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Differential adrenergic regulation of C/EBPα and C/EBPβ in brown adipose tissue

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We investigated the regulation of the expression of two members of the C/EBP family of transcriptional activators, C/EBPα and C/EBPβ, in brown adipose tissue in mice. Less than one hour of cold exposure led to dramatic changes in the expression of both genes. C/EBPα steady-state mRNA and protein levels were drastically and rapidly reduced whereas C/EBPβ mRNA and protein levels were induced severalfold. Also norepinephrine injection affected the expression of the transcription factors. Preconfluent cells in brown fat primary cultures responded to norepinephrine with a decrease in C/EBPα and an increase in C/EBPβ mRNA; in confluent cells the expression of both factors was increased. Thus, C/EBPα and C/EBPβ gene expression is under adrenergic control both in vivo and in vitro but the type of response is directed by the degree of differentiation of the cells.

Gene expression regulation; Differentiation; Transcription factor; C/EBPα; C/EBPβ; Brown adipose tissue; Mouse

1. INTRODUCTION

Recently, several transcription factors that belong to the CCAAT-Enhancer Binding Protein (C/EBP) family have been characterized and their corresponding genes cloned. These cloned genes include C/EBPα cloned from rat [1] and mouse [2], and C/EBPβ which was first cloned from man as NF-IL6 [3] and subsequently from rat and mouse, originally designated IL6-DBP, C/EBPβ, rNFIL-6, LAP, CRP II or AGP/EBP [4–9]. The C/EBP family members share extensive homology in their DNA-binding and dimerization domains; they belong to the 'basic leucine zipper' (bleuzip) class of transcription factors. Several reports, primarily resulting from in-vitro experiments on cell lines, indicate that C/EBPα and C/EBPβ may be associated with cell proliferation and cell differentiation [2,7,10–16].

In order to investigate the role of C/EBPα and C/EBPβ gene expression in a physiological context, we have here investigated the expression of these genes in brown adipose tissue. The brown fat cells in the tissue can be induced to proliferate and differentiate in response to a simple environmental stimulus: exposure to cold [17].

In this report we demonstrate that this physiological recruitment of brown adipose tissue leads to dramatic changes in both C/EBPα and C/EBPβ gene expression. Furthermore, such in-vivo studies, as wel as in-vitro studies in primary cultures of brown fat cells, indicate that the expression of the C/EBPα and C/EBPβ genes in this tissue may be strongly affected by norepinephrine, in good agreement with the fact that norepinephrine in these cells may promote both cell proliferation and differentiation [18]. The type of response appears to correlate with the different stages of cell growth.

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2. MATERIALS AND METHODS

2.1. *Animal experiments*

Mice (outbred NMRI, 2 months of age) were obtained from a local supplier (Eklunds, Stockholm). They were preacclimatized to 28°C for 1 week. Some mice were then transferred to 4°C and were studied after the indicated times in the cold. In some cases 1 mg/kg b.w. of norepinephrine bitartrate (Sigma) was injected i.p. 4 h before RNA isolation. Total RNA was isolated from interscapular brown fat as previously described [19,20] and analysed as described below.

2.2. *Cell isolation and culture*

Brown fat precursor cells were isolated and cultured as previously described [21]. The cells were plated in 25-cm^2 culture flasks (Bibby) or six-well multidishes (Corning) containing Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 4 nM insulin, sodium ascorbate 25 μg/ml, 10 mM HEPES, 4 mM glutamine, penicillin 50 IU/ml and streptomycin 50 μg/ml. The cells were grown at 37°C in an atmosphere of 8% CO_2 in air.

