±2.6	61±1.3	2.4±0.14	1.2±0.08	148±0.8
CE	191±2.9	194±2.7	119±5.5	62±2.8	2.2±0.08	1.1±0.05	160±1.6**
WC12	193±2.0	284±5.0	214±11.0	76±4.6	4.2±0.31	1.5±0.10	140±2.4
CA	191±1.8	251±3.6**	472±17.3**	188±5.5**	1.8±0.11**	0.7±0.05**	138±1.7

Each group consisted of 10 rats. WC8, warm controls 8 weeks of age; CE, acute cold-exposed (5°C, 24 h) rats; WC12, warm controls 12 weeks of age; CA, cold-acclimated (5°C, 4 weeks) rats; BAT, interscapular brown adipose tissue; BW, body weight at experiment; WAT, epididymal white adipose tissue. Mean±SE. Significant differences between groups were tested by Mann-Whitney test (** $p < 0.01$).

Fig. 1. Changes in composition of brown adipose tissue (BAT). Each group consisted of 10 rats. Results are presented as percentage of BAT weight. FFDM, fat-free dry matter. Other abbreviations are the same as in Table 1.

CA and WC12 (CA: 56.0 ± 2.9 , WC12: 67.2 ± 4.8 $\mu\text{U/ml}$). Acute cold exposure and cold acclimation increased insulin content of BAT ($\mu\text{U/g}$ BAT weight) by about 200% as compared with each control value (CE: 196 ± 6.7 , WC8: 61 ± 2.2 , $p < 0.01$; CA: 281 ± 16.2 , WC12: 91 ± 3.9 , $p < 0.01$) (Fig. 2).

Insulin binding to BAT plasma membranes

Figure 3 shows the specific binding of ^{125}I -insulin to plasma membranes of BAT. Insulin binding to plasma membranes of BAT from CE rats was higher than that from WC8 rats (CE: 3.39 ± 0.18 , WC8: 2.74 ± 0.18 fmol/mg protein, $p < 0.05$), while that from CA rats was lower than from WC12 rats (CA: 1.76 ± 0.14 , WC12: 2.58 ± 0.14 fmol/mg protein, $p < 0.05$). Figures 4A and 5A show the displacement curves of ^{125}I -insulin in the presence of various concentrations (10^{-11} – 10^{-7} M) of unlabeled insulin. The specific ^{125}I -insulin binding was consistently higher at all concentrations of insulin in CE than in WC8 (Fig. 4A), and lower at all concentrations of insulin in CA than in WC12 (Fig. 5A). The data of the displacement curves of ^{125}I -insulin were analyzed by Scatchard plots (Figs. 4B and 5B). Scatchard plots showed concave curves, suggesting the presence of two populations of binding sites. According to the graphic method described by Rosenthal [18], acute

Fig. 2. Insulin levels in plasma and brown adipose tissue. Each group consisted of 8–10 rats. Vertical bars represent mean $\pm$ SE. Significant differences between groups were tested by Mann-Whitney test (** $p < 0.01$). Other abbreviations are the same as in Table 1.

Fig. 3. Specific binding of ¹²⁵I-insulin to plasma membranes of brown adipose tissue. Vertical bars represent mean $\pm$ SE of 5–9 experiments performed in triplicate. Significant differences between groups were tested by Mann-Whitney test (* $p < 0.05$). Other abbreviations are the same as in Table 1.

Fig. 4. Displacement curve of ^{125}I -insulin of brown adipose tissue from acute cold-exposed (CE) and warm control (WC8) rats (A), and Scatchard plots (B). Each point represents mean $\pm$ SE of 5–10 determinations from triplicate samples. K_1 , high affinity constant of binding sites; K_2 , low affinity constants of binding sites; R_1 , the number of high affinity binding sites; R_2 , the number of low affinity binding sites. Other abbreviations are the same as in Table 1.

cold exposure for 24 h increased the number of binding sites by 100% in high affinity sites and by 80% in low affinity sites (Fig. 4B), while cold acclimation decreased the number of binding sites by 35% in high affinity sites and by 30% in low affinity sites of insulin receptors (Fig. 5B). Acute cold exposure and cold acclimation did not change affinity constants in both high affinity and low affinity sites (Figs. 4B and 5B).

