As the proliferation promoter noradrenaline induces expression of ICER (induced cAMP early repressor) in proliferative brown adipocytes, ICER may not be a universal tumour suppressor

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The CREM (cAMP-response-element modulator) gene product ICER (induced cAMP early repressor) has been proposed to function as a tumour (cell proliferation) suppressor. To investigate the generality of this concept, the expression pattern of ICER in brown adipocytes was followed; this was critical because brown adipocytes are one of few cell types in which cAMP is associated positively with cell proliferation but negatively with apoptosis. In response to the physiological stimulus of cold (which induces cell proliferation), ICER mRNA levels were increased in brown adipose tissue *in vivo*. In brown adipocytes in primary culture, ICER gene expression was induced by noradrenaline (norepinephrine) not only in the mature state (where noradrenaline potentiates differentiation), but also in the proliferative state of the cell cultures (where noradrenaline enhances cell proliferation). The induction was mediated via β-receptors and the cAMP/protein kinase A pathway. The induced ICER appeared to repress its own expression and that of the β2-adrenoceptor. It is thus evident that also in cell types in which cAMP induces proliferation, and even when these cells are in the proliferative state, ICER expression is induced by the same agents that stimulate proliferation. This can either mean that ICER is not a general tumour suppressor, or that brown adipocytes temporally or spatially avoid this role of ICER.

Key words: CREM, noradrenaline, norepinephrine, UCP1.

INTRODUCTION

From classical studies of cell proliferation, it is evident that cAMP is frequently inhibitory for cell proliferation and stimulatory for apoptosis [1,2]. Concerning the further cellular mediation of these anti-proliferative effects of cAMP, one of the products of the CREM (cAMP-response-element modulator) gene has been especially implicated: ICER (induced cAMP early repressor) [3]. ICER is expressed as a direct effect of the cAMP-induced activation of protein kinase A (cAMP-dependent protein kinase; PKA)-mediated phosphorylation of CREB (cAMP-response-element-binding protein) [3], and its expression is therefore a direct consequence of the increased cAMP level. The possibility has therefore arisen that ICER, being a repressor, could mediate the inhibitory effect of cAMP on cell proliferation. Indeed, when expressed ectopically, ICER inhibits DNA replication and proliferation in different systems [4–6]. This may be related to an inhibitory effect of ICER on cyclin A expression through transcriptional down-regulation [4]. ICER may also act as an anti-oncogene against, for example, c-*fos* [7]. It has thus been suggested that ICER is a tumour-suppressor gene product that mediates the anti-proliferative activity of cAMP [5]. This implies that tumour suppressor effects may be ascribed to ICER that are not directly linked to its accepted ability to competitively inhibit CREB-induced transcription [8] but rather could be related to the ability of ICER to form ternary complexes with other factors in the promoters of responsive genes [9,10]. Correspondingly, ICER has been discussed to be directly and positively involved in mediation of cAMP-induced apoptosis [7,11].

However, in a significant number of cell types, cAMP is not inhibitory for proliferation but rather induces or stimulates proliferation [12]. These cell types include, e.g., the well-studied thyrocytes [13], as well as somatotrophs [14] and lactotrophs [15]. Included in this group of cell types are also brown adipocytes [16]. It is evident that, in this group of cell types, the putative role of ICER as a general tumour suppressor can be examined critically. If the cAMP pathway also leads to ICER expression in these cell types, this would indicate that ICER in these cells should be devoid of a tumour suppressor effect (and then it would not be a general tumour suppressor). Alternatively, if ICER is a general tumour suppressor, ICER should not be expressed in these cell types under conditions in which cell proliferation is induced.

A basic question is therefore whether ICER expression is induced at all in this group of cell types. Until now, this has only been examined in thyrocytes, with somewhat divergent results [6,10,17,18]. Further, because thyrocytes respond to a recruiting stimulus with a simultaneous stimulation of proliferation and differentiation within the same cell [19], the potential role of ICER in the regulation of each of these processes is not easily delineated in this system.

In this respect, the role of ICER should be more easily elucidated in brown adipocytes because these cells in primary culture, in contrast with thyrocytes, spontaneously progress through a developmental pathway. Thus when brown adipocytes are in the undifferentiated state, β1-adrenergic stimulation leads to increased cell proliferation, mediated via increased cAMP levels [16,20]; when the cells become differentiated, the cells become refractory to the proliferative signal [16]. In parallel with this, in young brown adipocytes, cellular apoptosis is inhibited by adrenergic stimulation, via pathways including increased cAMP levels [21–23]; again, in differentiated cells, this no longer

Abbreviations used: CREM, cAMP-response-element modulator; ICER, induced cAMP early repressor; CREB, cAMP-response-element-binding protein; UCP1, uncoupling protein-1; RT-PCR, reverse transcriptase–PCR; PKA, cAMP-dependent protein kinase; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide.

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occurs [22]. Also, general cellular proteolysis is inhibited in brown adipocytes by cAMP [24].

Thus if ICER is directly and generally involved in regulation of cell proliferation as a tumour/proliferation suppressor and an apoptosis inducer, it may be anticipated that adrenergic stimulation would not induce ICER expression in brown adipocytes when the cells are in a proliferative state. Therefore we have examined in the present study ICER expression both in brown adipose tissue and in cultured brown adipocytes. We conclude that even under conditions in which physiological or adrenergic stimulation is associated with enhanced cell proliferation, ICER is markedly induced. Although temporal or spatial compartmentation may preclude the induced ICER from manifesting an anti-proliferative effect, the present studies make it less likely that ICER has a general physiological role as a tumour suppressor or apoptosis inducer.

