Cold exposure or chronic noradrenaline treatment induces an increase in the calmodulin-like immunoreactivity of brown adipose tissue of rats

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Cell proliferation is often associated with an increase in calmodulin, the ubiquitous intracellular calcium receptor of non-muscle cells. A long lasting increase in the proliferative activity of brown adipose tissue is induced by cold exposure in the rat. The present work showed that this phenomenon is also associated with a rapid and long lasting increase in the calmodulin content of this tissue. It was equally shown that this increase can be reproduced by noradrenaline administration.

Calmodulin (CaM), which is considered to be the major Ca\textsuperscript{2+} binding protein of non-muscle cells, is now accepted as the general mediator for most of the Ca\textsuperscript{2+} effects occurring in such cells (1,2). An increase in cytosolic Ca\textsuperscript{2+} is a control signal for the initiation of DNA synthesis and cell division (3), and there is increasing evidence that cytosolic CaM is involved in these events although its role is not fully understood (1,2). Thus, Ca\textsuperscript{2+}-CaM complex is highly implicated in the recent models proposed to explain the control of cell division (1,2,4,5). Furthermore, CaM concentration has been observed to increase in several situation where DNA synthesis and cell division is triggered off or enhanced. Examples include the transformation of various cell types (1), mitosis in non-transformed and synchronized CHO cells (in which CaM increases during the late G\textsubscript{1}-early S phases of the cell cycle) (1), meiosis in oocytes from *Xenopus laevis* (6), hepatoma (7,8), planarian regeneration (5), and in rat, regeneration of liver after partial hepatectomy (8). In the last situation, 'soluble CaM' (i.e. cytosolic CaM) increased without any changes in particulate CaM (i.e. CaM bound to membranes) (8).

Brown adipose tissue (BAT), the function of which is to produce heat during cold exposure and overfeeding (9), has been described in a large number of mammals. These two physiological stimuli induce hyperplastic growth of BAT in the rat which is associated with numerous metabolic and biochemical changes, including a specific increase in the mitochondria of the uncoupling protein responsible for the high thermogenic activity of BAT (10). The present experiments
were designed to determine whether the so-called 'BAT trophic response' is associated with an increase in the CaM level of this tissue, as proposed in the models cited above.

The major cellular events which constitute the BAT trophic response observed in cold-exposed animals and overfed rats are thought to be controlled by sympathetic innervation of the tissue through an increase in noradrenaline release (10). Recently it was observed that both the DNA content of BAT and the concentration of uncoupling protein in BAT mitochondria increased after chronic administration of noradrenaline using osmotic minipumps (11). Therefore, the effect of noradrenaline administration on CaM content in BAT was also studied using this technique.

**Materials and Methods**

**Animals and treatments**

Male Wistar rats (IFFA-CREDO, L'Arbresle, France) were fed stock diet, individually housed and kept at 26°C (control and noradrenaline-treated animals) or exposed to 5°C for various lengths of time (from 1 h to 14 d). All rats were 42 d old when sacrificed. Noradrenaline was administered using an osmotic minipump (Alzett model 2002) filled with (-) arterenol bitartrate (Sigma) 0.3 M protected by L-ascorbic acid (Prolabo) 0.1 M and tiron (Sigma) 0.02 M (11). The pumps delivered 0.1 μmol (approx. 20 μg) of (-) noradrenaline per hour and were implanted dorsally just behind the interscapular area (administration of L-ascorbic acid/tiron alone had no effect on the CaM content of BAT).

**Biochemical determinations**

As CaM is a soluble and heat resistant protein (12) the following method of extraction was employed: interscapular BAT was rapidly dissected out, weighed and homogenized (100 mg/ml) in ice-cold buffer pH 7.8 containing 50 mM Tris/HCl, 1 mM dithiothreitol and 1 mM EGTA (13) with a polytron homogenizer (2 x 10 s). The homogenates were centrifuged for 10 min at 700 g and the supernatants were collected and centrifuged for 30 min at 105 000 g. The 105 000 g supernatants were then heated at 90°C for 5 min, sonicated in an ice-cold vessel (2 x 10 s) and centrifuged again for 30 min at 105 000 g. These latter supernatants were stored at -80°C until assay of the 'soluble CaM'.

