Norepinephrine-induced synthesis of the uncoupling protein thermogenin (UCP) and its mitochondrial targeting in brown adipocytes differentiated in culture

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Synthesis of the brown adipocyte-specific mitochondrial uncoupling protein thermogenin (UCP) is demonstrated here in brown adipocytes differentiated in culture from precursor cells. By immunoblotting, no UCP was detectable in untreated multilocular adipocytes. The synthesis of UCP was stimulated by norepinephrine at physiological concentrations and was observable already after 2 h. It was evident from immunoelectron microscopy that the newly synthesised protein was targeted to the mitochondrial inner membrane, demonstrating the functional competence of these cultured cells.

Uncoupling protein; Thermogenin; Norepinephrine; Mitochondrial targeting; Nonshivering thermogenesis; Brown adipose tissue

1. INTRODUCTION

The recruitment process in brown adipose tissue is the basis for the enhancement of the thermogenic capacity in mammals adapted to cold and certain diets [1-4]. This process includes an increase in the amount of the rate-limiting protein for thermogenesis, the uncoupling protein thermogenin (UCP), in brown adipose tissue mitochondria. An involvement of the sympathetic nervous system in the control of the recruitment process has been implicated by in vivo studies. However, the exact delineation of this process has been hampered by the inherent limitations of in vivo systems.

Consequently, a cell culture system has been developed to study the growth and differentiation of brown adipose tissue [5]. After several days in culture the precursor cells have acquired many of the characteristics of the mature brown adipocyte [5-8]. However, we have not as yet demonstrated the synthesis of the brown adipocyte-specific uncoupling protein thermogenin (UCP) in these cells [9], which we have differentiated in culture from non-fetal precursor cells. Recently, culture conditions have been achieved which permit UCP gene expression in these cells, and it has been demonstrated that synthesis of mRNA coding for UCP can be induced by both α- and β-adrenergic agonists [10,11].

The aim of the present study was to determine whether these cultured cells had attained the competence to translate the UCP-mRNA into the uncoupling protein itself. Especially, we wanted to ascertain the effect of the physiological agonist norepinephrine on the synthesis and the targeting of this protein to the mitochondria.

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2. MATERIALS AND METHODS

2.1. *Cell isolation and culture*

Precursor cells from the brown adipose tissue of 4-week-old male mice (NMRI outbred strain) were isolated and cultured under identical conditions to Rehnmark et al. [11], that is, in a culture medium consisting of DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% newborn-calf serum, 4 nM insulin (Insulin Actrapid, Novo Industries), 10 mM Hepes, and 50 IU penicillin, 50 μg streptomycin and 25 μg sodium ascorbate per ml. The cells were grown at 37°C in an atmosphere of 8% CO_2 in air with 80% humidity. The medium was changed on day 1 and 3 after inoculation. On day 6, norepinephrine ((-)-Arterenol bitartrate, Sigma) was added to duplicate flasks as specified in the figure legends. After the indicated treatment period, the cells were harvested for immunoblotting and immunoelectron microscopy.

2.2. *Immunoblotting*

After the times indicated, cultured cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) (5 ml; pH 7.4) and harvested by scraping in SDS-PAGE sample buffer (500 μl; containing 62.5 mM Tris, 4% SDS, 5% 2-mercaptoethanol, 10% glycerol, and Bromophenol blue, pH 6.8). Aliquots (70 μl) of the total cell lysates were separated by 10% SDS-PAGE according to Laemmli [12]. Silver-staining of certain gels was performed as in Wray et al. [13].

Electrotransfer of the separated proteins to Hybond-C Extra nitrocellulose membranes (pore size 0.45 μm; Amersham) was carried out with a semi-dry electroblotter (JC Biotechnical Instruments) at 200 mA for 2 h, according to Kyhse-Andersen [14], with β-alanine (10 mM) in the cathode buffer [15]. After the electrotransfer, non-

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specific binding was quenched overnight with 1% bovine serum albumin (BSA) in 50 mM Tris, 150 mM NaCl and 0.5% Tween-20 (TST). After quenching (and between each antibody incubation), the blots were washed 3 × 5 min in TST. The blots were then probed for 2 h with antiserum (dilution 1:1000 in TST containing 0.1% BSA) raised in rabbits against rat UCP [16] and labelled for 1 h with goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (dilution 1:1000 in TST containing 0.1% BSA; Sigma). (In immunoblots performed with certain batches of this conjugated antibody, a non-specific reaction in the vicinity of molecular weight 30 kDa was observed.) The bands were visualised with a substrate mixture of β-naphthyl phosphate and Fast blue B according to Turner [17]. Colour development was stopped by rinsing with 70% alcohol and distilled water.