MATERIALS AND METHODS

Animals

Animals for experiments *in vivo* were adult male mice (> 4 weeks old) of the NRMI strain (Eklunds, Vallentuna, Sweden), given standard diet (B&K, Sollentuna, Sweden), and housed at 24 °C in the animal facility of the Wenner-Gren Institute (Stockholm University, Stockholm, Sweden) for a minimum of 4 days before being killed. When cold-exposure experiments were carried out, the mice were moved to a 4 °C room in separate cages for the indicated times.

Cell isolation and culture

Brown fat precursor cells were isolated from 4-week-old male NMRI mice principally as described in [25] and were cultured as described in [26]. The culture medium was Dulbecco's modified Eagle's medium supplemented with 10 % newborn calf serum (Life Technologies, Gibco-BRL), 4 nM insulin, 25 μg/ml sodium ascorbate, 10 mM Hepes, 4 mM glutamine, 50 i.u./ml penicillin and 50 μg/ml streptomycin. After plating (1 and 4 days), with rinsing on day 1, the medium was changed to new fresh prewarmed medium. The addition of agents was made without prior changing of the medium on the indicated day of experiment.

Cell harvesting, RNA isolation and Northern-blot analysis

The medium was discarded after the indicated time of treatment and total RNA was isolated with UltraSpec (BioTecx Lab., Houston, TX, U.S.A.) according to the instructions provided by the manufacturers. Briefly, the cells in a 6-well plate were collected in 0.8 ml of UltraSpec solution and vortexed, and the homogenate was stored for 5 min on ice. Chloroform (0.16 ml) was added, and the samples were vortexed for 15 s and put on ice for 5 min. The homogenate was centrifuged at 12000 ***g*** for 15 min and the upper aqueous phase was collected. The RNA was precipitated by adding an equal volume of propan-2-ol and then collected by centrifugation, followed by a washing step. RNA concentration was determined in a spectrophotometer (DU-50, Beckman Instruments) at 260 nm. Northern-blot analyses were performed principally as described previously [26]. Routinely, 8–10 μg of total RNA was loaded per well. The cDNA was labelled with [^{32}P]dCTP (Amersham Pharmacia Biotech) by Ready-To-Go DNA labelling beads (Amersham Pharmacia Biotech). The data were evaluated by exposing the membranes to a PhosphorImager screen and the screens were scanned with a Molecular Dynamics PhosphorImager. For quantification, the band with an apparent size of 2.0 kb was used.

cDNA probes

The cDNA used for detecting CREM transcripts was a 750 bp EST (expressed sequence tag) clone (GenBank accession number AA492985, IMAGE Consortium), confirmed by sequencing to correspond to a CREMτ sequence (Figure 1). The β2-adrenergic receptor cDNA was a human 896 bp *Eco*RV/*Bst*EII fragment [27]. The UCP1 (uncoupling protein-1) cDNA [28] and c-*fos* cDNA [26] probes were those used earlier.

Reverse transcriptase–PCR (RT-PCR) cloning and sequencing

Total RNA was isolated from either brown adipose tissue or from adipocyte cultures as above. Routinely, 400 ng of total RNA was subjected to RT-PCR by the use of the Access RT-PCR System (Promega). The specific amplification of ICER cDNAs was performed according to Bodor et al. [29], with the primer sequences 5′-ATGGCTGTAACTGGAGATGAAACT-3′ (ICER) and 5′-CTAATCTGTTTTGGGAGAGCAAAT-3′

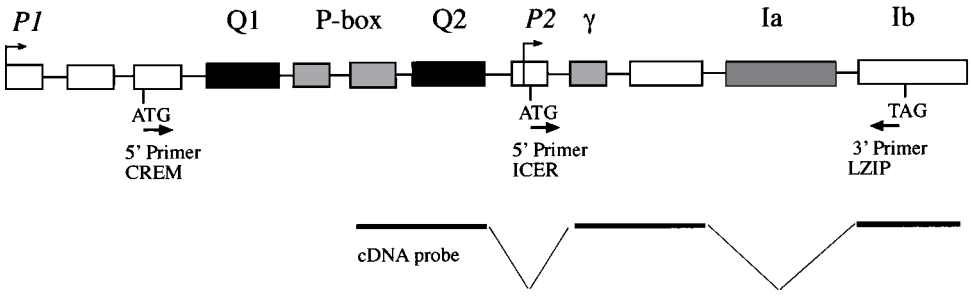

Figure 1 Schematic representation of the primers and cDNA probe used in the present study

In the structure of the CREM/ICER gene (based on [50]), the exons are indicated as boxes, including the glutamine-rich regions (Q1 and Q2), the phosphorylation region (P-box), the γ-exon (γ) and the DNA-binding domains (Ia and Ib). The arrows above the boxes indicate the transcription start sites, *P1* and *P2*, where *P2* is the one used for generating ICER transcripts. Below the gene are indicated the CREM-specific 5′ primer used (starting at the first CREM coding exon), the ICER-specific 5′ primer used (positioned in the ICER-specific exon), and the 3′ primer (LZIP), which was used with both 5′ primers, positioned in the exon of the second DNA-binding domain, Ib. Although both full-length transcripts (only detectable with the CREM primer) and short transcripts (only detectable with the ICER primer) may be considered as transcripts of the CREM gene, for simplicity we restrict here the meaning of CREM transcripts to those originating from the *P1* transcription site, and denote those originating from the *P2* site as ICER transcripts. Also indicated is the position of the cDNA probe used in Northern blotting. As seen, this probe detects both *P1* (CREM) and *P2* (ICER) transcripts.

