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Inducible cAMP early repressor (ICER) isoforms and neuronal apoptosis in cortical *in vitro* culture

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Abstract. CREB activation and CREB-dependent signaling pathways are crucial for neuronal survival. The term ICER (inducible cAMP early repressor) refers to four protein isoforms that are all endogenous, inducible antagonists of CREB. Jaworski and others (2003) have previously shown that one of those isoforms, ICER IIγ, is highly expressed in apoptotic neurons *in vitro* and its overexpression evokes neuronal death. In this study we investigated the role of all four ICER isoforms in cortical neuronal culture, comparing their expression level in serum-deprived/MK-801-treated neurons and their pro-apoptotic properties towards transfected cortical neurons. We have found that all four isoforms are induced upon pro-apoptotic treatment, and also that each of them separately evokes neuronal cell death following cortical culture transfection with the genes. The most efficiently induced, as well as the most effective in evoking neuronal cell death, were both ICER Iγ and IIγ isoforms.

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Key words: neurodegeneration, ICER, CREM, CREB, cell death, cortical neuronal culture

INTRODUCTION

The nuclear response to cAMP-mediated pathways involves a number of transcription factors. The best known of them is the cAMP response element (CRE) binding protein (CREB) and the whole family of proteins including CRE modulator (CREM) and activating transcription factor 1 (ATF1). *Creb* and *crem* genes encode multiple protein isoforms that can be either activators or repressors of cAMP dependent transcription. ICER (inducible cAMP early repressor) is one of the products of the *crem* gene, but it is unique among proteins of CREB/CREM/ATF family, because unlike the others, ICER expression is rapidly and transiently inducible by cAMP (Molina et al. 1993). Furthermore, ICER proteins share the DNA-binding and dimerization domain (containing basic leucine zipper module) with CREM, but lack the phosphorylation and transactivation domains. ICER proteins are powerful repressors of cAMP-driven transcription, acting either as homodimers or after heterodimerization with CREM/CREB proteins (Jaworski et al. 2003, Lamas et al. 1997, Stehle et al. 1993, Walker and Klaenhammer 1998).

ICER was first described as a negative CREM regulator in the pineal gland during day–night fluctuations of CREM expression, responsible for its returning to the steady-state level. At high levels it is predominantly expressed in tissues of neuroendocrine origin (pineal, pituitary and adrenal glands; Stehle et al. 1993). Increased levels of ICER transcripts were also observed in the cortex and hippocampus after treatment with kainate, NMDA and dizocilpine maleate (MK-801) (Konopka et al. 1998, Stehle et al. 1993, Storvik et al. 2000) at doses that induce neuronal apoptosis (Filipkowski et al. 1994, Hetman et al. 1997, Kaminska et al. 1994). Furthermore, Jaworski and coautors (2003) have shown that ICER IIγ overexpression induces neuronal cell death in the primary culture. The same study has shown that ICER IIγ is overexpressed in neurons *in vitro* treated to pro-apoptotic stimuli.

The term ICER refers collectively to 4 proteins, all produced from CREM/ICER gene by use of the internal promoter P2, localized in an intron of CREM/ICER gene (Molina et al. 1993, Stehle et al. 1993), as shown on Fig 1. ICER open reading frame is markedly shorter than CREM and consists of the exon γ, H and the DNA binding domain exon. The four known different isoforms of ICER result from alternative splicing and are named ICER I, ICER Iγ, ICER II and ICER IIγ. ICER I isoforms utilize DBDI (DNA binding domain I), while ICER II isoforms use DBDII. Additionally, both ICER I and II may be deficient in exon γ (ICER Iγ and ICER IIγ, respectively) (Fig. 1). The differences in structure of the isoforms may affect their function. For example, DBDI and DBDII may predestine a particular ICER isoform to form heterodimers with particular CREM or CREB proteins (Laoide et al. 1993).

