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Induction and degradation of the uncoupling protein thermogenin in brown adipocytes *in vitro* and *in vivo*

Evidence for a rapidly degradable pool

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The induction and degradation of the brown-fat-specific uncoupling protein thermogenin in brown fat cell cultures was investigated. Cultures were initiated with undifferentiated precursor cells from young mice and the amount of thermogenin was determined by immunoblotting. High levels of thermogenin could be induced by noradrenaline treatment in cells grown for more than 5 days in culture, and in such cell cultures continuously stimulated with noradrenaline, the thermogenin level continued to increase for at least a further 5 days. In cell cultures stimulated for only 24 h, the induced thermogenin was subsequently specifically and rapidly degraded, with a half-life of 20 h. As the half-life was prolonged by cycloheximide treatment, the degradation was apparently due to the induction of specific proteins after cessation of adrenergic stimulation. In cell cultures continuously stimulated with noradrenaline for 5 days, the induced thermogenin was degraded much more slowly after noradrenaline removal, with a half-life of 70 h. This half-life was unchanged by cycloheximide treatment, and the degradation after cycloheximide was in parallel with the degradation of protein in general, and was therefore non-specific. The prolongation of the half-life of thermogenin after the chronic treatment may be related to mitochondrial incorporation of thermogenin and consequent stabilization of the protein. The half-life of thermogenin in an *in vivo* situation of similar experimental design (the reacclimation of mice to warm after 5 days in the cold), was also long (about 7 days), and the loss was also non-specific, as it parallelled the loss of protein. Thus different molecular events are involved in thermogenin degradation when the protein is found in different functional pools.

INTRODUCTION

During adaptation to altered environmental conditions, a given tissue may undergo processes of atrophy or recruitment. For brown adipose tissue, the recruitment processes, which under different physiological conditions lead to an increased ability for heat production, have been the most studied [1,2]. The recent development of cell culture techniques for *in vitro* studies has permitted new insights into these recruitment processes [3–8].

However, those mechanisms which are initiated in order to enable an organism to adapt to a situation of lower demand on a tissue are equally important for the homeostasis of the organism and for the regulation of the differentiation status of a tissue. In brown adipose tissue, atrophy of the tissue is seen in several physiological conditions [2], but these processes have not as yet been studied *in vitro*. We have therefore conducted a series of experiments to monitor such events in brown fat cell cultures and to evaluate these *in vitro* results in the light of parallel *in vivo* processes. We have especially studied the expression of the brown-fat-specific uncoupling protein thermogenin, which is the rate-limiting factor for thermogenesis [9,10] and the only unique differentiation marker for brown adipocytes [11].

We conclude that in brown fat cell cultures, the presence of noradrenaline is essential not only to increase the level of the uncoupling protein thermogenin, but also to maintain this elevated level. In cells transiently adrenergically stimulated, the induced thermogenin is degraded specifically and rapidly; this degradation is apparently due to the induction of the synthesis of specific proteins involved in the degradation process. In cells chronically adrenergically stimulated, the half-life of thermogenin is much longer, and the degradation is non-specific. The half-life then approaches that observed in a parallel situation *in vivo*. Thus different molecular events are involved in thermogenin degradation in the early and late phases of tissue recruitment, probably related to the extent of thermogenin incorporation into the mitochondria.

MATERIALS AND METHODS

In vitro experiments

Cell isolation and cell culture. Brown adipose tissue precursor cells were prepared and inoculated as earlier described [4,8], except that multiwell plates were used. A 0.2 ml portion of pooled final cell suspension was inoculated per well (35 mm diameter culture wells, Corning cell wells). Routinely, about 40 wells were inoculated with a cell suspension prepared from eighteen 4-week-old NMRI outbred mice, obtained from a local supplier (Eklunds). To each well, 1.8 ml of culture medium was added. The medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn-calf serum, 4 nM-insulin (Insulin Actrapid, Novo Industries) and 10 mM-Hepes, and with 50 units of penicillin, 50 μg of streptomycin and 25 μg of sodium ascorbate (126 μM) [12] per ml. The cells were grown at 37 °C in an atmosphere of 8% CO_2 in air. On the first and third days after inoculation, the culture medium was removed and the cells were rinsed with 2 ml of DMEM before the addition of 2 ml of fresh medium. Where indicated, the cells were treated twice a day with noradrenaline (10 μM; freshly dissolved in water, 20 μl/well) from day 7, as described in the legends to the Figures.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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Harvesting. At the indicated time points, the culture medium was removed and the cells were rinsed twice with ice-cold phosphate-buffered saline (PBS; 137 mM-NaCl, 2.7 mM-KCl and 10 mM-phosphate buffer, pH 7.4). The cells were then scraped into 150 μl of PBS with a rubber policeman and transferred into Eppendorf tubes. The cell suspensions were sonicated for 20 s at 40 W (Sonifier cell disruptor B-12; Branson); the efficiency of the sonication procedure was checked by light microscopy. The resulting cell lysates were stored at −20 °C prior to protein measurement.