(LZIP, corresponding to the leucine zipper region), whereas amplification of CREM cDNAs was achieved with the primer sequence 5′-TGGAAACAGTTGAATCACAG-3′ (CREM) [30] and LZIP, all as depicted in Figure 1. The resulting products were resolved on a 1.2% agarose gel and visualized by ethidium bromide. The agarose gel was blotted on to a nitrocellulose membrane (Hybond-XL, Amersham Pharmacia Biotech) for Southern blotting and hybridized with the labelled CREM cDNA probe. The resulting image was scanned with a Molecular Dynamics PhosphorImager. Identification of the PCR products was carried out by automatic sequencing with the BigDye Terminator Kit (Perkin-Elmer).

RESULTS AND DISCUSSION

Physiological induction of CREM transcripts in brown adipose tissue

For the study of physiological control of proliferation *in vivo*, brown adipose tissue constitutes an excellent model system. Particularly, a simple physiological stimulus, that of cold exposure, initiates the process of recruitment in brown adipose tissue [31]. This process includes an increase in cell proliferation [32–34] and an inhibition of apoptosis [21–23]. In order to examine whether any CREM gene products were expressed in brown adipose tissue in intact animals during physiological induction of cell proliferation, cold-exposure studies were performed, and the RNA isolated from brown adipose tissue. These preparations were then analysed with a CREM probe. The probe utilized would detect all known transcripts of the CREM gene, including ICER (Figure 1).

In a preparation of brown adipose tissue RNA obtained from mice living under normal animal-house conditions (Figure 2A, left-hand lane), four different transcripts could be detected with the CREM probe. The approximate sizes of the transcripts observed were 5.8, 2.0, 1.4 and 1.2 kb. The sizes of the three lower bands were in agreement with predicted [35] and observed [3,11,30] sizes for ICER transcripts in other systems. The top band at $\approx$ 5.8 kb might be the result of unspecific hybridization. It is not a CREM transcript from the P1 promoter, because we could not confirm the presence of full-length CREM transcripts in the tissue by RT-PCR methods (see below).

Exposure of mice to a cold environment (which is associated with an increase in cell proliferation) rapidly increased the levels of the three tentative ICER mRNAs (Figure 2A). The levels of these three mRNAs were all increased in a similar fashion, reaching a maximum at 2 h and slowly returning to basal levels after 8 h of cold exposure (Figure 2B).

To establish whether the bands visualized by Northern blotting included CREM and/or ICER transcripts, RT-PCR was performed on total RNA from brown adipose tissue. As summarized in Figure 1, the CREM gene can be transcribed as full-length CREM transcripts from the P1 promoter (here referred to as CREM transcripts for simplicity) or as short transcripts from the alternative intronic P2 promoter; these transcripts represent (and will be referred to as) ICER transcripts. There are four known ICER transcripts: ICER-I and ICER-Iγ, each spliced to contain two DNA-binding domains, and ICER-II and ICER-IIγ, which only contain the DNA-binding domain encoded by exon Ib (Figure 1). To distinguish between the CREM and ICER transcripts in the RT-PCR experiments, we used the two upstream primers indicated in Figure 1.

When amplification was achieved with the CREM-specific primer, no products were visible, either in a control sample of RNA from brown adipose tissue, or in a sample prepared from mice exposed to cold for 2 h (Figure 3A). However, a product

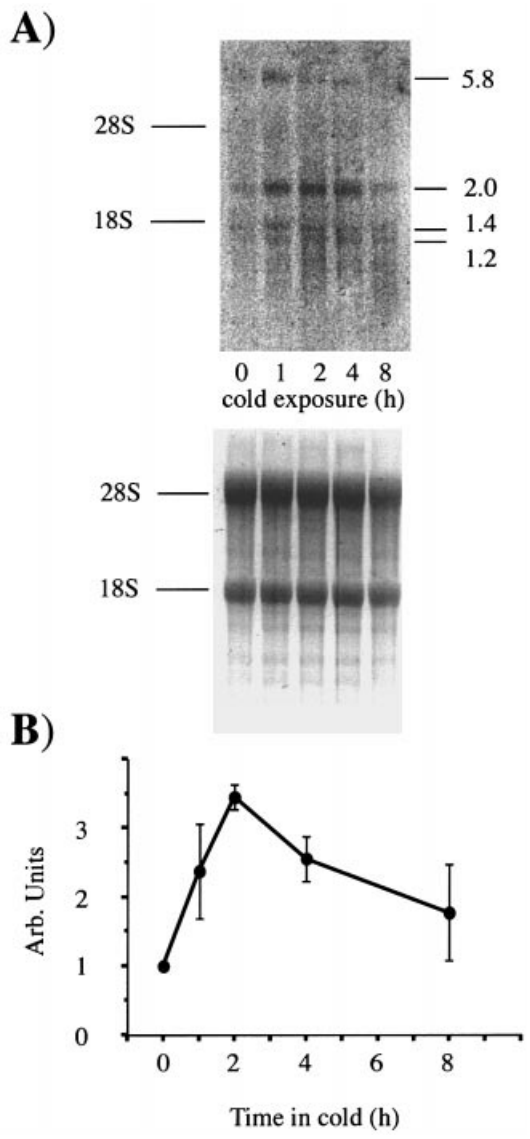

Figure 2 Cold-induced CREM transcripts in brown adipose tissue

Mice were exposed to cold (4 °C) for the indicated time periods and total RNA was isolated from brown adipose tissue. (**A**) Upper panel: Northern blot of samples of 20 μg of total RNA hybridized with the CREM cDNA probe. Lower panel: rRNA, visualized by ethidium bromide staining, presented as a negative image. (**B**) Time curve of CREM transcript levels in brown adipose tissue. The 2.0 kb band (**A**) was used for quantification. In each experimental series, the level at 0 h was set to 1 and the others adjusted accordingly. Points are means $\pm$ S.E.M. from three independent experiments. The significance of differences between values at 0 h and 2 h and at 0 h and 4 h were $P < 0.05$, as tested by Student's paired t test.

with a size of $\approx$ 1100 nt was detected in a reference RNA sample from testis, validating the functionality of the CREM-specific primer. The agarose gels were further subjected to Southern blotting and hybridized with the CREM cDNA probe. This procedure confirmed that there were no CREM transcripts in the brown adipose tissue samples, or in control or cold-exposed animals (Figure 3B). However, in the testis sample, several CREM products were detected, as anticipated [36]. Thus no full-length CREM transcripts were found in brown adipose tissue.