2.3. *RNA isolation and Northern blot analysis*

The cells were harvested as previously described [21] on the indicated days and total RNA isolated by ethanol precipitation in acid guanidine-HCl as previously described [19,20]. Total RNA was separated on 1.25% agarose gels containing 20 mM MOPS (pH 7.0), 6.6% formaldehyde, 50 mM NaOAc (pH 5.6) and 10 mM EDTA. The RNA was blotted to Hybond-N (Amersham) or Zeta-Probe blotting membrane (Bio-Rad) and the blots hybridized with the indicated cDNA probes. The probes used for hybridization corresponded to a 400 bp

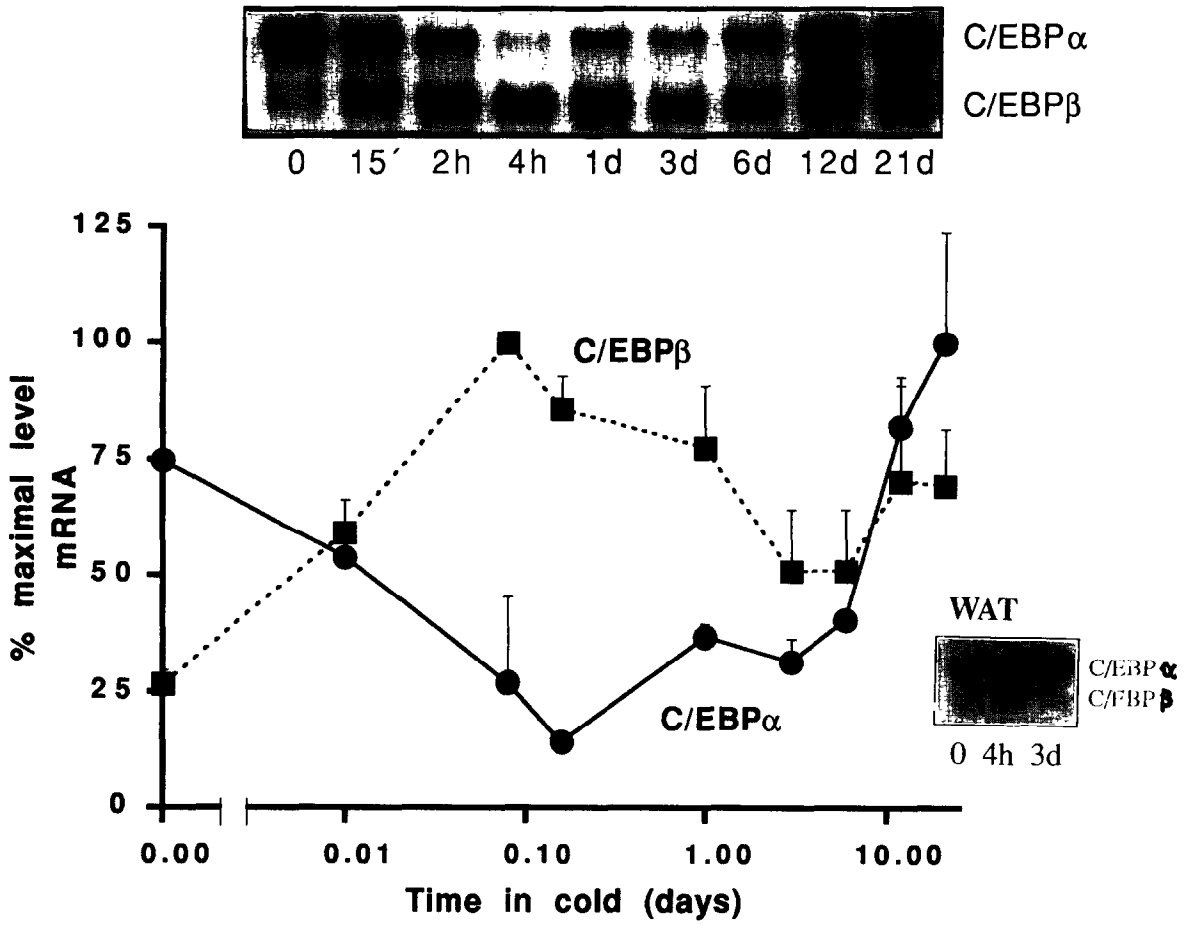

Fig. 1. Effect of cold exposure on the mRNA levels of C/EBPα and C/EBPβ in mouse brown adipose tissue. Four-week-old male mice were pre-acclimatized to 28°C for one week. The animals were then exposed to cold (4°C) for the indicated lengths of time. Total RNA was isolated from the interscapular brown adipose tissue. Ten μg of total RNA were run on each agarose gel lane, blotted to Zeta-Probe nylon membrane and hybridized with C/EBPα and C/EBPβ probes as described in section 2. The blot shows results from one experimental series. Values given in diagram are means ± S.E.M. from 2 (C/EBPα) or 3 (C/EPBβ) experimental series. (Insert) Effect of cold exposure on C/EBPα and C/EBPβ mRNA levels in epididymal white adipose tissue (WAT) from the same animals as the blots.

Pst–Sst fragment of the gene coding for the CCAAT-Enhancer Binding Protein (C/EBPα) [2] and to a full-length cDNA for the liver-enriched transcriptional activator protein (C/EBPβ) [5]. The cDNAs were labelled with [^{32}P]CTP by random priming (Boehringer Mannheim). The specific activity was typically more than 10^8 cpm/μg DNA.

2.4. *Hybridization*