Immunoblotting for thermogenin. Protein concentration was measured by the method of Bradford [13], with BSA as a standard. For immunoblotting, the samples were denatured by addition of about 10 μl (SDS/protein ratio of 4:1, w/w) of SDS/PAGE sample buffer (containing 62.5 mM-Tris base, 4% SDS, 5% 2-mercaptoethanol, 10% glycerol and Bromophenol Blue, pH 6.8). On every gel, a homogenate (20 μg of protein) of mouse liver prepared in 250 mM-sucrose, 10 mM-Tris/HCl and 1 mM-EDTA (pH 7.4) was run as a negative control. SDS/PAGE was carried out principally according to Laemmli [14], with 20 μg of protein per lane. Electrotransfer, blocking and development of the immunoblot were performed as described by Herron *et al.* [4], except that the final colour development was with an alkaline phosphatase-conjugated substrate kit containing Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad), according to the manufacturer's instructions. In some experiments an enhanced chemiluminescence method was used, in which membranes were incubated with goat anti-rabbit IgG (whole molecule)–peroxidase conjugate (Sigma; 1:5000 dilution) as a second antibody. The final development was then carried out with a peroxidase conjugate substrate kit containing luminol (Amersham), according to the instructions of the manufacturer. The films used were Kodak X-Omat AR films exposed for 40–300 s in a cassette.

For quantitative analysis, the bands were scanned with a Molecular Dynamics 300S computing densitometer and the results in each experimental series were expressed as percentages of the level of thermogenin found after 1 or 5 days of noradrenaline treatment, as indicated.

In vivo experiments

A total of 54 adult male NMRI mice (body weight about 40 g) were preacclimated to 28 °C for 1 week; then, 18 animals remained at 28 °C (controls), and 36 animals were transferred to 4 °C for 5 days. Of these animals, 18 were returned to 28 °C (cold, 5 days), while the other 18 remained in the cold (chronic cold). At the times specified, three animals from each experimental group were killed with CO_2, followed by cervical dislocation. The interscapular brown fat depot was dissected out, weighed and homogenized in Tris/sucrose buffer (1.5 ml; 250 mM-sucrose, 10 mM-Tris/HCl and 1 mM-EDTA, pH 7.4) with a Teflon/glass homogenizer (10 strokes; Thomas Laboratories). The tissue homogenates were transferred to Eppendorf tubes and centrifuged in an Eppendorf centrifuge at 2600 rev./min (600 *g*) to remove debris and triacylglycerols. After removal of the floating fat layer, 1 ml of the infranatant was transfered to a second Eppendorf tube and frozen at −20 °C, prior to protein measurement and immunoblot determinations as described above.

RESULTS AND DISCUSSION

Induction and degradation of thermogenin in brown fat cells differentiating in culture

Regulation of the amount of the uncoupling protein thermogenin in brown adipose tissue has attracted considerable interest because of the central role of this protein in thermogenesis. We and others have shown that it is possible, by addition of noradrenaline to brown fat cells differentiated in culture from undifferentiated precursor cells, to evoke a marked expression of the gene for thermogenin, when followed as either thermogenin mRNA [7,8] or as the amount of thermogenin itself [4,5,15,16]. The thermogenin thus synthesized can be incorporated into the mitochondria [4,17].

Confluence-dependent thermogenin expression. For the present study, we initially determined the degree of cell differentiation which would best be suited to study thermogenin induction and degradation in greater detail. This was necessary, as earlier studies have indicated that the cells in culture, while initially progressing through phases of increased differentiation, may with time apparently spontaneously dedifferentiate [8,18].