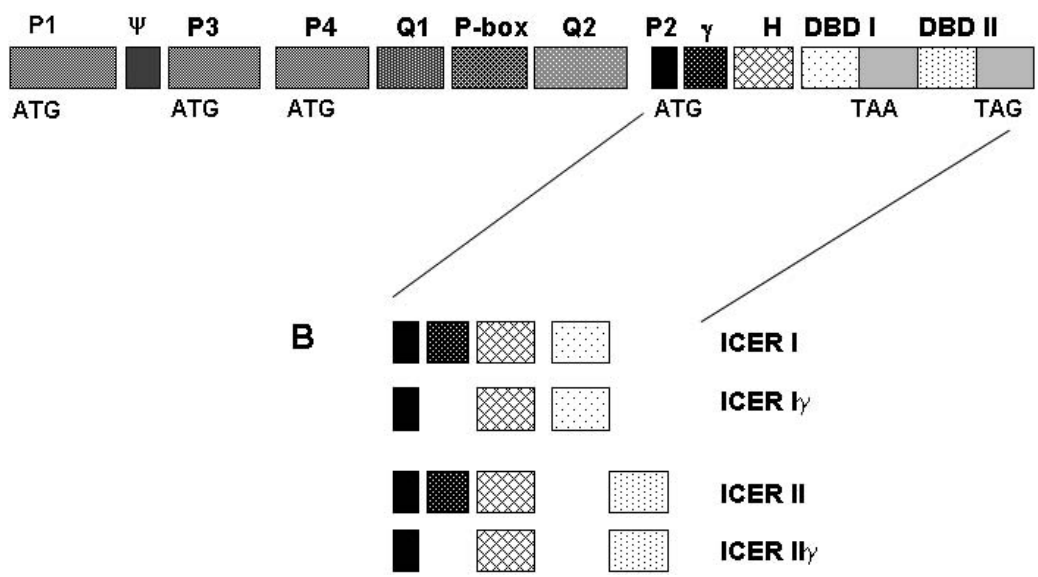

Fig. 1. Organization of CREM/ICER gene and ICER proteins. (A) Schematic representation of CREM/ICER gene structure. (P1), (P3), (P4) CREM promoters; (P2) ICER promoter. Activation of inducible P2 promoter drives expression of ICER transcript. (ATG) Start of the transcription; (TAA), (TAG) stop codones. (DBDI), (DBDII) DNA binding domains I and II; (Ψ), (Q1), (Q2), (γ) exons. (B) ICER isoforms protein structure.

Among the four isoforms known to date, only ICER IIγ was thoroughly investigated in the context of neuronal cell death. Jaworski and colleagues (2003) have shown that ICER IIγ is upregulated in apoptotic neurons *in vitro* and that overexpression of ICER delivered via adenoviral vectors evokes apoptosis in three different types of neuronal culture. The role of remaining ICER protein variants apparently has not yet been investigated. Therefore, we set out to study whether there are any particular differences among the four known ICER isoforms in the *in vitro* neuronal culture as far as neuronal cell death is concerned.

METHODS

Primary neuronal culture

Primary cultures of cortical neurons were obtained from newborn Wistar rats (Hetman et al. 1999). Briefly, dissociated cortical neurons were plated in 24-well dishes or 6-well dishes at density 0.5×10^6 or 2.0×10^6, respectively, and cultured in basal Eagle medium (BME) supplemented with 10% heat-inactivated bovine calf serum, 35 mM glucose, 1 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin and maintained in humidified incubator with 6.5% CO_2 at 37°C. Plates were coated with poly-D-lysine. Cytosine-D-arabinofuranoside (AraC, 2.5 μM; Sigma) was added to culture on the second day after seeding to inhibit proliferation of non-neuronal cells. This procedure results in a population consisting of at least 90% neurons at DIV5 (5 days *in vitro*) (Hetman et al. 1999).

Quantification of apoptosis by nuclear morphological changes

To visualize nuclear morphology, cells were fixed in 4% paraformaldehyde and stained with 2.5 mg/ml DNA dye Hoechst 33258 (bis-benzimide, Sigma). Cell morphology was analyzed by use of fluorescent microscopy (Olympus).

ICER isoforms cloning

To induce ICER expression three Wistar rats were subcutaneously injected with cycloheximide 3 mg/kg (see Konopka et al. 1995) in 100 μl of 0.9% NaCl. Forty-five minutes after the injection, the rats were sacrificed and visual cortices were isolated. For RNA isolation TRIZOL reagent (Invitrogen) was used. The remaining DNA was removed by digestion with DNase I (Sigma). RNA was reverse transcribed by use of Super Script reverse transcriptase (Invitrogen), in the presence of oligo-dT. The cDNA was then amplified in PCR reaction with the following set of primers: 5' ICER ATGAATTCACAAGACCACTCTGTATGCAAAA, 3'ICER for I and Iγ isoform ATTCTAGATTACTCTGCTTTATGGCAATAAAGG, 3'ICER for II and IIγ isoform ATTCTAGACTAATCTGTTTTGGGAGAGCAAATG. Amplified DNA was then digested with EcoRI and XbaI (Promega) together with the expression vector pEF1α containing the promoter of human Elongation Factor 1. Ligation was performed using Promega T4 DNA ligase, overnight at 15°C.