The blots were prehybridized in 50% formamid, 5 × SSC, 50 mM NaP, (pH 6.5), 5 × Denhart, 0.5% SDS, 100 μg/ml herring sperm DNA at 42°C for 1 h and hybridized at 42°C for at least 16 h in the same solution containing the labelled cDNA probes. After hybridization, the membranes were washed 2 × 30 min at room temperature in 2 × SSC, 0.2% SDS, and 2 × 60 min at 50°C in 0.1 × SSC, 0.2% SDS. The resulting autoradiograms (Kodak) were evaluated by scanning with a laser densitometer (Molecular Dynamics 300A computing densitometer).

2.5. *Nuclear extracts and immunoanalysis*

Brown adipose tissue nuclei were isolated from 2-month-old mice which had been exposed to 4°C for 16 h. Control animals of the same age were kept at 28°C. 40–50 mg of brown adipose tissue per animal was dissected and used to isolate nuclei as described [22].

Nuclear extracts from both groups were prepared as described [2]. The nuclear extracts were fractionated on a 12% SDS-polyacrylamide gel (23 μg protein per lane) and electroblotted onto a nitrocellulose filter. Immunoblot analysis was performed with a rabbit polyclonal antibody directed towards a peptide corresponding to the basic region of the mouse C/EBPα protein which was further purified on a C/EBPα-peptide column. C/EBPβ (IL6-DBP) antibodies (a generous gift from Dr. Cortese) had been raised against a bacterially expressed rat recombinant protein [6]. C/EBPα and C/EBPβ nuclear proteins were detected at 1:500 and 1:1,000 (respectively) dilution of the primary antibodies and visualized by an enhanced chemiluminescent detection system (Amersham) with horseradish peroxidase-conjugated swine anti-rabbit IgG antibodies (Dakopatts) as the secondary antibodies.

Silver staining was performed on parallel lanes with 11 μg protein per lane with a kit used according to the manufacturer's instructions (Amersham).

3. RESULTS

3.1. *Physiological regulation of C/EBPα and C/EBPβ expression in brown adipose tissue*