The 700 and first 105 000 g pellets were pooled, diluted with buffer to the original volume, sonicated, heated as just described, re-sonicated and centrifuged for another 30 min at 105 000 g. This final set of supernatants was used to assay 'particulate CaM' (8). CaM was also extracted from rat liver for purposes of comparison.

Total heat-stable proteins of the 105 000 g supernatant (HSP) were assayed according to the method of Lowry (14) in the supernatants used to assay soluble CaM. Both soluble and particulate CaM were determined by radioimmunoassay in 15 μl aliquots of the corresponding supernatants using a NEN (Boston, Massachusetts, U.S.A.) calmodulin 125I RIA kit. The stock unlabelled CaM (NEN)
solution was prepared in extraction buffer and heated at 90°C for 5 min. As samples all standards and blanks contained 15 µl of buffer. Both the preheated CaM provided with the NEN kit and our own preheated CaM as well as serial dilutions of BAT extracts produced parallel displacements of the 125I CaM/CaM antibody complex.

The rate of thymidine incorporation into DNA of BAT was also determined in cold-exposed rats (for technical data, see Fig. 1).

Results and Discussion

Soluble CaM content in BAT and liver

Three times less CaM-like radioimmunoreactivity was found in interscapular BAT than in liver (Table 1) when results were expressed per g fresh tissue. This lower 'concentration' of CaM in BAT was probably attributable to a larger cytosolic volume in hepatocytes as compared to that in brown adipocytes, which are filled with lipid droplets and mitochondria. Furthermore, the extracted CaM/extracted HSP ratio was very similar in BAT and liver, and even slightly greater in BAT (761 ± 72 µg/g vs 508 ± 61 µg/g, P ≤ 0.05).

Effect of cold exposure on the soluble CaM level in BAT

A first batch of rats was used to determine the effects of cold exposure for 1 h to 7 d on the soluble CaM level of BAT. Table 1 shows that the BAT trophic response to cold was associated with an increase in the soluble CaM content per organ. This increase was detectable after 1 h of cold exposure (x 1.3; P ≤ 0.05) and was maintained for 7 d. The rate of increase in CaM content was greater than that of BAT growth. Thus CaM concentration in BAT from cold-exposed rats were greater than in control tissue, irrespective of the duration of exposure at 5°C (1 h at 5°C, + 32%; 5 h, + 43%; 2 d, + 66%; 7 d, + 44%).

The possibility that the higher values observed in cold-exposed rats were artefacts due to differences in rates of CaM extraction cannot be excluded a priori. However, this hypothesis is markedly weakened by the fact that HSP concentrations were almost unaffected by cold exposure, while CaM concentrations increased.

