Effect of Immobilization Stress on *In Vitro* and *In Vivo* Thermogenesis of Brown Adipose Tissue

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**Abstract** Repetitive intermittent stress such as immobilization has been shown to induce an improved cold tolerance through an enhanced capacity of nonshivering thermogenesis (NST), causing positive cross adaptation between nonthermal stress and cold. In the present study, effect of 3-h-daily immobilization stress for 4–5 weeks was investigated on *in vitro* and *in vivo* thermogenesis of interscapular brown adipose tissue (BAT). *In vitro* thermogenesis was measured in the minced tissue blocks incubated in Krebs-Ringer phosphate buffer with glucose and albumin at 37°C, using a Clark-type oxygen electrode. The stressed rats showed less body weight gain during the experiment. The BAT weight, its protein and DNA contents were significantly greater in the stressed rats. Basal, noradrenaline- and glucagon-stimulated oxygen consumptions were significantly greater in the stressed rats. *In vivo* thermogenesis was assessed by the changes of temperatures in colon \((T_{col})\), BAT \((T_{BAT})\), and tail skin \((T_{sk})\) induced by noradrenaline or glucagon infusion in the anesthetized rats. Noradrenaline and glucagon increased the \(T_{BAT}\) and the extent of increase was greater in the stressed rats. These results indicate that cross adaptation between nonthermal stress and cold may be mediated through an enhanced thermogenic activity of BAT.

**Key words**: immobilization stress, brown adipose tissue, thermogenesis, noradrenaline, glucagon.

Most mammals, especially small ones such as rats and mice, have been shown to acclimate to cold by means of enhanced nonshivering thermogenesis (NST) unrelated to muscular contraction. It has been well established that a major site of NST is the unique thermogenic tissue, brown adipose tissue (BAT) [1]. Stimulation of thermogenic activity of this tissue during cold acclimation has been indicated to be mediated by sympathetic catecholamines and hormonal factors such as thyroidal, adrenocortical, and pancreatic hormones [2]. It was further de-
monstrated that intermittent repetitive nonthermal stress such as immobilization could enhance cold tolerance through an enhanced NST, causing positive cross adaptation to cold [3]. Since more densely developed BAT mitochondrial cristae were observed in the stressed rats as in the cold-acclimated ones [4], this cross adaptive phenomenon is likely to be developed, at least in part, by an enhanced BAT activity. The aim of the present study was to investigate the thermogenic capacity of BAT from the stressed rats in in vitro and in vivo conditions.

MATERIALS AND METHODS

Preparation of animals. Adult male Wistar strain rats, weighing about 180 g, were kept in metal cages (5 rats/cage) and given standard laboratory chow (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. They were placed under the artificial lighting from 7:00 a.m. to 7:00 p.m. at 25 ± 1°C and about 50% RH. They were divided into two groups. One group was subjected to stress for 4–5 weeks by 3-h-daily immobilization with wire mesh on wooden board as described elsewhere [5] and the other group was served as controls who were subjected only to handling. Experiments were performed 24 h after the last immobilization stress.

Measurement of in vitro tissue oxygen consumption. The isolated brown adipocytes have been used in in vitro studies. However, it was shown that the less active cells are preponderantly recovered during isolation [6]. Therefore, we used the finely minced tissue blocks instead of isolated cells in order to assess in vitro thermogenic response of BAT. Rats were sacrificed by cervical dislocation and the interscapular BAT was excised. The BAT was cleaned of any adhering tissue, weighed and minced into approximately 1 mm tissue blocks. These minced tissue blocks were incubated for 2 h at 37°C in Krebs-Ringer phosphate buffer (pH 7.4) continuously gassed with air, containing half the recommended concentration of CaCl₂, 5 mM glucose and 4% bovine serum albumin (Armor Co., Fraction V, dialyzed for 24 h through cellulose membrane against phosphate buffer). About 30 mg of the preincubated tissue was incubated in the water-jacketed chamber and stirred in 2 ml of the same buffer gassed with oxygen, and the oxygen consumption was measured using Clark-type oxygen electrode (Rank Brothers, Cambridge) at 37°C. After the basal oxygen consumption was obtained, usually in 20 min, 1 μg/ml of noradrenaline (NA; Arterenol, Hoechst) or glucagon (G; Sigma Chemical Co.), which has been shown to give the maximum response [7], was added in the incubation medium. Maximum increase in oxygen consumption was observed in 10 to 20 min after the administration of NA or G.

Composition of BAT. A small amount of BAT was examined for its composition by the method of Folch et al. [8]. The tissue lipids were extracted in the chloroform-methanol (2:1 v/v) by shaking the minced BAT for 4 h. The lipids were measured by drying the extracted solution at 60°C for 1 h. The fat-free dry matter (FFDM), mostly protein, was measured by drying the tissue residue at
110°C for 4 h.