For quantitative analysis, the bands were scanned with a Molecular Dynamics 200A Laser Densitometer and the results expressed as a percentage of the mean maximum value.

2.3. *Immunoelectron microscopy*

To the cells in the culture medium, fixative (2 ml) containing 2% glutaraldehyde, 1% paraformaldehyde, 100 mM phosphate buffer and 100 mM sucrose was added, mixed and then discarded. Fixative (4 ml) was again added to the cells for 4 h, removed and then replaced by 100 mM phosphate buffer containing 100 mM sucrose. The cell monolayer was scraped as sheets and these placed in 100 mM phosphate buffer in order to wash away the fixative. Thereafter, the cells were embedded, sectioned and examined by immunoelectron microscopy according to Lončar et al. [18] with the same antiserum as above.

2.4. *Materials*

Rat UCP was isolated essentially according to Lin and Klingenberg [19] with modifications by Cannon et al. [16]. Purified rat UCP was concentrated prior to SDS-PAGE by centrifuge-filtration with an Ultrafree-MC filter unit with 10 kDa cut-off (Millipore). Homogenates from mouse brown adipose tissue and liver were prepared in 250 mM sucrose, 10 mM Tris-HCl and 1 mM EDTA (pH 7.4).

Molecular weight markers were purchased from Biorad. The source of the materials used for cell isolation, culture and harvesting was as given previously in Néchad et al. [5], and for immunoelectron microscopy as in Lončar et al. [18].

3. RESULTS AND DISCUSSION

An effect of norepinephrine on the rate of transcription of the UCP gene, determined as the amount of UCP mRNA in brown adipocytes matured in culture, has recently been observed [10,11]. In order to ascertain that such cells are fully competent in translation and targeting processes, we have here investigated the effect of norepinephrine on UCP synthesis in these cells.

3.1. *Effect of norepinephrine treatment on UCP expression*

Precursor cells were grown in culture for 6 days under conditions identical to those of Rehnmark et al. [11]; a time optimal for demonstration of norepinephrine-induced expression of the UCP gene (UCP mRNA synthesis). The cells were then treated with norepinephrine for 24 h in order to allow sufficient time for activation of synthetic processes. Total cell lysates were then made from control cells and from the norepinephrine-treated cells, the proteins separated by SDS-PAGE and the protein patterns examined, after silver-staining, for changes in protein expression (Fig. 1). No obvious differences in protein pattern were observed, indicating that norepinephrine had no general effect on steady state protein levels in the cultured cells. However, there was a weak tendency for certain bands, in the region of approximate molecular mass 30 kDa, to be increased in density by the norepinephrine treatment.

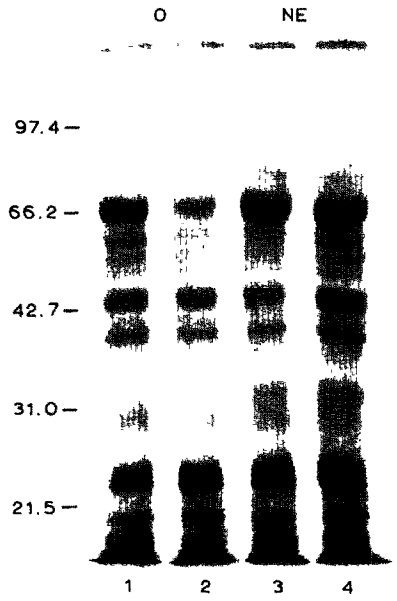

Fig. 1. The effect of norepinephrine on steady-state protein levels in cultured precursor cells from mouse brown adipose tissue. Cultured cells were grown for 6 days and then treated for 24 h with 10 μM norepinephrine (added at 0 and 7 h). The cells were harvested after the treatment period and total cell lysates prepared as in section 2. After SDS-PAGE, the gel was silver-stained. Four parallel cultures, two untreated (lanes 1 and 2) and two treated with 10 μM norepinephrine (lanes 3 and 4), are shown. The positions of the molecular weight markers are indicated.