In order to examine whether the expression of C/

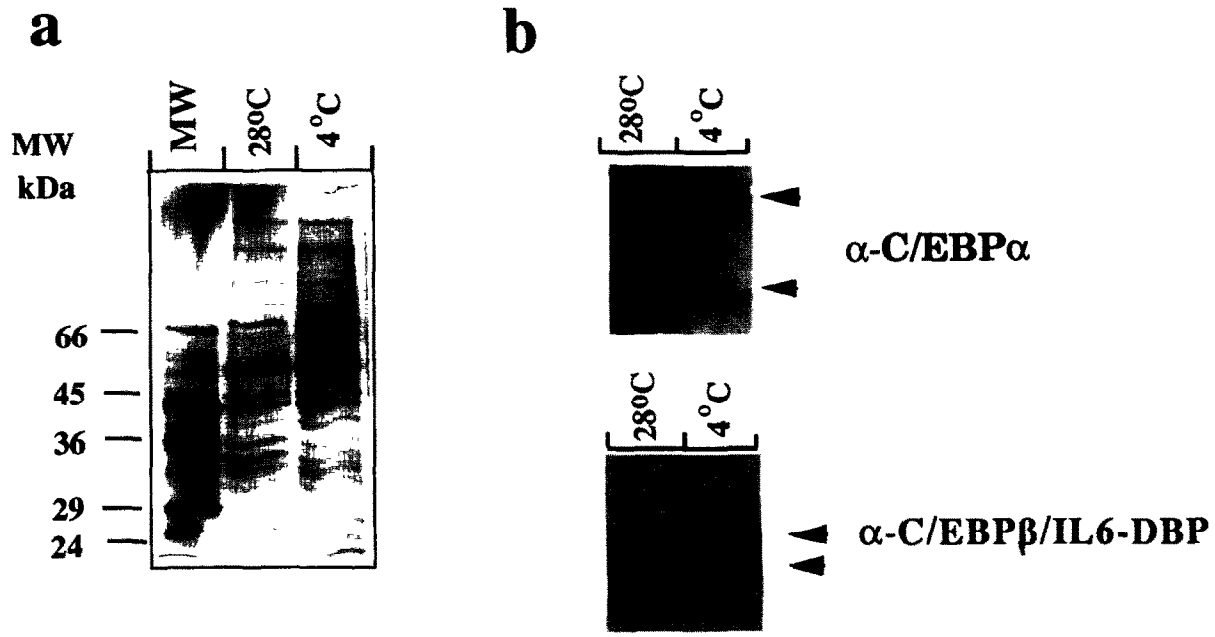

Fig. 2. Effect of cold exposure on the protein levels of C/EBPα and C/EBPβ in mouse brown adipose tissue. Equal amounts of nuclear extracts from mice exposed to 28°C and cold (4°C) were fractionated on a 12% SDS-PAGE and analyzed (a) by silver staining (11 μg per lane) or (b) by immunoblot analysis.

EBPα and C/EBPβ genes levels was regulated in brown adipose tissue during the recruitment phase, RNA from mice exposed to 4°C for different lengths of time was analysed.

The results presented in Fig. 1 show that there was a relatively high expression of C/EBPα mRNA in non-activated brown adipose tissue. The expression of C/EBPα mRNA was decreased about fivefold already within 4 h of cold exposure (Fig. 1). The decrease was very pronounced until the 6th day of cold exposure. After 12 days of cold exposure the expression of C/EBPα mRNA was back to the initial level seen in control animals. It then remained at this level even after 6 months of cold exposure (not shown).

There was a relatively low expression of C/EBPβ mRNA in non-activated brown adipose tissue (28°C). In contrast to the decrease in C/EBPα-expression, C/EBPβ mRNA was rapidly increased and the highest level was detected already within the first hours of cold exposure (Fig. 1). The amount of C/EBPβ mRNA was markedly elevated even after 6 months of cold exposure, compared to the level at 28°C (not shown).

These observations in brown adipose tissue should be contrasted to the effect of cold exposure on the expression of these genes in white adipose tissue (Fig. 1, insert). In this tissue, the mRNA levels of both genes were unaffected by cold exposure; this was also the case in liver (not shown).

To investigate whether the effects of cold exposure observed at the mRNA level for C/EBPα and C/EBPβ corresponded to changes at the corresponding protein level, we performed immunoblot assays. As shown in Fig. 2b, exposure of mice to 4°C for 16 h resulted in large changes in the levels of the two proteins. As previously demonstrated in other tissues [10,23], antibodies against C/EBPα detect two polypeptides of apparent molecular mass 43 kDa and 34 kDa, corresponding to the intact protein and a possible degradation product, respectively (Fig. 2b). The immunoblot demonstrated that exposure to cold reduced the amount of C/EBPα about three times, in good correlation with the reduction at the mRNA level seen from the mRNA data under the same conditions.

C/EBPβ antibodies detected two polypeptides as observed elsewhere [5]. The amount of C/EBPβ protein was induced about two to three times. This was similar to the induction observed at the mRNA level.