In contrast, performing RT-PCR with the ICER-specific primer resulted in the appearance in brown adipose tissue of one cold-induced band with a size of $\approx$ 750 nt (Figure 3C). Also, a

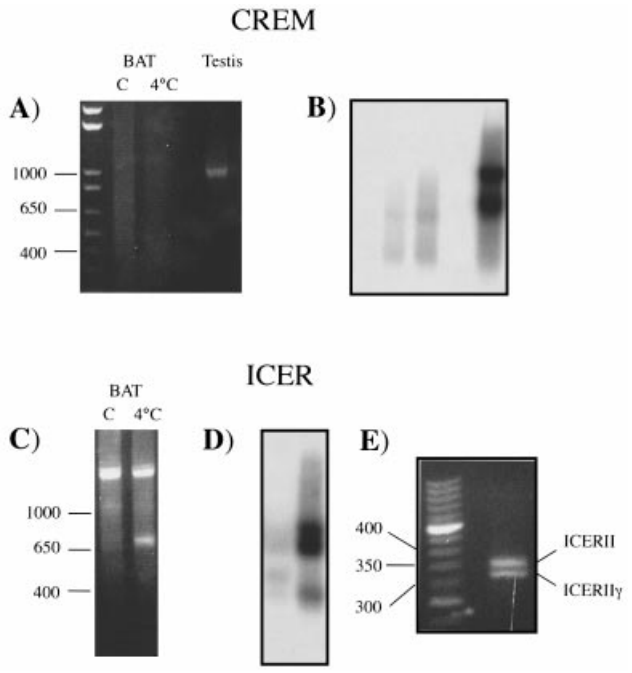

Figure 3 Detection of CREM and ICER transcripts in brown adipose tissue

Total RNA from control and cold-exposed mice was subjected to RT-PCR with the indicated primers. (**A**) and (**C**) show visualization of PCR products by ethidium bromide-containing agarose gel electrophoresis. Base-pair standards are shown on the left in (**A**). BAT indicates lanes containing PCR products derived from 400 ng of total RNA from brown adipose tissue subjected to RT-PCR as described in the Materials and methods section. The mice were either untreated (C) or exposed to cold (4 °C) for 2 h. In (**A**) RT-PCR was performed with the CREM-specific 5′ primer, whereas in (**C**) the ICER-specific 5′ primer was used. The lane labelled Testis contains 100 ng of total RNA from mouse testis examined in parallel with the BAT samples. (**B**) and (**D**) are Southern blots of the agarose gels in (**A**) and (**C**) respectively, hybridized with the CREM cDNA probe. (**E**) Re-amplification of the $\approx$ 350 bp band seen in (**D**). ICERII and ICERIIγ refer to the transcripts defined in [50].

product with an approximate size of 1500 nt was amplified, but when the gels were subjected to Southern blotting (Figure 3D) the product at 1500 nt did not hybridize with the CREM cDNA probe. Therefore, the 1500 nt band was considered a non-specific PCR-amplification product. The $\approx$ 750 nt band hybridized with the CREM probe as two bands (Figure 3D) and additional cold-induced ICER transcripts, smaller than 400 nt, were also observed in the Southern blot. The nature of the signal of $<$ 400 nt was examined by isolating DNA from that region of the gel and subjecting it to a second round of PCR with the use of the same primers. When this product was resolved on a new agarose gel, two bands could be identified (Figure 3E), with sizes of 370 and 330 nt. Further characterization by sequencing confirmed them to be ICER-II and ICER-IIγ (results not shown).

Taken together, the transcripts induced in the brown adipose tissue of cold-exposed mice probably consisted of all four ICER transcripts: ICER-I, ICER-Iγ, ICER-II and ICER-IIγ; no detection of full-length CREM transcripts could be made. In accordance with this, a polyclonal anti-CREM antibody did not detect any protein(s) in either samples of brown adipose tissue or in samples from cultured brown adipocytes (results not shown).

ICER transcripts in brown adipocyte cultures

The above experiments *in vivo* demonstrated induced ICER gene expression under conditions in which the proliferation process was initiated physiologically. This could still be compatible with a general proliferation-repressing role of ICER, provided that the induced ICER expression was restricted to cells in the differentiated state.

To analyse whether ICER expression was coupled in this way to the developmental state of the cells, it was therefore feasible to use primary cultures of brown adipocytes. These cultures progress from a proliferative state to a differentiated state [16,37]. *In vivo*, the cold-induced cell proliferation in brown adipose tissue is mediated via β-adrenergic stimulation [34,38]; in parallel with this, proliferation in young cells in cell culture is also enhanced via β-adrenergic stimulation [16].

Therefore, to examine whether ICER was only induced in differentiated cells and not in proliferating cells, we followed the effect of noradrenaline (norepinephrine) stimulation on ICER mRNA levels during the transition period from the proliferative to the differentiated state in the cultured brown adipocytes (Figure 4A).

In untreated cells, there was no detectable ICER mRNA on any of the culture days examined (Figure 4A). However, when noradrenaline was added to the cell cultures, there was a strong induction of CREM transcripts. The induction was seen on all the days examined and, if anything, tended to be higher in cells in the proliferative state (days 3 and 4) than in cells in the differentiated state (days 6 and 8). Thus the ability of noradrenaline to induce ICER gene expression was not inhibited in the proliferation phase, indicating that the suggested role of ICER as an anti-proliferative agent is not a general one.