Table 1. Effects of exposure to 5°C on the calmodulin (CaM) content of interscapular BAT of rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interscapular BAT weight (mg)</th>
<th>Heat stable proteins of the 105 000 g supernatant (HSP) (mg/g tissue⁻¹)</th>
<th>Soluble CaM (µg/g tissue⁻¹)</th>
<th>Soluble CaM (µg/g tissue⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (liver)</td>
<td>51.6 ± 3.2 **</td>
<td>1.8 ± 0.1</td>
<td>26.2 ± 4.1 **</td>
<td>508 ± 61 *</td>
</tr>
<tr>
<td>0</td>
<td>208 ± 8</td>
<td>11.3 ± 1.5</td>
<td>2.4 ± 0.2 **</td>
<td>943 ± 80 *</td>
</tr>
<tr>
<td>5 h</td>
<td>210 ± 24 NS</td>
<td>12.1 ± 1.3 NS</td>
<td>11.4 ± 0.8 *</td>
<td>1023 ± 64 *</td>
</tr>
<tr>
<td>5 h</td>
<td>236 ± 10 **</td>
<td>12.0 ± 0.8 NS</td>
<td>2.9 ± 0.1 **</td>
<td>1023 ± 64 *</td>
</tr>
<tr>
<td>2 d</td>
<td>244 ± 14 *</td>
<td>16.4 ± 1.6 *</td>
<td>3.5 ± 0.2 **</td>
<td>871 ± 64 NS</td>
</tr>
<tr>
<td>7 d</td>
<td>363 ± 28 **</td>
<td>11.4 ± 0.8 NS</td>
<td>4.5 ± 0.2 **</td>
<td>1087 ± 66 **</td>
</tr>
<tr>
<td>0</td>
<td>183 ± 27 (4)</td>
<td>7.95 ± 0.8 (4)</td>
<td>0.85 ± 0.02 (4)</td>
<td>585 ± 36 (4)</td>
</tr>
<tr>
<td>14 d</td>
<td>317 ± 28 **</td>
<td>9.4 ± 0.8 NS</td>
<td>4.6 ± 0.9 **</td>
<td>1542 ± 335 **</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. NS, not significant; * P < 0.05, ** P < 0.01 vs control values, i.e., BAT at 0 h (Mann-Whitney U-test). Except where indicated otherwise, n = 6 rats per treatment.
A second batch of animals was exposed to 5°C for 2 weeks. Comparison of these results with those of the previous experiments was difficult since the trophic response of BAT was smaller. Nevertheless, the CaM concentration in BAT was elevated and remained so (+ 212% vs the corresponding control value). Whether CaM concentration is raised in BAT as long as rats are kept to 5°C or whether adaptation and a tardy return to control values occurs requires further study.

**Effect of noradrenaline administration on the CaM level in BAT**

The results obtained with the third batch of rats (Table 2) confirmed the effects of cold-exposure on CaM level in BAT (µg soluble CaM per g tissue: + 91% after 5 h at 5°C, + 105% after 7 d); they also showed, as previously described (11), that chronic administration of noradrenaline stimulates BAT growth (BAT weight: + 27% after a 7 d treatment). In addition, soluble CaM content per organ was enhanced (+ 300%). The increases in BAT weight and CaM content were smaller than in rats exposed to 5°C for 7 d but the concentration of soluble CaM was equally elevated (+ 126% after NA treatment vs + 106% after 7 d cold exposure). Thus it can be concluded that, like most of the modifications induced in this tissue by cold exposure (10), the increase in CaM concentration in the BAT of cold-exposed rats was the result of sympathetic activation and noradrenaline release. However, it is not known whether the effects of noradrenaline or cold-exposure on the level of CaM in BAT were also due to an increase in the synthesis of this protein. Still, it should be noted that the variations in the concentration of 'particulate CaM' fraction (Table 2) did not accord with the hypothesis of CaM redistribution between 'particulate' and 'soluble' pool: particulate CaM per g tissue did not decrease in cold-exposed rats and even increased in noradrenaline-treated rats, and the two treatments induced an increase in total (soluble + particulate) CaM concentration.

**Possible roles for CaM in BAT trophic response to cold**

These observations provide another example of the association between cell proliferative activity and the increase in CaM concentration. As in other models of cell proliferation (1,2,4,5,8), it can be hypothesized that this increase plays an important part in the control of the cell multiplication triggered by cold exposure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interscapular BAT wt. (g)</th>
<th>Total heat stable proteins of the 105 000 g supernatant (HSP) (µg gum tissue-1)</th>
<th>Soluble CaM (µg organ-1)</th>
<th>Particulate CaM (µg gum tissue-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>199 ± 15</td>
<td>11.0 ± 1.2</td>
<td>1.3 ± 0.3</td>
<td>6.5 ± 1.3</td>
</tr>
<tr>
<td>5 h at 5°C</td>
<td>198 ± 15 NS</td>
<td>8.7 ± 1.0 NS</td>
<td>2.43 ± 0.5</td>
<td>12.4 ± 1.5</td>
</tr>
<tr>
<td>7 d at 5°C</td>
<td>391 ± 37 NS*</td>
<td>12.25 ± 0.4 NS</td>
<td>5.2 ± 0.5</td>
<td>13.3 ± 0.2</td>
</tr>
<tr>
<td>NA 10⁻⁷</td>
<td>252 ± 28 NS**</td>
<td>9.0 ± 1.5 NS</td>
<td>3.7 ± 0.3</td>
<td>14.7 ± 0.6</td>
</tr>
</tbody>
</table>