We then investigated whether there was a specific effect of norepinephrine on the amount of the brown adipocyte-specific uncoupling protein thermogenin (UCP); this was performed by immunoblotting for UCP. Fig. 2A shows an immunoblot for UCP in cultured brown adipocytes under conditions similar to those above. In untreated cultures, no signal (band) for UCP was seen (lane 1). However, when the cells were stimulated with as little as 10 nM norepinephrine for 24 h, there was a visible signal for UCP (lane 2). Concentrations of 0.1 μM and above gave further inductions of the signal (lanes 3–5). It should be noted that the bands ran at a molecular mass of 32 kDa and closely paralleled to the signal corresponding to purified rat UCP (lane 8).

Fig. 2B shows the results of scanning an immunoblot derived from the same experiment as the one depicted in Fig. 2A. The effect of the norepinephrine treatment on UCP expression is clearly shown by this dose-response curve. These results show that norepinephrine is necessary for the synthesis of detectable amounts of

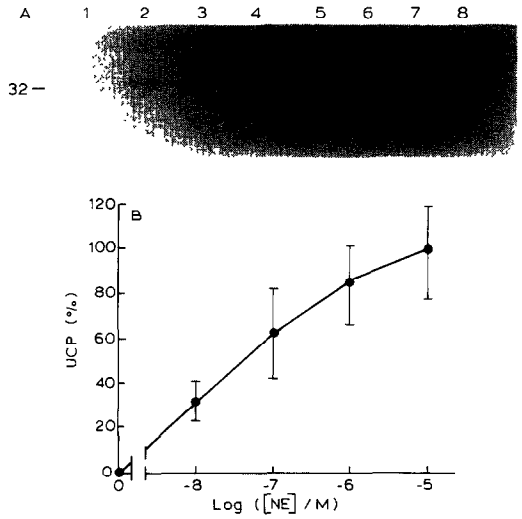

Fig. 2. Norepinephrine-induced UCP expression in cultured precursor cells from mouse brown adipose tissue. (A) Immunoblot from a 10% SDS-PAGE of total cell lysate of cell grown in culture for six days. Cells were treated for 24 h with norepinephrine (added at 0 and 7 h) at the following concentrations: 0 (lane 1), 10 nM (lane 2), 0.1 μM (lane 3), 1.0 μM (lane 4) and 10 μM (lane 5). '32' indicates the 32 kDa molecular weight position according to calculation from molecular weight markers. As controls, mouse brown adipose tissue homogenate (lane 6; 100 μg), mouse liver homogenate (lane 7; 100 μg) and purified rat UCP (lane 8; 5.6 μg) were run. The blot was developed with antiserum against rat UCP. No other bands were visible on the blot. (B) Graph of data compiled after densitometric scanning of an immunoblot derived from the same experiment as the one depicted in (A). Each point represents the mean (and individual values) for UCP analysed in the two parallel culture flasks. The results are expressed as a percentage of the value at 10 μM norepinephrine (NE).

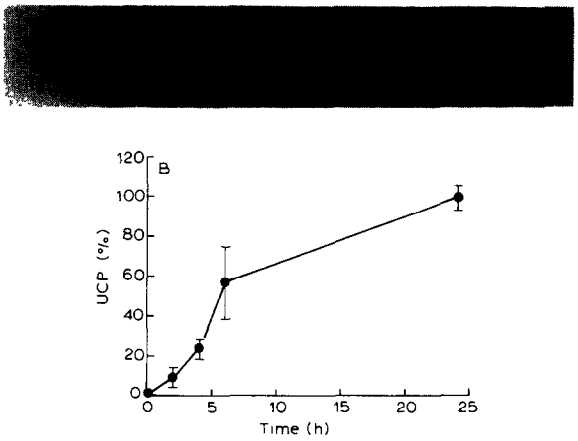

Fig. 3. Time course for norepinephrine-induced UCP expression in cultured precursor cells from brown adipose tissue. (A) Immunoblot from an SDS-PAGE performed as in section 2. Norepinephrine (10 μM) was added to the culture flasks and the cells harvested at the times shown. (Norepinephrine was added again after 7 h to the flasks analysed at 24 h). (B) Graph of the data obtained by scanning of immunoblots as in (A). In two independent experimental series, duplicate flasks were analysed and for each series the mean value calculated relative to the mean value after 24 h. The points shown are the means ± SE from the two experimental series (2 h value only from one series).