ICER expression kinetics

A time course analysis of the effect of noradrenaline in brown adipocyte cultures showed that the induction was rather fast but transient, reaching a maximum at around 2 h and then returning to the basal level within 8 h (Figure 4B). The time curve is identical with that observed in the tissue in the cold-exposure experiments (Figure 2B). This transient pattern of inducibility has the temporal characteristics of an immediate-early gene, as has been pointed out previously in other systems [3].

Confirmation of the nature of the transcripts found in cell cultures was carried out with the use of RT-PCR. No basal or noradrenaline-induced full-length CREM transcripts could be observed (Figures 5A and 5B), whereas detectable ICER transcripts (Figures 5C and 5D) showed a similar pattern to that found in whole tissue (Figures 3C and 3D). Thus the transcripts detected by Northern blots in brown adipose tissue represent *bona fide* ICER transcripts, indicating that the presence of such transcripts is not inhibitory for proliferation.

Receptor mediation and intracellular messengers for ICER induction

In brown adipocytes, the response to noradrenaline can be mediated via both α1-adrenergic and β-adrenergic receptors, which in turn couple to different intracellular second-messenger pathways [39]. It is the β-adrenergic cAMP-mediated pathway that has been linked to stimulation of cell proliferation [16]. Selective agonists were therefore employed to examine which pathways were involved in the control of ICER expression in brown adipocytes.

The α1 agonist cirazoline was without effect on ICER mRNA levels (Figure 6). This was also the case with the calcium ionophore A23187, an elevator of cytosolic calcium levels, and with the phorbol ester PMA, an activator of protein kinase C (Figure 6; both A23817 and PMA mimic α1-adrenergic down-

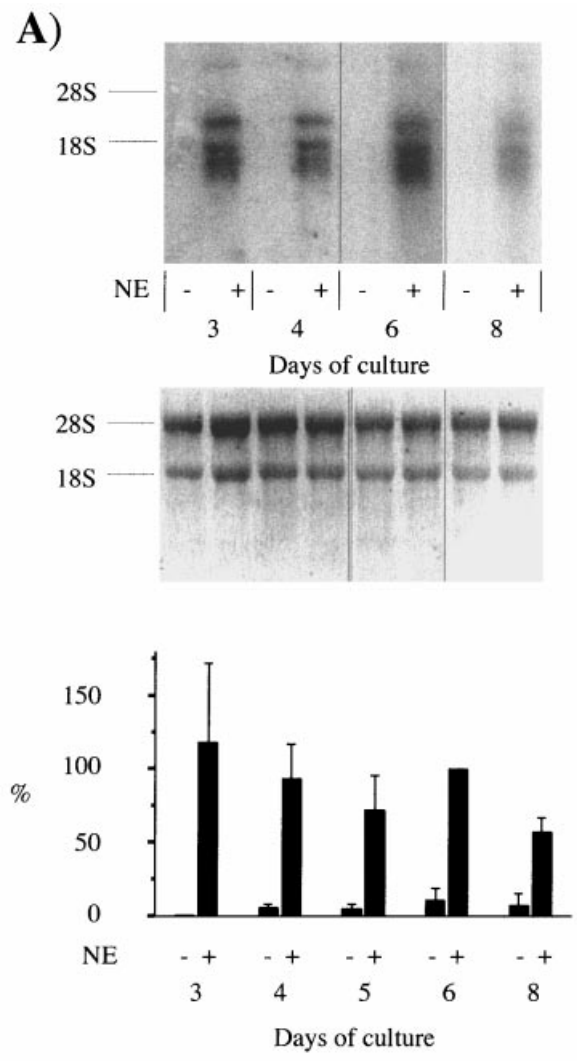

Figure 4 Time courses of noradrenaline-induced ICER expression in primary cultures of brown adipocytes

Brown pre-adipocytes were isolated from mice and grown in primary culture until the days indicated, as described in the Materials and methods section. (**A**) Upper panel: Northern blot of cultures stimulated or not with 1 μM noradrenaline (NE) for 2 h on the indicated days of culture and hybridized with the CREM cDNA probe. Middle panel: rRNA, visualized by ethidium bromide staining. Lower panel: bar graph from two to three independent experiments shown as means $\pm$ S.E.M. In each experimental series, the response to noradrenaline on day 6 was set to 100%. Significance of differences between values of untreated and noradrenaline-treated cultures, set to 100% at every day, was tested by Student's paired *t* test: $P < 0.05$. (**B**) Graph from day-6 cultures stimulated with 1 μM noradrenaline for the times indicated. Each point is based on two to three experiments in duplicate and shown as the mean $\pm$ S.E.M. In each experimental series, the ICER mRNA levels found after 2 h were set to 100%. Values at 0 h and 2 h were significantly different, as tested by Student's paired *t* test: $P < 0.01$.