Thus, these protein results correlated well with the changes observed at the mRNA levels.

3.2. *Effect of norepinephrine on C/EBPα and C/EBPβ expression in vivo*

Norepinephrine injections into an animal can activate brown adipose tissue and induce several features of the recruitment process [24] including stimulation of expression of the brown fat-specific expression of the uncoupling protein (UCP) gene, both in rats [25] and mice [26]. This effect of norepinephrine injection is supposedly a mimicking of the effect of release of norepinephrine from the sympathetic nervous system in the physiologically stimulated animal.

Therefore, the effect of norepinephrine injection on

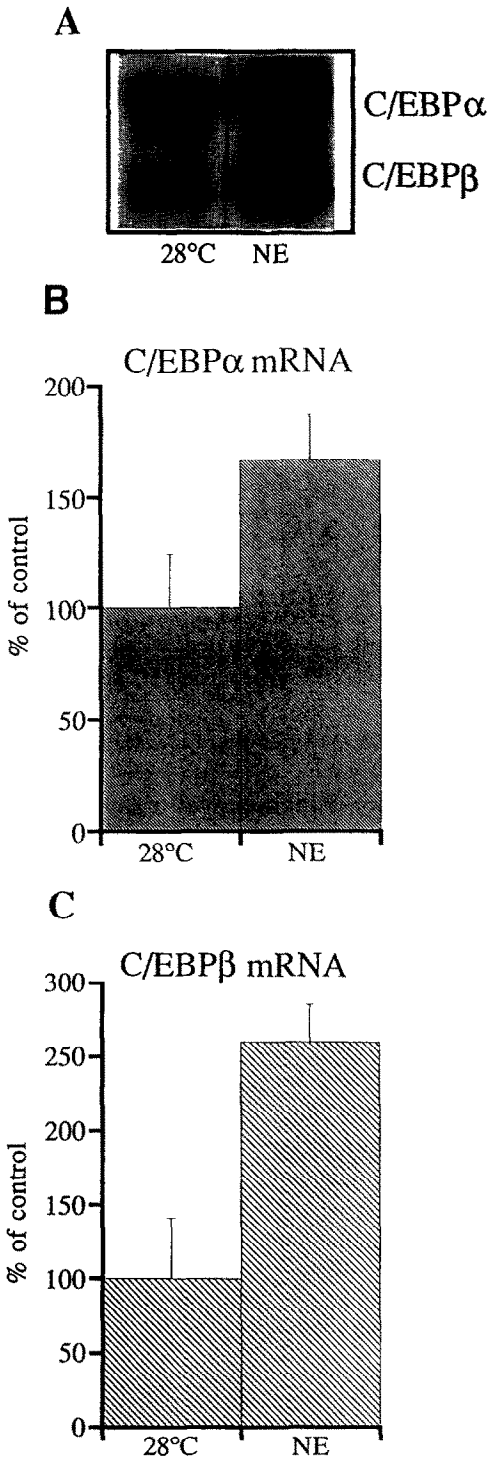

Fig. 3. Effect of norepinephrine injection on the mRNA levels of C/EBPα and C/EBPβ in mouse brown adipose tissue. Four-week-old male mice were acclimatized to 28°C for one week. The animals were then injected with norepinephrine or not treated (28°C). After 4 h, total RNA was isolated from the interscapular brown adipose tissue. (A) Northern blot analysis of C/EBPα and C/EBPβ mRNA levels. The autoradiograms were evaluated by laser scanning. The mean values of C/EBPα mRNA (B) and C/EBPβ mRNA (C) levels observed in control animals (28°C) were set to 100% and the C/EBPα and C/EBPβ mRNA levels in the other samples expressed relative to this value. Values are means ± S.E.M. from one experiment performed in triplicate.

C/EBPα and C/EBPβ gene expression was examined in physiologically unstimulated mice at 28°C. As shown in Fig. 3, 4 h after a single injection of norepinephrine, there was a slight increase in C/EBPα mRNA level and a more pronounced increase in C/EBPβ mRNA level. Thus, norepinephrine was able to affect C/EBPα and C/EBPβ gene expression.