UCP in the cultured brown adipocytes and that maximal effects are seen with norepinephrine concentrations in the micromolar range. Recently, we have shown that under identical culture conditions, UCP mRNA levels increased due to increased transcription, in response to norepinephrine, with a maximum signal occurring at 0.1 μM [11]. Taken together, it seems therefore highly likely that the UCP observed here after norepinephrine treatment, arises from translation of UCP transcripts, these being, in turn, the product of norepinephrine-induced nuclear transcription of the UCP gene.

Previous in vivo studies [20–23] have implied that both UCP mRNA and UCP synthesis are apparently stimulated by norepinephrine treatment. However, this is the first demonstration that norepinephrine in itself is able to activate not only the transcription of the UCP gene, but also the synthesis of the UCP protein itself, and that the effect of norepinephrine treatment in vivo is indeed directly on the brown adipocyte itself. A fully functional in-vitro system for the study of UCP gene expression is thus now available.

To elucidate the kinetics of the UCP induction, a dose of 10 μM norepinephrine was used, since this dose gave an effective stimulation of UCP expression after the 24 h time-period (see Fig. 2B). The norepinephrine was added to brown adipocytes grown as above and the cells harvested for immunoblotting after different lengths of treatment. Fig. 3A shows an immunoblot depicting the increasing density of the UCP signal with time ater the addition of norepinephrine. There was a visible UCP signal as early as 2 h after the addition of norepinephrine, while the strongest signal appeared after 24 h. The results of scanning immunoblots developed after two time course experiments are shown in Fig. 3B. The UCP amount in the cultured cells rose most rapidly during the first 6 h. We have recently shown that specific UCP mRNA levels begin to rise within 15 min of norepinephrine addition to cells grown under identical culture conditions [11]. This strongly suggests that UCP gene activation precedes and is causal to the increased UCP protein levels.

The kinetic results presented in Fig. 3B may be compared to in vivo results of Ricquier et al. [24]. In those experiments, UCP levels were estimated after different times of cold exposure of rats by densitometrical analysis of electrophoretically-separated mitochondrial proteins from brown adipose tissue. Within 2 h of cold exposure, the authors observed a small but significant increase in the relative UCP level. Therefore, the results presented in Fig. 3 show that the cell culture system used here is able to temporally mimic an important event occurring in brown adipose tissue in response to a cold stress, namely, the induced synthesis of UCP. Thus, for the study of the molecular mechanisms of recruitment