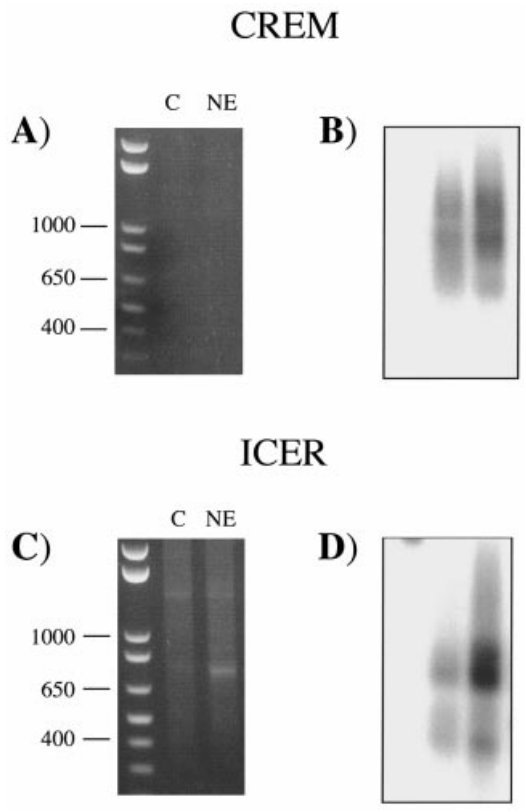

Figure 5 Detection of CREM and ICER transcripts in brown adipocyte cultures

Cultures of brown adipocytes were either left untreated or treated with 1 μM noradrenaline (NE) for 1 h on day 6. Total RNA was isolated and subjected to RT-PCR. (**A**) and (**C**) show visualization of PCR products by ethidium bromide-containing agarose gel electrophoresis. Base-pair standards are shown on the left. The culture lanes contain 400 ng of total RNA from adipocyte cultures subjected to RT-PCR, as described in the Materials and methods section. In (**A**) RT-PCR was performed with the CREM-specific 5′ primer, whereas in (**C**) the ICER-specific 5′ primer was used. (**B**) and (**D**) show Southern blots of the agarose gels in (**A**) and (**C**) respectively, hybridized with the CREM cDNA probe.

stream signals). Thus in these cells, Gq/G11-coupled pathways are not involved in the increased ICER expression, in contrast to the case in pancreatic cell cultures [40]. However, the potency of the β-adrenergic agonist isoprenaline in inducing ICER transcripts was at least as good as that of noradrenaline (Figure 6B). In agreement with this, the adenylate cyclase activator forskolin caused induction to the same extent as noradrenaline (Figure 6). This implies that the noradrenaline-induced ICER gene expression is completely under the control of β-adrenergic receptors and intracellular cAMP levels. This is therefore the same pathway as that utilized for activation of cell proliferation [16].

The further relay of a cAMP signal is generally believed to be via PKA, and this kinase has also been implicated in the induction of ICER expression [3]. To test PKA involvement in the noradrenaline-induced expression of ICER transcripts, we used the PKA inhibitor H-89 {*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulphonamide} [41]. H-89 fully inhibited

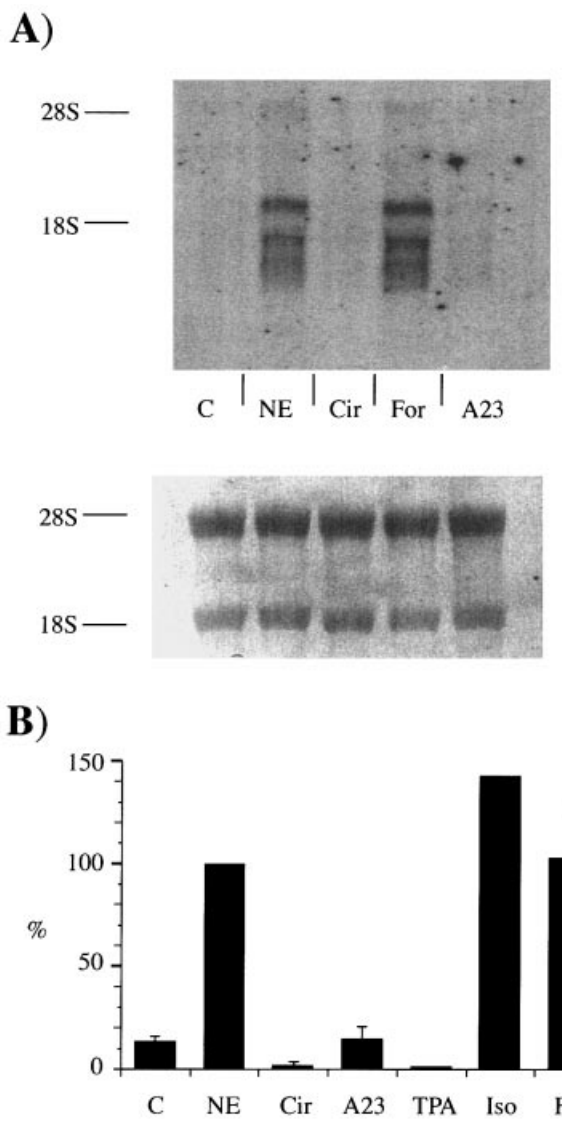

Figure 6 Mediation of noradrenaline-induced ICER gene expression

Cultures of brown adipocytes were stimulated with various agents for 2 h on day 6, and total RNA was isolated. (**A**) Upper panel: Northern blot showing the effects of 1 μM noradrenaline (NE), 1 μM cirazoline (Cir), 1 μM forskolin (For) and 1 μM A23187 (A23) or no treatment (C) on CREM transcript levels after 2 h of treatment. Lower panel: rRNA, visualized by ethidium bromide staining. (**B**) Graph of day-6 cells stimulated with the indicated agents: control (C; $n = 4$); 1 μM noradrenaline (NE; $n = 4$); 1 μM cirazoline (Cir; $n = 2$); 1 μM A23187 (A23; $n = 3$); 500 ng/ml PMA (TPA; $n = 1$); 1 μM isoprenaline (Iso; $n = 1$); 1 μM forskolin (For; $n = 2$). In each experiment, duplicate wells were examined. Results are given as means $\pm$ S.E.M. In each experiment, the response to noradrenaline was set to 100%. Noradrenaline treatment led to a significant increase in ICER mRNA levels, tested by Student's paired t test ($P < 0.001$), but cirazoline, A23187 and PMA did not. The level after isoprenaline or forskolin treatment was not significantly different from that after noradrenaline treatment.

noradrenaline-induced ICER expression (Figure 7). Further experiments also demonstrated that H-89 fully inhibited the forskolin-induced ICER expression (results not shown), indicating that the effect of H-89 was indeed on PKA and not through antagonism of the β-receptor (in contrast with [42]). Therefore, the signalling pathway activated by noradrenaline presumably involved PKA activation.