Cell transfection

Cortical neurons, cultured in 24-well plates (5×10^5 cells/well) were transiently transfected at DIV3 with 0.5–1.0 μg of total DNA using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. As the transfection control pTRACER vector (Invitrogen) was used, containing the eGFP gene under control of EF1α promoter. Cells were exposed to the mixture of plasmid DNA and Lipofectamine 2000 (1:2 ratio) in serum free medium (BME), supplemented with 35 mM glucose, 1 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Afterwards, the saved conditioned medium was returned to neurons, which were then cultured for 48 h and then fixed with 4% paraformaldehyde (20 min, room temperature).

Serum deprivation

Serum deprivation was performed with cortical neurons at 5 days *in vitro* as described previously (Hetman et al. 1999). Briefly, the cells were washed twice with serum-free BME and incubated with serum-free BME supplemented with 35 mM glucose, 1 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1 mM cytosine arabinoside (all from Sigma) in the presence of the NMDA receptor antagonist MK-801 (10 mM, Sigma). Control cells were washed similarly and then incubated in serum-containing conditioned medium.

RT-PCR analysis

For RT-PCR analysis total RNA was isolated from 2×10^6 by use of TRIZOL reagent (Invitrogen). The remaining DNA was removed by digestion with DNaseI (Sigma). RNA was reverse transcribed by use of Super Script reverse transcriptase (Invitrogen), in the presence of oligo-dT. The cDNA was then amplified in PCR reaction with the set of ICER primers described above. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used for verification of the amount of template used for reaction (GAPDH 5' AACTACATGGTCTACATGTT and GAPDH 3' GTGGTGCAGGATGCATTGCT).

Statistical analysis

Every experiment was done in triplicate. Data show either mean or representative example (Fig. 2). Statistical significance was assessed using *post-hoc* ANOVA. Differences were considered statistically significant if $P<0.05$.

RESULTS

Serum deprivation-induced apoptosis and ICER expression

It was previously described that serum deprivation in the presence of NMDA receptor antagonist MK-801 evokes apoptotic cell death in cortical neurons cultured *in vitro* (Hetman et al. 1997). We analyzed the levels of all four ICER mRNAs in these cultures. We found that all ICER isoforms are induced in serum deprived, MK-801 treated cortical neurons. However isoforms Iγ and IIγ are induced strongly, especially at 1 hour after the treatment. Compared to the γ isoforms expression of I and II, isoforms are apparently less inducible. On the other hand, serum deprivation did not change the expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fig. 2).

Overexpression of ICER Iγ and IIγ isoforms induces apoptosis stronger than isoforms I and II

We developed gene constructs, encoding each isoform of ICER protein under control of EF1α promoter. Every construct was co-transfected into primary neuronal culture with a vector carrying eGFP under EF1α promoter to visualize the transfected neurons. After 48 hours the cells were fixed and stained with a DNA dye, Hoechst 33258. Cell morphology was analyzed with fluorescent microscopy. GFP-positive cells, considered as co-transfected with ICER, were counted and percentage of apoptotic cells was calculated (Fig. 3A, B). As shown on the Fig. 3C, all four ICER isoforms induce a statistically significant increase of apoptotic neuronal death, compared with controls transfected with empty vectors.

DISCUSSION

The main findings from this study are that all four ICER isoforms, I, Iγ, II, and IIγ are induced when cortical neurons in culture are treated by pro-apoptotic deprivation of serum and blockade of NMDA receptors. Most importantly, overexpression of each of them individually results in neuronal cell death *in vitro*. Notably, both in inducing to higher levels, as well as more potent in evoking neuronal death, are gamma isoforms. Furthermore, the functional data were obtained with application of a cell transfection protocol in contrast to the previous studies (Jaworski et al. 2003), implicating ICER IIγ in neuronal apoptosis that employed adenoviral delivery of the gene.

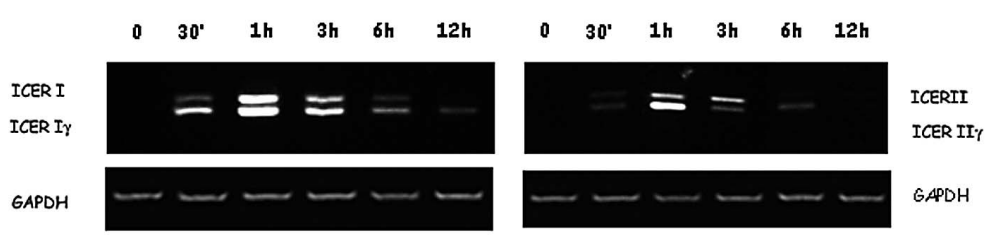

Fig. 2. Serum deprivation-induced apoptosis and ICER expression. Rat neonatal cortical cultures were serum-deprived and treated to MK-801 (see Methods for details) to induce apoptotic neuronal cell death and then subjected to total RNA extraction to analyze with RT-PCR for expression of each ICER isoform. As a control for the amount of template amplification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA analysis was used.