Measurement of tissue DNA and protein content. The excised BAT was homogenized with 1% sodium dodecyl sulfate (Sigma Chemical Co.) and incubated at 37°C for 1 h. Then, the DNA concentration of crude homogenates was measured fluorometrically using bisbenzimidazole (Hoechst 33258, Polysciences, Inc.) as described by Brunk et al. [9] with calf thymus DNA as standard. Protein was measured by the method of Lowry et al. [10] with bovine serum albumin as standard.

In vivo temperature measurements. The animal was anesthetized with urethan (120 mg/100 g, i.p.) at 25°C. NA or G was infused in a dose of 2 μg/(0.005 ml·min) into the left jugular vein for 10 min. The thermistor thermometer (Takara Kogyo, Tokyo) was inserted 5 cm into the colon for measuring colonic temperature (T_col), attached to the proximal tail skin for skin temperature (T_ski), or placed beneath the interscapular BAT for BAT temperature (T_BAT). These temperatures were recorded continuously with Takara Thermistor. G was dissolved in 0.01 N HCl and diluted with saline. NA was also diluted with saline. Acid saline or saline was infused as the vehicle control respectively.

Statistical analysis. The results were expressed as mean±SE. Data in thermogenic response obtained from each group before, during, and after infusion were tested for significance of changes by paired t-test. The others were tested by Student’s t-test.

RESULTS

Body and tissue weights (Table 1)

The stressed rats gained less body weight as compared with the controls, as described in the previous report [3]. The interscapular BAT weight significantly increased in the stressed rats both in terms of total tissue pad and weight per unit body weight, while the epididymal white adipose tissue (WAT) significantly decreased both in terms of total tissue mass and weight per unit body weight. The weight of adrenal glands was similar in these two groups in terms of total weight, but significantly increased in the stressed rats in terms of weight per unit body weight.

Composition, protein and DNA contents of BAT (Table 2)

The FFDM and water significantly increased, and the lipid fraction decreased remarkably in the stressed rats as compared with those in the controls. Protein content was significantly higher in the stressed rats as compared with that of controls. DNA content was also significantly higher in the stressed rats.

In vitro thermogenic response of BAT

Basal oxygen consumption was significantly increased in the stressed rats both in terms of per mg tissue and per total tissue pad, from 313±26.1 pmol O₂/(min·mg
Table 1. Body and tissue weights.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Interscapular BAT</th>
<th>Epididymal WAT</th>
<th>Adrenal glands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>At experiment</td>
<td>(mg)</td>
<td>(g)</td>
</tr>
<tr>
<td>Controls (8)</td>
<td>178 ± 4</td>
<td>276 ± 6</td>
<td>163 ± 10.7</td>
<td>3.85 ± 0.23</td>
</tr>
<tr>
<td>Stressed rats (8)</td>
<td>174 ± 2</td>
<td>207 ± 4</td>
<td>211 ± 6.3</td>
<td>2.15 ± 0.11</td>
</tr>
<tr>
<td>p vs controls</td>
<td>NS</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Numbers in the parentheses indicate the number of animals. BAT, brown adipose tissue; WAT, white adipose tissue. Mean ± SE. NS: not significant.

Table 2. Composition, protein and DNA contents of brown adipose tissue (BAT).

<table>
<thead>
<tr>
<th></th>
<th>Composition (%)</th>
<th>Protein content (mg/tissue pad)</th>
<th>DNA content (μg/tissue pad)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FFDM</td>
<td>Lipid</td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (10)</td>
<td>8.2 ± 0.3</td>
<td>61.1 ± 0.8</td>
<td>30.8 ± 0.8</td>
</tr>
<tr>
<td>S (11)</td>
<td>10.9 ± 0.2*</td>
<td>50.9 ± 0.4*</td>
<td>38.2 ± 0.5*</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of animals. C, controls S, stressed FFDM, fat-free dry matter Mean ± SE. *Significantly different vs C (p < 0.001).
Fig. 1. Noradrenaline (NA)- and glucagon (G)-stimulated increases in *in vitro* oxygen consumption of brown adipose tissue (BAT) from controls (C) and stressed rats (S). ***p vs C<0.001. Vertical line indicates SE. Each point consists of 10 to 12 samples.