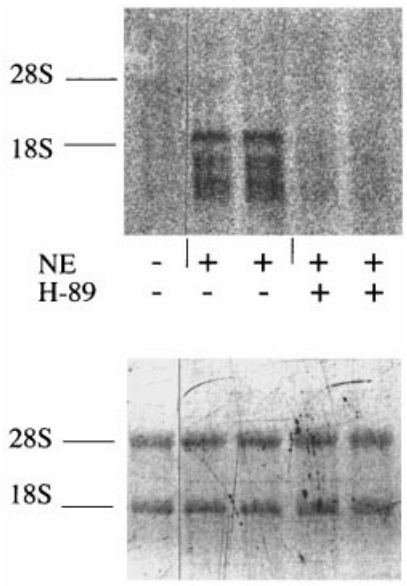

Figure 7 Effect of the PKA inhibitor H-89 on noradrenaline-induced ICER gene expression

Northern blot showing the effect of pre-treating brown adipocyte cultures with 50 μM H-89 for 30 min prior to stimulation with 1 μM noradrenaline (NE) for 2 h. The upper panel shows ICER transcripts and the lower panel shows rRNA.

ICER as a repressor of transcription

Even though ICER is clearly expressed in brown adipocytes due to β-adrenergic stimulation, it may still be the case that this ICER mRNA is not translated in brown adipocytes, or the resulting ICER may be inactive in these cells. We have attempted to obtain some evidence for functional activity of ICER.

The established function of ICER proteins as repressors of transcription is generally described as a negative cAMP-induced feedback mechanism [3], i.e. newly synthesized ICER proteins will inhibit cAMP-response-element-mediated transcription by competition with phosphorylated CREB. Considering the significance of cAMP-regulated gene expression in brown adipocytes [39], it may be anticipated that ICER thus could play an important but until now unrecognized role in these cells. If ICER represses the expression of any given gene, it would be expected that inhibition of protein synthesis would alleviate this repression. Therefore, some candidate genes were tested as ICER targets by examining whether the protein-synthesis inhibitor cycloheximide would augment and/or prolong the noradrenaline-induced expression of these genes (in parallel with what has been shown earlier for other genes [7]).

A prime candidate is ICER expression itself, demonstrated here in brown adipocytes to be under cAMP/PKA control and demonstrated earlier in other systems to be feedback-inhibited by the ICER proteins themselves [3]. Cycloheximide treatment alone did not induce ICER expression, whereas noradrenaline, as observed above, resulted in induction (Figure 8A). However, when cycloheximide was added together with noradrenaline, the response became vastly potentiated and was converted from being transient to remaining augmented during the period of study. This augmentation of gene expression is in accordance with essentially similar observations in other systems [11,43,44]. ICER transcription itself is thus probably a target for ICER control in brown adipocytes.

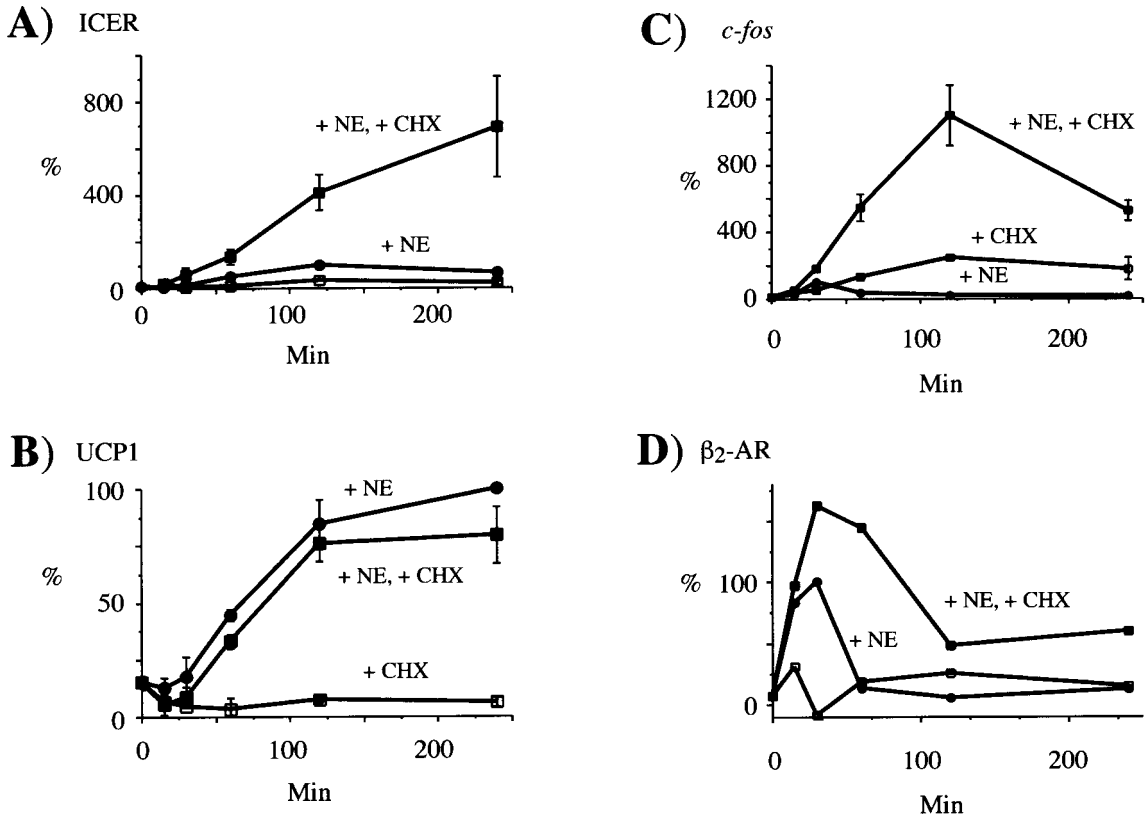

Figure 8 Effect of inhibition of protein synthesis on the expression profiles of the ICER, UCP1, c-*fos* and β2-adrenoceptor genes