We therefore added noradrenaline to brown fat cell cultures on days 4–7 after inoculation; i.e. on the days just preceding and including confluence. A concentration of 10 μM-noradrenaline

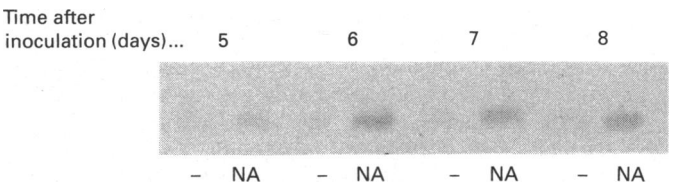

Fig. 1. Immunoblot showing the effect of time in culture on noradrenaline-induced thermogenin expression in cultured precursor cells from brown adipose tissue

Cultured cells were treated with noradrenaline (NA; final concentration 10 μM; added twice daily) or with water (−) for 24 h before harvesting on the indicated days. Cellular proteins were immunoblotted for thermogenin as described in the Materials and methods section. No other bands were visible in the blot. Similar results were obtained in two independent experimental series.

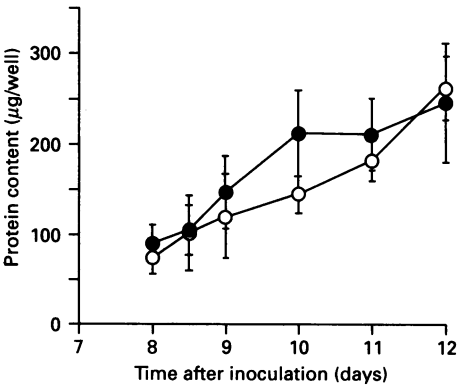

Fig. 2. Effect of time in culture and noradrenaline treatment on protein content of cultured brown fat cells

Cells were grown in culture for 7 days and then treated with noradrenaline (●; final concentration 10 μM, added twice daily) or with water (○; controls) for 24 h. After this 24 h period, the medium was discarded, all wells were rinsed once with 2 ml of DMEM, and 2 ml of new medium was added. The noradrenaline-treated wells again received noradrenaline and the other wells received water. Noradrenaline or water was always added twice daily. At the indicated time points, the medium was discarded from all wells and the cells from two wells per treatment were harvested for protein measurement and immunoblotting as described in the Materials and methods section. The remaining cells received fresh medium and a readdition of either noradrenaline or water. The data shown are the means ± S.E.M. from five independent experimental series.

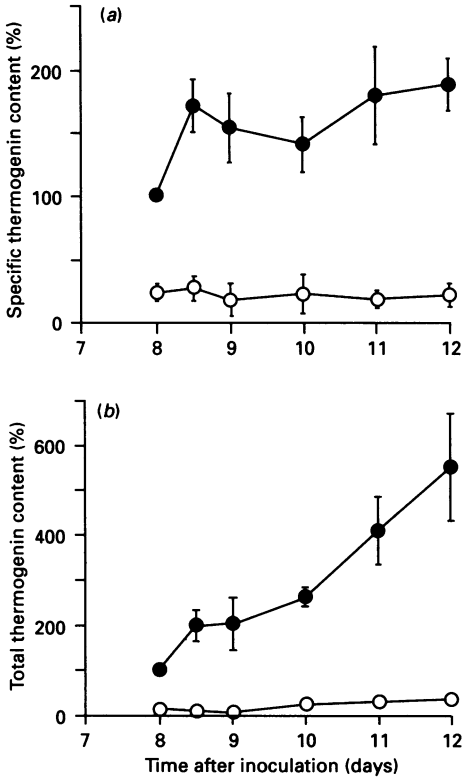

Fig. 3. Effect of noradrenaline treatment on thermogenin content

The cells were those described in Fig. 2. ●, Noradrenaline treatment; ○, control (water). The data were obtained by scanning of immunoblots for thermogenin, as described in the Materials and methods section. In five independent experimental series, individual wells were analysed and the values calculated relative to the initial value after 24 h of noradrenaline treatment in that series. The points shown are the means ± S.E.M. from the five experimental series; where not visible, the S.E.M. was smaller than the size of the symbol. (*a*) Specific thermogenin content (per mg of protein), (*b*) total thermogenin content, calculated from the data in Figs. 2 and 3(*a*).