3.3. *C/EBPα and C/EBPβ expression in primary cultures derived from brown adipose tissue*

To examine the possibility of studying the regulation of C/EBPα and C/EBPβ gene expression in an in-vitro system, we have examined the expression of these genes in our well-established brown fat cell culture system. In this cell culture system, freshly isolated precursor cells from brown adipose tissue grow and differentiate in vitro. In the preconfluent phase, the cells have the morphological and biochemical appearance of undifferentiated cells, but in the confluent phase, the cells undergo adipose conversion [27], and advance in differentiation to reach a stage where brown-fat-specific gene expression can be demonstrated [18,28]. We here present results from these two contrasting phases of cell development: preconfluent, undifferentiated cells (here: 3 days in culture) and confluent, differentiated cells (6 days).

Preliminary experiments indicated that the basal expression of C/EBPα did not change markedly between these two phases (not shown but cf. Fig. 4). In this respect, our results with these natural precursor cells contrasted markedly with earlier results from studies on cell lines; thus, in 3T3-F442A cells, 3T3-L1 cells and TA1 cells, adipose conversion is associated with a much enhanced expression of C/EBPα [7,10,14,29,30]. Also the basal expression of C/EBPβ was practically unaffected by adipose conversion; this observation is fairly similar to that seen in 3T3-L1 cells [7].

In order to investigate if the observed effect of norepinephrine injection in vivo was an effect directly on the brown fat cells, we examined the effect of norepinephrine stimulation on the brown fat cell cultures. The results presented in Fig. 4A show that norepinephrine had contrasting effects on C/EBPα expression when applied to preconfluent and confluent cells: in the proliferating preconfluent cells, it had a notable suppressing effect on C/EBPα gene expression whereas it tended to have a promoting effect in the differentiating confluent cells.

In contrast, the effect of norepinephrine on C/EBPβ gene expression was not associated with the developmental state of the cells: both in preconfluent and confluent cell cultures norepinephrine led to an enhanced expression of the C/EBPβ gene (Fig. 4).