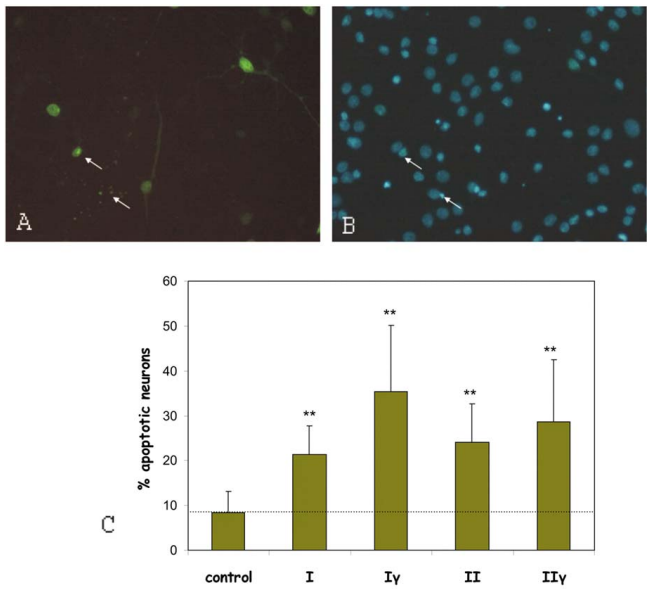

Fig. 3. Effect of ICER overexpression on neuronal viability in *in vitro* culture. Cortical neurons were co-transfected with eGFP and each ICER isoform, individually. Arrows point to apoptotic neurons (A) and corresponding Hoechst 33258 stained nuclei (B). Apoptotic neurons were counted among the transfected (eGFP-positive) cells. The histogram shows the number of dying cells as a percentage of the total number of the transfected cells (C). Result is the mean of 3 experiments, each done in triplicate. Error bars show SD value.

The expression pattern, as well as functional activity of all four proteins collectively named ICER, was apparently never previously characterized. Morales and others (2003) have shown that all four isoforms of ICER are upregulated in forskolin-induced granulosa cells. They did not note any differences between expression levels among these isoforms. The other studies have apparently focused only on a single ICER protein, most often the IIγ isoform (Jaworski et al. 2003, Mioduszewska et al. 2003, Razavi et al. 1998).

Isoforms of ICER differ in their structure by containing different DNA-binding domains (either DBDI or DBDII), as well as by having or lacking the γ domain. By analogy to the effects of different DBDs' utilization by CREM proteins, one can speculate that different ICER isoforms can form heterodimers more efficiently with particular isoforms of CREB or CREM. However, there is apparently nothing presently known about differential functions of ICER isoforms in any biological system.

We show herein that ICER variants lacking the γ domain (Iγ and IIγ) are more efficient in inducing neuronal apoptosis. Moreover, transcripts corresponding to the same proteins are more robustly induced in apoptotic neurons. Thus, one may suggest that the γ-domain has the more important impact on the ICER activity. Because there are virtually no other data on function and regulation of all four isoforms of ICER, it is difficult explain the association of γ isoforms with neuronal apoptosis. One may, however, speculate that shorter versions of ICER proteins may be able to make more stable heterodimers with CREB family proteins leading to greater downregulation of the anti-apoptotic CREB-driven gene expression. Clearly, this important issue needs further investigation.

It is also important to point out that we show herein, for the first time, functional analysis of ICER overexpression using a transfection (lipofection) procedure. Previous study, implicating ICER IIγ in neuronal apoptosis, has employed adenoviral expression vector (Jaworski et al. 2003). Since adenoviral vectors elicit very high expression levels of the proteins of interest, they are very useful experimental tools. However, adenovectors may induce cellular toxicity and elicit immune response (for review see: Bangari and Mittal 2006). Cell transfection approaches produce relatively little cytotoxicity and hence the data concerning neuronal apoptosis following ICER transfection are important in further supporting involvement of ICER in evoking neuronal cell death.

CONCLUSION

Our findings emphasize the crucial role of ICER in induction of neuronal apoptosis. Exploring the biological roles of ICER proteins, especially downstream effectors of each of the ICER isoforms, may have important consequences for our understanding of brain physiology and pathology, including neurodegeneration, as well as advancing knowledge on other tissues and diseases such as, e.g., diabetes type II (Abderrahmani et al. 2006).

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