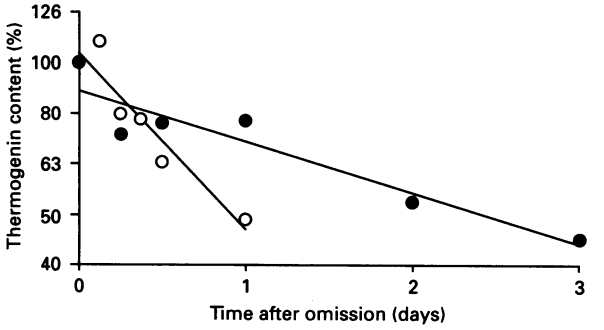

Fig. 4. Comparison of the half-lives of thermogenin after 1 and 5 days of noradrenaline treatment

Cell were grown in culture for 7 days and then treated for 1 or 5 days with noradrenaline (final concentration 10 μM, added twice daily). Thereafter, the cells were rinsed with DMEM and maintained in medium without noradrenaline. The cells were harvested at the indicated time points after noradrenaline omission and the thermogenin content was determined as described in the Materials and methods section. For each of the five culture series investigated, the content of thermogenin on culture day 8 (after 1 day of noradrenaline treatment) or day 12 (after 5 days of noradrenaline treatment) was set to 100% and the thermogenin content in each series was expressed relative to this. The values shown here are thus means from five independent series; S.E.M. values were about 8% of the mean. Lines were drawn according to least-squares analysis and the half-life calculated from the slope (note the logarithmic *y*-axis). ○, Noradrenaline treatment for 1 day ($t_{\frac{1}{2}} = 20$ h; $r = 0.94$); ●, noradrenaline treatment for 5 days ($t_{\frac{1}{2}} = 70$ h, $r = 0.95$).

(added twice daily) was used, since this was the optimal concentration for thermogenin induction in cells cultured for 6 days [4]. The cell cultures were analysed on the following day for the amount of thermogenin (Fig. 1). As can be seen, only a faint band of thermogenin was found in the untreated cells. However, particularly in cells older than 5 days, the addition of noradrenaline led to a conspicuous expression of thermogenin. This confluence-associated thermogenin inducibility is in good agreement with earlier observations in which thermogenin mRNA levels were measured [8].

Based on these results, it was decided to perform the study with cells which were treated with noradrenaline from day 7 after inoculation.

Thermogenin induction. Since our earlier results indicated that the induced thermogenin level had not reached a plateau after a 24 h noradrenaline stimulus [4], we decided to treat the cultures with noradrenaline for 24 h as in [4], and for longer periods of up to 5 days. Protein content and thermogenin were then determined at various time points and compared with values from cells which had received no adrenergic stimulation at all.

As seen in Fig. 2, the total amount of protein in these cell cultures was about 90 μg/well at day 8, and after this the cells continued to accumulate protein at a rate of about 40 μg/day. This increase in protein is probably mainly due to an increased amount of protein per cell [19], but some cell proliferation does also take place after apparent confluence, although at a much lower rate than before confluence [19]. It was not possible to alter the protein accumulation rate by noradrenaline treatment, in accordance with earlier observations [19].

As seen in Fig. 3(*a*), there was only a low level of specific thermogenin expression in the control cells which had not received any noradrenaline treatment, and in these experiments there was no tendency to any spontaneous increase in the expression of this differentiation marker with prolonged culture time. After 24 h of exposure to noradrenaline (from day 7 to day 8), the thermogenin expression had increased more than 4-fold, and continued exposure of the cells to noradrenaline elevated this level even further. This induction was even more marked when the results are presented as the total amount of thermogenin per well (Fig. 3*b*). The total amount of thermogenin continued to increase with undiminished velocity over the 5 days investigated.

Thermogenin degradation. As seen in Fig. 3(*a*), after 1 day of noradrenaline treatment the specific level of thermogenin, although markedly increased, was only half its final value, and the cells had thus not reached their maximal level of expression. We found it of interest to investigate if the half-life of thermogenin in this transition state was different from that of this protein in the cells which were chronically treated and had achieved an apparent new steady state. The degradation of thermogenin was thus measured after cessation of noradrenaline stimulation in cells which had been treated for either 1 or 5 days. As seen in Fig. 4, the rate of degradation of thermogenin was markedly different in the two states. The half-life of thermogenin in the acutely stimulated cultures was relatively short (20 h), but in the chronically noradrenaline-treated cells it was prolonged to 70 h.