Effect of insulin on in vitro thermogenesis of BAT

Basal oxygen consumption. Initial resting *in vitro* oxygen consumption of BAT was defined as basal oxygen consumption, and its stable level was usually obtained within 20 min. Basal oxygen consumption from WC8 rats was not affected

Fig. 5. Displacement curve of ^{125}I -insulin of brown adipose tissue from cold-acclimated (CA) and warm control (WC12) rats (A), and Scatchard plots (B). Each point represents mean $\pm$ SE of 5–7 determinations from triplicate samples. Other abbreviations are the same as in Fig. 4.

by insulin. Basal oxygen consumption from CE rats in the absence of insulin was about two-fold higher than that from WC8 rats, and it was suppressed by insulin to 70% (Fig. 6). Basal oxygen consumption from WC12 rats was not affected by insulin. Basal oxygen consumption from CA rats was significantly lower than that from WC12 rats, and not affected by insulin (Fig. 7).

NA-induced oxygen consumption. NA (0.01–1 $\mu\text{g/ml}$) increased *in vitro* oxygen consumption of BAT in a dose-dependent manner in each group (Figs. 6 and 7). NA-induced oxygen consumption of BAT from WC8 rats at a concentration of 0.01 $\mu\text{g/ml}$ was suppressed by insulin, but not at higher concentrations of NA, while that from CE rats was suppressed by insulin at all concentrations of NA. Maximum value in CE was higher than that in WC8 (Fig. 6). NA-induced oxygen consumption from WC12 rats was suppressed by insulin at all concentrations of NA, while that from CA rats was significantly lower than that from WC12 and not affected by insulin at any concentration of NA (Fig. 7).

Fig. 6. Effect of insulin on *in vitro* oxygen consumption of brown adipose tissue from acute cold-exposed (CE) and warm control (WC8) rats. Each point represents mean $\pm$ SE of 9–12 samples. Effect of insulin on *in vitro* oxygen consumption was tested by Wilcoxon signed-ranks test (*, ** $p < 0.05$, 0.01, respectively). Differences between CE and WC8 in the absence of insulin were tested by Mann-Whitney test (#, ## $p < 0.05$, 0.01, respectively). Other abbreviations are the same as in Table 1.

DISCUSSION

Previous report showed that insulin content of BAT increased in cold-acclimated rats [13]. In this study, we confirmed the previous finding and demonstrated that acute cold exposure for 24 h also increased insulin content of BAT. Furthermore, the ^{125}I -insulin binding study revealed that the number of insulin binding sites of BAT from CE rats increased and that from CA rats was decreased as compared with respective warm controls (Figs. 4 and 5). These findings suggested that there were different mechanisms of the increase in BAT insulin content between acute cold-exposed and cold-acclimated animals, and that insulin might exert different actions on BAT functions between CE and CA rats. Insulin has been proved to have many effects on BAT functions. Insulin affects the thermogenesis [6–12], proliferation and differentiation [19], glucose and lipid metabolism [6–7, 20–23] of BAT.

The lower lipid fractions of BAT in CE and CA as compared with those in respective warm controls are considered to result from the increase in utilization of lipid (Fig. 1). It is interestingly noted that lipid percentage is significantly lower in WC8 than in WC12, and in CE than in CA ($p < 0.01$). The result may indicate greater utilization of lipid in WC8 than WC12, and in CE than in CA. In any case,

Fig. 7. Effect of insulin on *in vitro* oxygen consumption of brown adipose tissue from cold-acclimated (CA) and warm control (WC 12) rats. Each point represents mean $\pm$ SE of 7–11 samples. Effect of insulin on *in vitro* oxygen consumption was tested by Wilcoxon signed-ranks test (* $p < 0.05$). Differences between CA and WC12 in the absence of insulin were tested by Mann-Whitney test (## $p < 0.01$). Other abbreviations are the same as in Table 1.