4. DISCUSSION

4.1. *C/EBPα and cell proliferation*

We here report a dramatic but transient decrease in

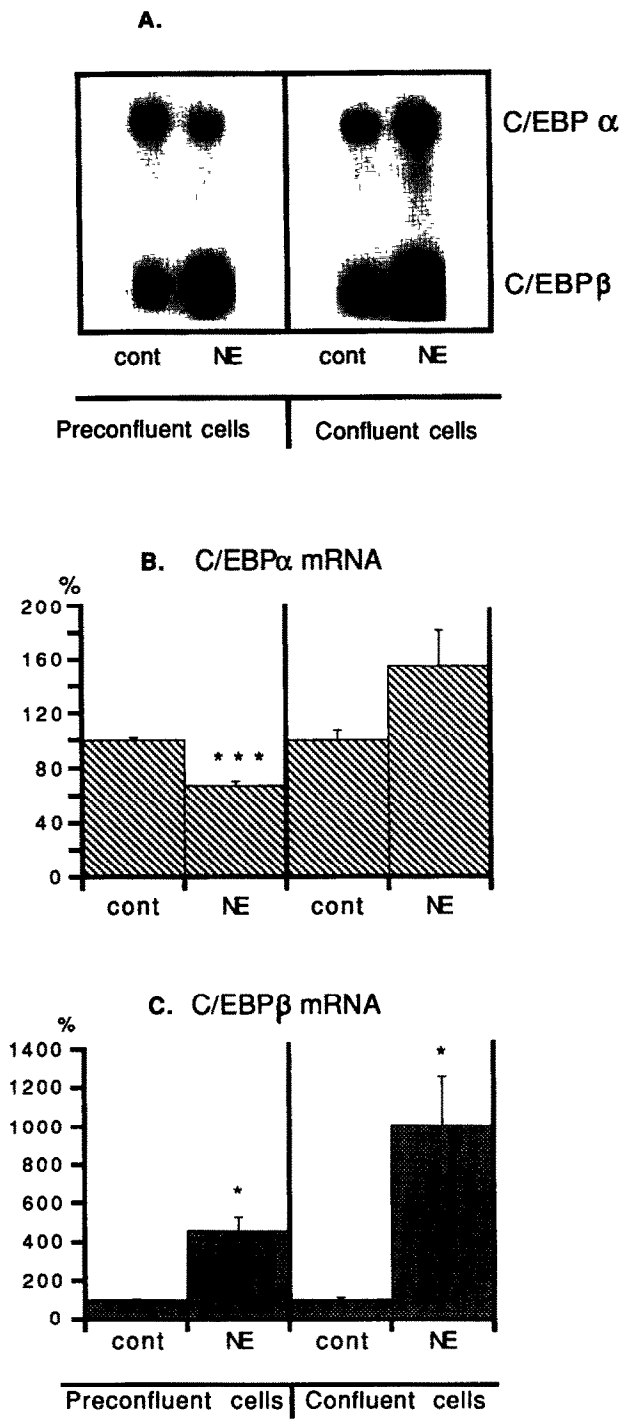

Fig. 4. Effect of norepinephrine on the mRNA levels of C/EBPα and C/EBPβ in cultured brown fat cells. Brown fat precursor cells were grown in culture for 3 days (pre-confluent cells) or 6 days (confluent cells). On the respective day, 0.1 μM norepinephrine was added (NE) or not (cont) to the cultures, 1 h before the cells were harvested. (A) Northern blot analysis of C/EBPα and C/EBPβ mRNA levels. Densitometric evaluation of the autoradiograms, showing the C/EBPα (B) and the C/EBPβ (C) mRNA levels in the brown fat cell cultures. The values were normalized to the intensity of C/EBPα and C/EBPβ mRNA levels in the controls. Statistical evaluation was performed by Student's paired *t*-test on 7 independent experiments, assayed in single or duplicate flasks (*** < 0.001; * < 0.05).

C/EBPα expression during the initial phase of the recruitment process in brown adipose tissue. As cold acclimatization leads to a persistent increase in the total capacity of the tissue for heat production, it is unlikely that the transient decrease in C/EBPα gene expression is directly related to the achievement of a persistently more differentiated state. Rather, the transient nature of the C/EBPα response makes it more likely that it is a reflection of a known transient phenomenon during the recruitment phase: the initiation of cell proliferation.

When mice are exposed to cold, DNA synthesis in brown adipose tissue is rapidly but transiently stimulated [31–33]. It is very remarkable that the expression pattern for C/EBPα observed here in the cold-exposed mice (Fig. 1) is a mirror image of the rate of DNA synthesis under the same conditions [33]. Thus, it would seem from the in-vivo experiments that C/EBPα expression is strongly negatively correlated with cell proliferation.

Seemingly, this conclusion is not supported by the observations in the brown fat cell culture system: the level of C/EBPα mRNA was not markedly lower in the rapidly proliferating preconfluent cells than in the nearly dormant confluent cells. However, our observations of a dramatic switch in the effect of adrenergic stimulation on C/EBPα gene expression tie the in-vivo and the in-vitro observations together. Thus, in preconfluent cultured cells, norepinephrine stimulation led to a decrease in the C/EBPα mRNA level, whereas in the confluent cells, the C/EBPα mRNA level was increased. Such a switch in responsiveness between preconfluent and confluent brown fat cells has earlier been observed for other phenomena: norepinephrine can promote cell proliferation in the preconfluent cultures [18,34] and cell differentiation (including gene expression) in the confluent cultures [18,21,28,35]. As the stem cells in the tissue are induced to proliferate by the exposure of the animals to cold, these cells in vivo are probably initially and transiently in the same phase as the preconfluent cells in vitro, and as the cells in vivo are exposed to an endogenous stimulation with norepinephrine, released from the sympathetic nervous system, they respond in the same way as the cells in vitro: with a decreased expression of the C/EBPα gene. Conversely, when the cells in vivo are in a dormant phase (as in the animals at 28°C), they respond to a single norepinephrine injection (which is apparently not sufficient to induce cell proliferation) with a tendency to an increased C/EBPα gene expression (Fig. 3), just as the confluent cells in vitro respond to norepinephrine stimulation with a tendency to an increased C/EBPα gene expression (Fig. 4).