Effect of inhibition of protein synthesis on thermogenin half-life. In order to investigate whether the degradation of thermogenin was due to the induction of active degradative processes, cell cultures in which thermogenin synthesis had been stimulated with noradrenaline for 1 or 5 days were treated for 12 h with the protein synthesis inhibitor cycloheximide [20], at a concentration

Table 1. Effects of cycloheximide on thermogenin degradation

Cells were grown in culture for 7 days as described in the Materials and methods section. All cultures were treated with noradrenaline (NA; final concentration 10 μM, twice a day) for 1 or 5 days, and the noradrenaline was then removed (0 h). Vehicle (−) or cycloheximide (Cyclohex; final concentration 50 μM, +) was then added. At 0 or 12 h after the cessation of noradrenaline treatment, the cells were harvested and analysed for protein and thermogenin content as described in the Materials and methods section. Results are means ±S.E.M. of 4–5 wells (from 4–5 independent experiments). * indicates a statistically significant effect of the cycloheximide addition ($P < 0.05$, Student's paired t test).

NA (days)	Cyclohex	Total protein (μg/dish) 0 h	Total protein (μg/dish) 12 h	Thermogenin (%) 0 h	Thermogenin (%) 12 h
1	−	67±7	90±10	100	63±6
1	+	78±13	54±11*	100	91±10*
5	−	300±33	345±40	100	76±9
5	+	292±34	228±40*	100	71±6

($50\ \mu$M) which in other cell systems has been shown to inhibit protein synthesis without producing acute cell toxicity [21]. In both the acutely and the chronically noradrenaline-treated cells which had not received cycloheximide, the protein content increased during the following 12 h (Table 1). In the cells which were cycloheximide-treated, the total protein content had already diminished after 12 h in both cases.

Concerning the thermogenin content, there was a remarkable difference in the effects of cycloheximide between acute and chronic noradrenaline treatment. In agreement with the above results in Fig. 4, in the cells in which the noradrenaline treatment was terminated in the absence of cycloheximide, the total amount of thermogenin was clearly decreased in the 12 h after cessation of stimulation in both cases. However, in the cycloheximide-exposed cells there was no decrease in thermogenin content at all in the acutely noradrenaline-treated cells, despite some loss of total protein. In the cycloheximide-exposed, chronically noradrenaline-treated cells, the thermogenin content decreased markedly, but this occurred in parallel with total protein loss. Thus there was an interesting correspondence between the difference in half-life between the acutely and chronically noradrenaline-treated cells and their susceptibility to cycloheximide.

The rapid degradation seen after the cessation of adrenergic stimulation in the acutely stimulated cells was clearly dependent upon uninhibited protein synthesis. It would therefore seem likely that specific proteins involved in the degradation process were induced by the cessation of the adrenergic stimulus. The nature of these proteins is not established; they could be proteases of the type described by Desautels *et al.* [22]. In contrast, the slow degradation observed in chronically noradrenaline-stimulated cells was not dependent on ongoing protein synthesis, and was thus not due to induced degradative proteins.

Thus the difference between thermogenin degradation in the two cases is not merely kinetic but also functional. It is likely that the newly synthesized thermogenin, which is found in the acutely treated cells, is more susceptible to degradation than that which has become fully incorporated into the mitochondria. Such a pool of newly synthesized thermogenin, with a high susceptibility to degradation, is probably also found in the chronically treated cells, but as this pool is relatively small compared with the large pool of fully incorporated thermogenin, it is not experimentally visible. Only by analysis in the transition period is this functionally interesting pool observable.

Thus the data presented above would indicate that a major part of the newly synthesized thermogenin is found in a more labile pool where it is exposed to the action of the degradative systems. With time (probably as a larger fraction becomes fully incorporated into the mitochondria) the protein is stabilized, and is protected against these degradative systems.