insulin would be involved in the replenishment of lipid in BAT utilized during cold exposure. It is well established that glucose serves as a precursor for newly synthesized fatty acids. Insulin enhances glucose uptake [20, 21] and lipogenesis [22, 23] in BAT, and cold exposure potentiates the effect of insulin on BAT glucose uptake [21] and fatty acids synthesis [23]. Enhanced insulin action may be essential for regulation of glucose and lipid metabolism in CE rats, though plasma insulin level was reduced by acute cold exposure (Fig. 2). It is inferred that the increase in the number of insulin binding sites of BAT from CE rats contributes to an enhanced BAT glucose uptake and lipogenesis. Insulin binding sites have been evidenced to be regulated by plasma insulin level. Several reports show that insulin receptors of white adipocytes in streptozotocin-induced hypoinsulinemic rats are increased [24, 25]. Therefore, the decrease in plasma insulin and the increase in plasma glucose in CE rats (Fig. 2 and Table 1) may induce the increase in insulin binding sites of BAT to keep up with the level of extracellular ligands. In the present study, insulin (10^{-7} M) suppressed the basal as well as NA-induced oxygen consumption of BAT from CE rats (Fig. 6), while insulin had no effect on *in vitro* BAT thermogenesis from WC8 rats except NA-induced oxygen consumption at a concentration of $0.01 \mu\text{g/ml}$. These findings indicate an enhanced effect of insulin on *in vitro* BAT thermogenesis by acute cold exposure. The increase in BAT insulin content in CE rats resulting from the increase of insulin binding sites of BAT

plasma membranes would be favorable to supply energy substrate through its lipogenic action.

Insulin content of BAT from CA rats was higher than that from WC12 rats despite the decrease in the number of insulin binding sites (Figs. 2 and 5). This finding suggests that immunoreactive insulin of tissue extract in CA is present mainly in intracellular space. Insulin binds to specific cell surface receptors, internalized in the intracellular space, and then locates intracellular organelles such as lysosome, Golgi apparatus [26] and nuclei [27, 28]. Geiger *et al.* [29] claimed that receptor-mediated endocytosis (internalization) is a major mechanism of down-regulation of insulin receptors. It is thus likely that the decrease in the number of insulin binding sites of BAT from CA rats results from a high rate of internalization of insulin, and that the high rate of internalization of insulin accumulates immunoreactive insulin in intracellular space in CA rats. Whether intracellular insulin has any significant roles in BAT remains obscure. It has been shown that insulin acts as a growth factor in BAT. Klaus *et al.* [19] revealed that insulin was essential in brown adipocyte differentiation in cell culture. Miller [30] showed that microinjection of insulin into the cytoplasm of *Xenopus laevis* oocytes resulted in an increased RNA and protein synthesis. Cold acclimation increased the weight and DNA content of rat BAT, indicating hyperplasia of the tissue [31]. Such extensive hyperplasia is supposed to contribute to the greatly increased thermogenic capacity of BAT in cold. It is inferred that increase in intracellular insulin of BAT is associated with protein synthesis, growth, and differentiation of BAT during cold acclimation. Insulin did not affect basal oxygen consumption in both WC8 and WC12, and suppressed NA-induced oxygen consumption from WC 12 rats at all concentrations of NA, but not in WC8. The inconsistent results between the warm control groups may be due to the different states of BAT at different ages. Figure 1 showed that lipid composition of BAT was different between WC8 and WC12, being lower in WC8 than in WC12. Basal oxygen and maximal NA-induced oxygen consumption in the absence of insulin tended to be higher in WC8 than those in WC12 (basal oxygen consumption: WC8, 360 ± 20.2 , WC12, 311 ± 28.4 ; maximal NA-induced oxygen consumption: WC8, $1,380 \pm 14.3$, WC12, $1,148 \pm 92.7$ pmol O₂/(min·mg BAT)). These findings suggested that the BAT activity in WC8 was higher as compared with that in WC12. It is thus likely that an inhibitory effect of insulin on *in vitro* BAT thermogenesis was partly cancelled in WC8. Basal and NA-induced oxygen consumption in the absence of insulin from CA rats were lower than those from WC12 rats as previously evidenced [16]. It has been suggested that the poor responsiveness to NA on *in vitro* BAT thermogenesis in CA rats is, at least in part, due to the decrease in β -adrenergic receptors in brown adipocytes during cold acclimation [32, 33]. Insulin did not affect both basal and NA-induced oxygen consumption in CA rats. Therefore, the number of insulin and β -adrenergic receptors in BAT plasma membranes may decrease simultaneously in CA rats.

The present study demonstrated that insulin status (tissue content and binding

to plasma membranes) in BAT and the tissue responsiveness to insulin on *in vitro* BAT thermogenesis were modified during cold exposure and cold acclimation, indicating that it was closely associated with the regulation of BAT function in various facets. However, further studies would be needed to elucidate the precise role of insulin in BAT functions.

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