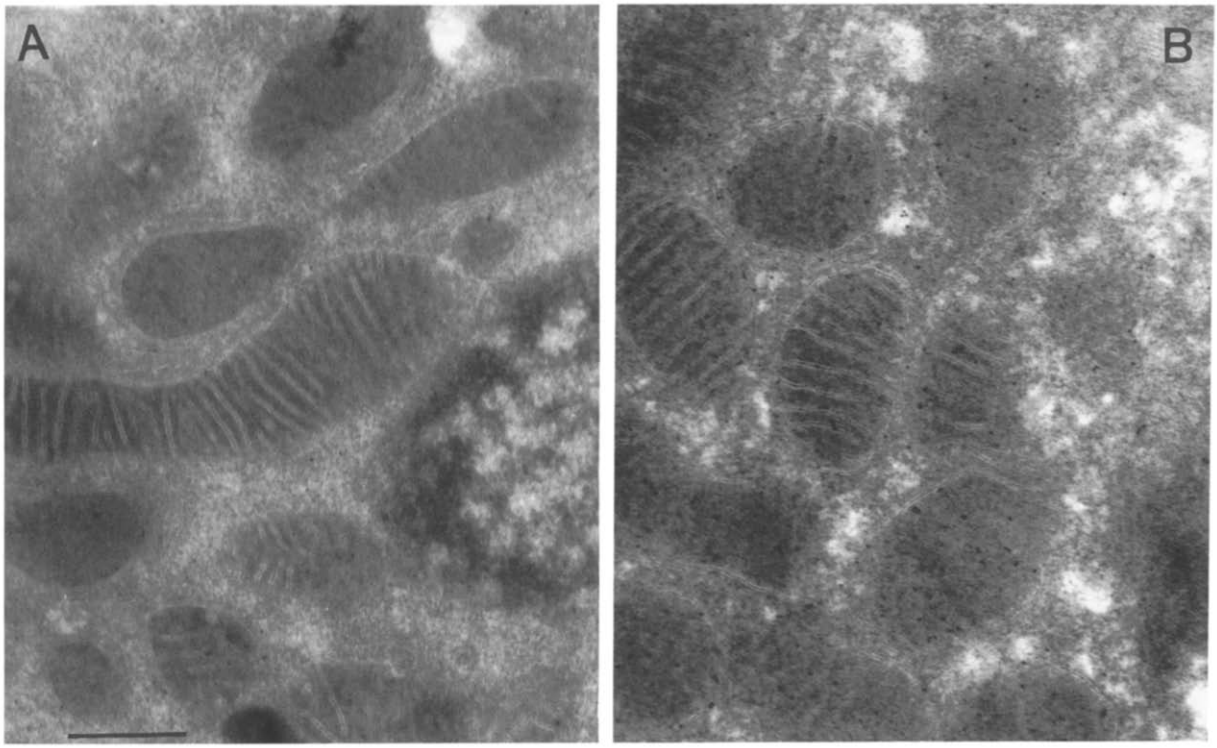

Fig. 4. The effect of norepinephrine on intracellular targeting of UCP in cultured precursor cells from brown adipose tissue. Immunoelectron microscopy was performed as described in section 2. Cultured cells, grown for 6 days, were either untreated (A) or treated for 24 h with 10 μM norepinephrine (added at 0 and 7 h) (B). After this period of stimulation, the cells were fixed, sectioned and examined. Antiserum to rat UCP and a protein A-gold conjugate were used to visualise the protein. Length of bar 1 μm (same magnification on both micrographs).

in brown adipose tissue, this culture system represents a biologically accurate model. Since no UCP is detectable before the norepinephrine treatment, even small increases in UCP level can easily be detected, and the cell culture system is therefore more sensitive than the in vivo system.

3.2. *Incorporation of UCP into mitochondrial membranes*

Since the synthesis of UCP in the brown adipocyte cultures was induced by norepinephrine treatment, the question arose as to whether the UCP synthesised was transferred to its functional location, i.e. the mitochondrial inner membrane. Therefore, the fate of the newly synthesised protein was investigated.

Cells were grown as above, treated with 10 μM norepinephrine for 24 h and then fixed for immunoelectron microscopy. Fig. 4 shows the results of this immunocytochemical investigation of the localisation of UCP in untreated (A) and norepinephrine-treated cultured cells (B). In the untreated cultured cells (A), almost no electron-dense dots corresponding to UCP were seen. This finding was completely in agreement with the immunoblot results (Figs. 2 and 3). In contrast, in the case of the norepinephrine-treated cells (B), numerous dots were observed, clearly located over the mitochondria and apparently over the mitochondrial cristae. This is identical to the localization seen in tissue obtained from animals in which the brown adipose tissue was in a recruited state [18,25–27]. Thus, it would seem that the newly synthesised UCP had been targeted to its functional site. Although not quantified in the present investigation, it was our impression that the morphology of the mitochondria had changed during this treatment, from being elongated in the untreated cells to becoming more rounded after norepinephrine treatment. Whether this is secondary to the insertion of UCP or is an independent effect of norepinephrine treatment is not known.

Based on the rapid time-course for insertion of UCP shown in this experiment it would seem that the UCP is incorporated into pre-existing mitochondria, rather than having to await the synthesis of a new mitochondrial population.

4. CONCLUSIONS

We present here evidence that while UCP was undetectable in untreated brown adipocytes differentiated in culture, synthesis of the protein could be induced by norepinephrine. The newly synthesised UCP was targeted to the mitochondrial inner membrane. Thus, the potency of norepinephrine to promote the process from the activation of the UCP gene [11],

through to the synthesis and final insertion of the protein into the mitochondrial membrane has now been demonstrated.

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Note added in proof: In a parallel study with brown adipocytes differentiated under similar conditions, Houštěk et al. have recently also been able to demonstrate the synthesis of UCP (in Structure, function and biogenesis of energy transfer systems (E. Quaglieriello et al., eds.) Elsevier 1990, pp. 87–90).