Thus, concerning C/EBPα we conclude that the expression of the gene in response to adrenergic stimulation is directed by the stage of the cell cycle in which the cells are present. We would like to point out that this is the first indication that C/EBPα gene expression may be functionally under adrenergic control. Earlier studies

have demonstrated a suppressive effect of glucocorticoids or tumor necrosis factor on C/EBPα gene expression [14,36].

Earlier the relationship between C/EBPα gene expression and cell differentiation (here: adipose conversion) has only been experimentally addressed in in-vitro studies with cell lines. That C/EBPα expression is essential for adipogenesis has been indicated by demonstrating that antisense C/EBPα mRNA reduces adipogenesis in 3T3-F442 cells [14]. Whether expression of C/EBPα in itself can promote adipogenesis is not so clear: overexpression of C/EBPα may or may not be sufficient for stimulated adipogenesis in 3T3-L1 cells [15,23]. Our results, both from in- vivo studies and from in-vitro studies on primary cultures (i.e. not cell lines) of brown fat cells do not contradict an essential role for C/EBPα expression during adipogenesis but they do not support a strong positive correlation between the degree of C/EBPα expression and adipose conversion.

4.2. *C/EBPβ and cell differentiation*

The expression of C/EBPβ during the recruitment process in animals was dramatically different from that of C/EBPα. Thus, the level of C/EBPβ mRNA was increased only minutes after cold exposure and remained elevated during the entire time of cold acclimatization (Fig. 1). In this persistent positive response, the gene resembles the response of several structural genes, the function of which are intimately associated with the thermogenic function of the tissue. This is especially true for the expression of the uncoupling protein (UCP) [19,20,37] but also for the genes for lipoprotein lipase and glycerol-3-phosphate dehydrogenase in the tissue [20,38]. Also, directly parallel with these structural genes, a very much enhanced C/EBPβ gene expression can be induced via adrenergic stimulation, both in vivo (Fig. 3) and in vitro (Fig. 4). Indeed, it is likely that the enhanced expression of C/EBPβ in vivo is fully induced by the enhanced endogenous adrenergic stimulation of the cells occurring in the cold-exposed animals.

There is, however, a feature which is notably different between the adrenergic control of C/EBPβ expression and of the expression of the structural genes mentioned: whereas the ability to respond with an enhanced (or indeed any) expression of the structural genes is dependent of the cells having advanced to a differentiated state, the ability to enhance C/EBPβ expression adrenergically is not differentiation dependent; it occurs equally well in preconfluent as in confluent cells (Fig. 4).

Thus, concerning C/EBPβ we conclude that the expression of the gene in response to adrenergic stimulation is not directed by the stage of cell cycle in which the cells are present; indeed the ability to respond to norepinephrine is found even in undifferentiated cells, and the increased level of expression found in recruited tissue is more likely a reflection of the endogenous adrenergic stimulation than of an increased degree of differentiation in these cells.

We would like to point out that this is the first demonstration that this gene may be functionally under adrenergic control. Earlier studies have demonstrate an induction in other cells in vivo through cytokines [3].

4.3. *Conclusions*

In conclusion, we have here demonstrated a physiologically controlled regulation of the expression of C/EBPα and C/EBPβ. The genes demonstrate a clear difference in their response to adrenergic stimulation: the response of C/EBPα shows a switch during the differentiation process whereas the response of C/EBPβ is differentiation-independent. The exact correlation between the responses in vivo during physiological stimulation and the responses in vitro in primary cells differentiating in cell culture makes the cell culture system an excellent tool for studies both concerning the intracellular pathways controlling the expression of these genes and for elucidating their role in the differentiation and proliferation processes.

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