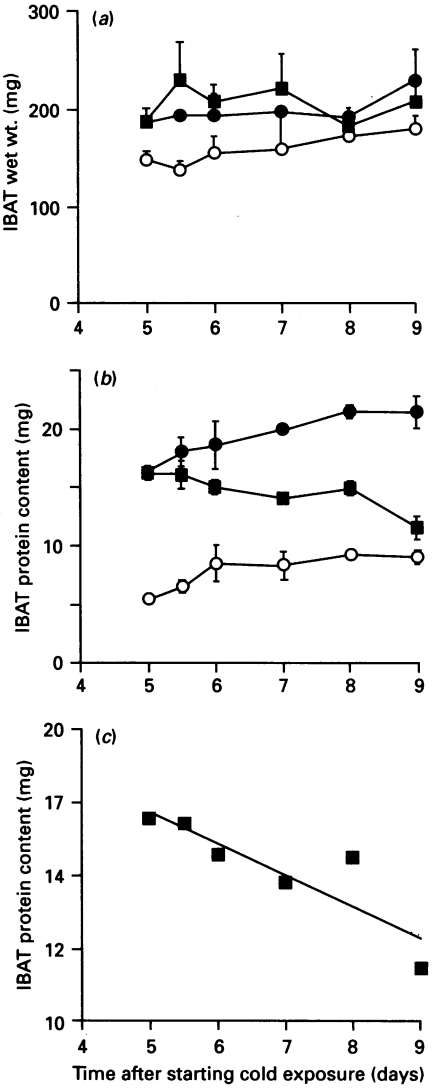

Fig. 5. Effect of cold treatment and deacclimation on brown adipose tissue wet weight (*a*) and protein content (*b*, *c*)

Mice which had remained at 28 °C (○), or which had been exposed to cold for 5 days before being returned to 28 °C (■) or which had remained in the cold (●) were killed at the indicated time points and the wet weight and protein content of the interscapular brown fat depot (IBAT) were determined. Results are means ±S.E.M. from three animals. In (*c*), the data from the 5 day cold curve are replotted with a logarithmic y axis; the decay was analysed by the least squares method ($r = 0.88$) and the half-life calculated from the slope to be 9 days.

Induction and degradation of thermogenin in brown adipose tissue *in vivo*

In order to further evaluate the findings concerning thermogenin induction and degradation in cell cultures, their relationship to similar processes *in vivo* was studied. Information on thermogenin degradation *in vivo* is not fully concordant [23–25], and we have therefore investigated these processes *in vivo* under physiological conditions which most closely resembled those used for the cell culture studies above, and using the same

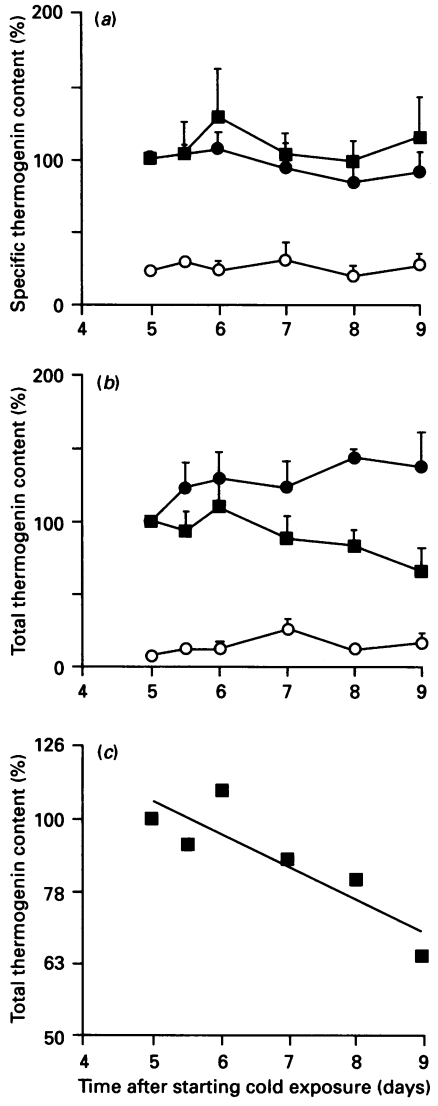

Fig. 6. Effect of cold treatment and deacclimation on thermogenin content in brown adipose tissue