BAT) and 57±4.6 nmol O$_2$/((min·tissue pad)) for the controls to 486±38.7 pmol O$_2$/((min·mg BAT)) and 116±11.9 nmol O$_2$/((min·tissue pad)) for the stressed rats, respectively. The previous report [7] indicated that both G and NA gave the maximum response in 1 µg/ml in the same *in vitro* incubation medium. Thus, this dose of G or NA was used in the present study to examine the responsiveness of BAT. Figure 1 shows the changes in the oxygen consumption per mg tissue induced by G or NA addition to the chamber. The maximum response was obtained 10–20 min after addition of G or NA. NA as well as G gave the greater response in the BAT from the stressed rats as compared with that from the controls. The maximum increase induced by G was 479±79.3 pmol O$_2$/((min·mg BAT)) and 87±14.7 nmol O$_2$/((min·tissue pad)) for the controls, and 1,082±76.3 pmol O$_2$/((min·mg BAT)) and 252±21.2 nmol O$_2$/((min·tissue pad)) for the stressed rats. This was also the case for the NA-stimulated increases, 533±64.0 pmol O$_2$/((min·mg BAT)) and 98±11.7 nmol O$_2$/((min·tissue pad)) for the controls, and 1,213±85.2 pmol O$_2$/((min·mg BAT)) and 283±24.3 nmol O$_2$/((min·tissue pad)) for the stressed rats. The maximum increases did not significantly differ between G and NA stimulation in either controls or stressed rats.

*In vivo temperature responses*

$T_{BAT}$ was higher than $T_{col}$ in both control and stressed rats under all experimental conditions. Initial $T_{BAT}$ and $T_{sk}$ showed no significant difference between two groups. $T_{col}$ was significantly higher in the stressed rats as compared with that in controls (Table 3). The saline and acid saline infusion caused no significant
Table 3. Temperatures before infusion of noradrenaline and glucagon.

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{col}}$ (°C)</th>
<th>$T_{\text{BAT}}$ (°C)</th>
<th>$T_{\text{sk}}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (26)</td>
<td>36.9±0.08</td>
<td>37.2±0.08</td>
<td>29.7±0.2</td>
</tr>
<tr>
<td>Stressed rats (28)</td>
<td>37.3±0.09*</td>
<td>37.3±0.09</td>
<td>29.4±0.2</td>
</tr>
</tbody>
</table>

$T_{\text{col}}$: colonic temperature; $T_{\text{BAT}}$: temperature of interscapular BAT; $T_{\text{sk}}$: temperature of tail skin. Numbers in the parentheses indicate the number of animals. Mean±SE. *Significantly different vs the controls ($p<0.01$).

Fig. 2. Changes in brown adipose tissue (BAT) temperature ($T_{\text{BAT}}$) from preinfusion value. Noradrenaline (NA) or glucagon (G) was infused from the time zero to 10 min. *$p$ vs preinfusion value $<0.05$, **$p$ vs controls $<0.05$. Each point consists of 7 to 9 samples. Legends are the same as in Fig. 1.

changes in $T_{\text{col}}$, $T_{\text{BAT}}$, and $T_{\text{sk}}$. It was previously shown that NA infusion in a dose of 2 μg/min elevated $T_{\text{BAT}}$ significantly higher in the cold-acclimated anesthetized rats than the warm controls [11]. G infusion in 2 μg/min also caused the maximum rise in $T_{\text{BAT}}$ in the controls (data not shown). In the present study the NA or G infusion in the same dosage increased $T_{\text{BAT}}$ and the extent of increase was greater in the stressed rats. Furthermore, the extent of rise in $T_{\text{BAT}}$ was greater for NA as compared with those for G (Fig. 2). NA-induced increase in $T_{\text{col}}$ was greater in the stressed rats as compared with that in the controls (Fig. 3). $T_{\text{col}}$ also increased by G infusion in the controls, but not in the stressed rats (Fig. 3). $T_{\text{sk}}$ increased by G in both controls and stressed rats (Fig. 4). The extent of increase in $T_{\text{sk}}$ tended to be greater in the stressed rats as compared with that in the controls, and $T_{\text{sk}}$ increased earlier in the stressed rats as compared with that in the controls: 4 min for the stressed rats, 8 min for the controls after G infusion (Fig. 4). In contrast to G infusion, NA infusion did not affect $T_{\text{sk}}$ in either the stressed rats or the controls during infusion (Fig. 4). However, it was interestingly noted that NA increased $T_{\text{sk}}$ both in the stressed rats and the controls after cessation of NA infusion. This
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Fig. 3. Changes in colonic temperature ($T_{col}$) from preinfusion value. Legends are the same as in Figs. 1 and 2.

Fig. 4. Changes in tail skin temperature ($T_{sk}$) from preinfusion value. Legends are the same as in Figs. 1 and 2.

extent of increase did not significantly differ between the stressed rats and the controls.