Cell cultures were treated with either 1 μM noradrenaline alone (NE, ●) or 50 μM cycloheximide alone (CHX, □) or with noradrenaline and cycloheximide combined (■) for the indicated times. Total RNA was then isolated and subjected to Northern blotting. (**A**) ICER mRNA levels. In each experimental series, the response to noradrenaline alone at 120 min was set to 100%. (**B**) UCP1 mRNA levels. In each experimental series, the response to noradrenaline alone at 240 min was set to 100%. (**C**) c-*fos* mRNA levels. In each experimental series, the response to noradrenaline alone at 30 min was set to 100%. For (**A**)–(**C**), the data are from two independent experiments in duplicate and expressed as means ± S.E.M. (**D**) β2-Adrenergic receptor mRNA levels. The data are means from one experiment in duplicate. The response to noradrenaline alone at 30 min was set to 100%.

The most distinctive cAMP-regulated gene in brown adipocytes is, however, undoubtedly that encoding the unique, tissue-specific UCP1 (thermogenin) [45], the expression of which is under β-adrenergic/cAMP control, probably due to multiple cAMP-response-element sequences in its promoter [46]. Also concerning UCP1, inhibition of protein synthesis with cycloheximide was in itself without effect on the (very low) endogenous mRNA levels. Addition of noradrenaline led, as described previously [37], to a very marked increase in expression that was maintained over time (Figure 8B). However, the combined treatment with noradrenaline and cycloheximide was without effect, as compared with that seen after noradrenaline treatment alone. From this, it could be concluded (i) that noradrenaline-induced expression of the UCP1 gene is not dependent on any inducible factor (as concluded earlier [47]), and (ii) that UCP1 is not a likely candidate for being an ICER target.

The apparent absence of an effect of ICER on UCP1 gene expression may principally be said to be in accordance with the increase in UCP1 expression with time; ICER targets would be expected to show transient expression patterns. Two other genes have, however, been shown to be transiently induced by noradrenaline in brown adipocytes: c-*fos* [26] and the β2-adrenoceptor [48].

Concerning c-*fos*, we found, principally in agreement with our earlier observations [26], that cycloheximide itself induced gene expression (Figure 8C). Although cycloheximide also greatly augmented the effect of noradrenaline on c-*fos* expression, the effect of cycloheximide itself makes it impossible to conclude whether the augmentation is mediated partly via ICER.

In contrast, however, the expression pattern observed for the β2-adrenoceptor was very clear (Figure 8D). No effect of cycloheximide itself was observed, and noradrenaline led to the earlier reported transient increase in gene expression [48]. The combined presence of cycloheximide and noradrenaline led to a response that was both augmented and extended, as compared with that to noradrenaline alone. Thus the β2-adrenoceptor represents a probable target for ICER in brown adipocytes.

The above observations are therefore compatible with the induced ICER being active in brown adipocytes. The remarkable difference between the apparent susceptibility of the ICER and β2-receptor genes to ICER and the lack of effect on the UCP1 gene may call for further study. All three genes have been reported to contain cAMP-response-element sequences in their promoters [3,46,49]. However, subtle differences in the actual sequences or in their localization may shift the balance of these elements more or less towards ICER binding, as compared with CREB binding.

Conclusions

Based on a series of studies in different systems, as detailed in the Introduction, an interesting hypothesis has been formulated:

that ICER in itself functions as a tumour suppressor and perhaps as an inducer of apoptosis [5,7,11]. Here we used the brown adipose tissue system to test the generality of this tenet. These investigations led to the first detection of ICER mRNA in this system; ICER mRNA was observed both in brown adipose tissue *in situ* and in primary cultures of brown adipocytes. Although ICER expression is probably tissue-specific, its inducibility also in brown adipose tissue may mean that its expression is more widespread than anticipated originally, provided that suitable inducers are used.

In brown adipocytes, ICER was β-adrenergically induced; the induction was mediated via cAMP and PKA and indications were obtained that functional ICER was present in the stimulated cells. However, the noradrenaline-mediated induction of ICER expression was equally intense in cells in the proliferative phase, when noradrenaline stimulates proliferation and inhibits apoptosis, as in the differentiated phase, when noradrenaline does not have these effects.

This inducibility of ICER in cells in a proliferative phase with an agent that promotes proliferation and inhibits apoptosis in these cells is clearly not easily compatible with the hypothesis ascribing to ICER a general functional role in tumour suppression and in induction of apoptosis. Several possibilities may be discussed concerning this apparent incompatibility. One is that the question can be raised as to whether ICER really has a general tumour-suppressor function or whether certain cell types may have escaped from susceptibility to ICER in this respect. Another possibility is that temporal (or spatial) compartmentation of ICER in cell types such as brown adipocytes may preclude ICER from exhibiting its tumour-suppressor effect at time points when it would inhibit proliferation; i.e. cell proliferation would not occur until ICER transcription had ceased and ICER protein had been degraded.

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