Specific (*a*) and total (*b*) thermogenin content was measured in the brown fat obtained from the mice described in Fig. 5. ○, Controls (mice remaining at 28 °C); ■, mice exposed to cold for 5 days and then returned to 28 °C; ●, mice remaining in the cold. Results are means + S.E.M. from three animals at each point. In (*c*), the 5 day cold data from (*b*) were replotted on a logarithmic scale and the half-life was calculated from a least-squares fit of the data points to be 7 days ($r = 0.88$).

sensitive immunoblot technique for thermogenin determination. The *in vivo* model used consisted of mice which were cold-exposed, a treatment which leads to noradrenaline stimulation of the tissue [26]. Cold exposure for 5 days was chosen for these experiments, as it is known that the thermogenin level is clearly different from controls at the end of this period [27].

As seen in Fig. 5(*a*), the wet weight of the interscapular brown adipose tissue depot was about 130 mg in control animals, and it did not change during the following 4 days. There was only a small increase in wet weight due to 5 days of cold exposure, and irrespective of whether the animals remained in the cold or were returned to 28 °C, the wet weight of the tissue remained slightly elevated but not markedly different between these two groups.

However, as expected, marked effects of treatment were observed on the protein content of the tissue (Fig. 5*b*). The protein content remained below 10 mg in the control animals, but in animals after 5 days in the cold this value had increased 3-fold. When the mice remained in the cold the protein content tended to continue to rise, whereas in the mice returned to 28 °C the protein content slowly diminished, approaching control levels after 4 days. When this was analysed as an exponential decay (Fig. 5*c*), a half-life of about 9 days for total protein content during deacclimation was estimated.

Very marked differences between the groups were observed when the specific thermogenin content was analysed. There was only a low specific content (per mg of protein) of thermogenin in the control animals, but after 5 days in the cold the specific content had increased 4-fold (Fig. 6*a*). It remained at this level during the subsequent 4 days, irrespective of whether the animals remained in the cold or were reacclimated to 28 °C.

The total thermogenin content was low in the animals which had not been exposed to cold, but it had increased at least 12-fold during the 5 days in the cold, and it tended to continue to increase during subsequent days in the cold (Fig. 6*b*). In the animals which were returned to 28 °C the thermogenin content slowly decreased, but even after 4 days it was still much higher than in the controls. When these data were analysed as an exponential decay (Fig. 6*c*), the half-life of thermogenin during the deacclimation phase was calculated to be about 7 days, i.e. not significantly different from that of total protein. Thus, as was also evident from Fig. 6(*a*), the specific content of thermogenin (per mg of protein) did not change during the deacclimation period, i.e. there was no selective degradation of thermogenin in the *in vivo* experiment.

It may be noted that despite differences in the detailed treatment of animals and the use of different methods for thermogenin determination, the qualitative and quantitative outcome of this experiment *in vivo* is similar to that of Desautels *et al.* [23]. Thus during deacclimation, thermogenin and protein loss parallel each other. The protein half-life during the deacclimation phase is also similar to the half-lives obtained by Bukowiecki & Himms-Hagen [28] who, in labelling experiments, monitored total protein turnover in the tissue (in rats) in physiologically stable conditions.

Conclusions

Thermogenin expression in cultured brown fat cells can be markedly and rapidly increased by noradrenaline stimulation. We have found here that for a pool consisting of newly synthesized thermogenin, a very high degradation rate is observed when the noradrenaline stimulation is removed. It would seem that the high degradation rate of this pool is the result of induction of certain proteins involved in the degradative process [29], although nothing can be currently concluded about their nature. In contrast to the rapidity with which the pool of newly synthesized thermogenin was degraded, the thermogenin which was accumulated during prolonged noradrenaline stimulation was much more stable, and a specific degradation system did not seem to be activated upon noradrenaline removal. Thus the thermogenin degradation mechanisms in the two conditions seem to be different in principle. For newly synthesized thermogenin a specific degradation mechanism must be evoked, as degradation occurs without a decrease in the protein content. For thermogenin in the chronically noradrenaline-treated cells and *in vivo*, degradation parallels the general rate of protein turnover and a selective mechanism need not be evoked. It may therefore be speculated that incorporation of thermogenin into mitochondria would appear to be a rate-limiting step, and that a post-translational mechanism exists which ensures the rapid degradation of newly synthesized molecules should the physiological stimulation cease.

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