DISCUSSION

We have shown previously that an intermittent and repetitive chronic stress by means of immobilization could induce positive cross adaptation between stress and cold and improve cold tolerance through an enhanced NST [3]. The phenomenon
would appear to be mediated by an increased BAT activity, since BAT mitochondria in the stressed rats resemble those in the cold-acclimated ones [4]. The present study apparently evidences this view directly at BAT level. It has been reported that various nonthermal stressful stimuli could stimulate multiple neuroendocrine systems as cold; increased secretions of catecholamines, adrenocorticoids, and glucagon, etc. [12]. Therefore, it is likely that multiple humoral responses occur in both the stressed and the cold-exposed rats, resulting in the development of cross adaptation between cold exposure and nonthermal stress through an enhanced thermogenic activity of BAT. Since food intake does not differ between the stressed rats and the controls [3], large energy dissipation, at least in part, by BAT thermogenesis may cause less body weight gain in the stressed rats. In BAT composition, FFDM, protein and DNA contents significantly increased and the lipid fraction decreased markedly in the stressed rats as in the cold-acclimated ones, as previously reported [13, 14]. This suggests that the repetitive immobilization stress also causes an increase in BAT cellularity and facilitates mobilization of lipids of BAT as cold acclimation.

\( T_{col} \) was greater in the stressed rats, as shown also in a previous report [15], but \( T_{BAT} \) did not significantly differ between the stressed rats and the controls. The basal oxygen consumption was greater in BAT from the stressed rats. The reason for this discrepancy remains unknown. \( T_{BAT} \) was measured by the thermometer beneath the interscapular BAT through the incision of the skin in the anesthetized rats. Thus, the anesthesia might modify \( T_{BAT} \). It is also likely that the chronic intermittent immobilization stress induces an enhanced thermogenesis not only in BAT, but also in other thermogenic organs such as liver, skeletal muscle, etc. However, it was apparently shown in the present study that \( T_{BAT} \) was elevated by NA and G infusion, and the increments of \( T_{BAT} \) were greater in the stressed rats. These results are consistent with those of the in vitro BAT thermogenesis. It was further noted that the increment of \( T_{BAT} \) was greater for NA infusion than for G infusion, though the increases in in vitro oxygen consumption did not differ between G and NA administration. The reason remains to be explained. Both NA and G infusions increase the blood flow through BAT in vivo, but the extent of increase is greater for NA as compared with that for G [16]. The greater blood flow may result in the greater elevation of \( T_{BAT} \) in the NA-infused stressed rat. It is, therefore, inferred from these results that NA is involved as a main thermogenic factor in an enhanced NST of BAT in the stressed rats and the controls as well. G infusion caused greater elevation of \( T_{sk} \), while NA infusion did not change it during the infusion, though \( T_{sk} \) was markedly elevated after the cessation of NA infusion. NA is known to cause a generalized constriction of blood vessels, including skin ones. Therefore, this phenomenon may be regarded as a rebound and compensatory response to an increased metabolism induced by NA as indicated by increases in \( T_{BAT} \) and \( T_{col} \). In contrast to NA infusion, \( T_{sk} \) increased during glucagon infusion, and the extent of increment of \( T_{sk} \) was greater as compared with that of \( T_{BAT} \) in both the stressed rats and the controls. This result indicates a direct effect.
of G on $T_{sk}$. It was previously reported that intrasplenic administration of glucagon increased the skin blood flow [17]. In the present study, the extent of increase in $T_{sk}$ did not differ between the controls and the stressed rats, but the onset of rise in $T_{sk}$ during G infusion was earlier in the stressed rats as compared with that in the controls. Therefore, immobilization stress may enhance the vasodilating action of G, facilitating heat loss, and causing less increase in $T_{col}$ of the stressed rats. It is suggested that G enhances not only BAT thermogenesis, but also general heat loss. The excess increase in body temperature may be unfavorable for the organism to survive under stressful conditions. Thus, this effect of G may be regarded as a defense mechanism to prevent an excessive temperature rise induced by an enhanced BAT thermogenesis. Taken altogether, the present findings from in vitro and in vivo studies may indicate that cross adaptation between stress and cold is developed through an enhanced thermogenic activity of BAT. Such stress-induced activation of thermogenic tissue, BAT, may serve to raise metabolic rate and cope with a stressful situation. Moreover, BAT has been shown to release a considerable amount of FFA to the circulation [18]. Thus, an increased release of energy substrate, FFA, from BAT to other organs may be useful for the organism to adapt to the stressful environment. This presumption may explain partly why $T_{col}$ was higher in the stressed rats than in the controls in spite of similar $T_{BAT}$ in these two groups; more energy substrate from BAT may be used in the other organs to increase thermogenesis.

REFERENCES


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