Same legend as for Table 1. Except where indicated otherwise n = 6 rats per treatment. Note that the effects of cold-exposure on CaM content per g or per organ were larger in this experiment than in the first experiment (Table 1).
Fig. 1. Time-course of the increase in DNA and [3H]thymidine incorporation into DNA of BAT of cold-exposed rats compared to the time-course of the increase in CaM concentration. Rats (other than those used for CaM determinations) were exposed to 5°C for 2, 6 or 12 d. They received i.p. [3H] thymidine (CEA, France) 48 h before sacrifice. BAT homogenates were delipidated and DNA was extracted and assayed as previously described (20). The radioactivity of an aliquot was determined by liquid scintillation counting. The results are presented as means ± S.E.M.; n = 11 (0 h and 2 d) or 6 rats (7 and 12 d). Note that the curve of the variation in thymidine incorporation into DNA of BAT shows agreement with the data of Cameron and Smith (15) obtained using the DNA synthetic index method after autoradiography of histological preparations. They observed an increase in cell division in BAT after 1 day's exposure to 6°C. This reached a maximum after 4 d and returned to control levels after 16 d. The CaM values presented for comparison are the pooled data from Tables 1 and 2 (the slight decrease observed at 7 d was not statistically significant).

The increase in CaM concentration in regenerating liver is large (concentration x 3) and transient (several hours) and is followed by proliferative activity which is also short lived (8). The increase in CaM levels in the BAT of cold-exposed rats is rapid (but weaker than in liver) and long-lasting. However, whilst the proliferative peak for regenerating liver lasts for approx. 8 h (8), that for BAT of rats chronically exposed to 6°C lasts for approx. 400 h (15). This characteristic of BAT response to cold is illustrated by the time-course of in vivo incorporation of tritiated thymidine into DNA of BAT of cold-exposed rats (Fig. 1). The increase in CaM levels of BAT appeared to be lasting and was probably related to the cell proliferation which was sustained for a number of weeks.

It cannot be determined at present whether the increase in CaM levels only occurs in proliferating preadipocytes (mature brown adipocytes do not divide) or whether it occurs in all the cell types of this tissue (mature adipocytes, vascular cells, nerve fibers, etc.).
Since many regulating pathways are calcium dependent and since BAT of cold-exposed rats exhibits extensive metabolic and biochemical modification (10), the possibility that this increase in CaM may be also involved in the control of other intracellular processes cannot be excluded. Moreover, Raasmaja et al. (16) recently observed that the density of α1-adrenergic receptors was enhanced in BAT by cold exposure. These authors suggested that the physiological significance of α1-adrenergic pathways for BAT function is increased in cold-adapted rats. The function of BAT α-adrenergic receptors is incompletely understood (while that of its β-adrenergic receptor is well documented; see ref. 17). However, as described in other tissues, stimulation of BAT α1-receptors induces cytosolic release of Ca2+ from internal stores (18). It is therefore suggested that there is a functional relation between the increase in Ca2+ receptor protein and the rise in α1-receptor density in the BAT of cold-exposed rats.

Conclusion

The results show that activation of BAT is associated with an increase in the ubiquitous Ca2+ receptor CaM. This increase occurs rapidly and is as rapid as the specific rise in uncoupling protein which is responsible for the thermogenic activity of this tissue (19). Hence determination of the exact purpose of the increase in the CaM content of the BAT of cold-exposed rats and of the mechanisms underlying noradrenaline control of this unique intracellular binding protein are pertinent to the understanding of BAT function and development.

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References