Stress-induced expression of transcriptional repressor ICER in the adrenal gland

Maria Agnese Della Fazia[1], Giuseppe Servillo, Nicholas S. Foulkes, Paolo Sassone-Corsi*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS-INSERM-ULP, B.P. 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

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Abstract Second messenger cyclic AMP plays a central role in signalling within the hypothalamo-pituitary-adrenal (HPA) axis. Changes in gene expression are central to long-term adaptations made in response to stress in the adrenal gland. Here we demonstrate that expression of the cAMP inducible transcriptional repressor, ICER (Inducible cAMP Early Repressor), is rapidly and powerfully induced in response to surgical stress in the rat adrenal gland. Hypophysectomisation blocks stress-induced ICER expression. Finally we demonstrate that injection of the pituitary hormone ACTH (Adrenocorticotropin Hormone) induces robust ICER expression in the adrenal cortex. Thus, induction of the transcriptional repressor ICER is coupled to the HPA axis response to stress.

Key words: Inducible cAMP early repressor; cAMP-responsive element modulator; Adrenocorticotropin hormone; Adrenal gland; Surgery; Stress

1. Introduction

The ability to respond appropriately to environmental stress is fundamental to the survival of all plants and animals. In mammals, the hypothalamo-pituitary-adrenal (HPA) axis plays a central role in the physiological response to stress by transducing nervous stimuli into hormonal signals [1]. Steroid hormone production by the cells of the adrenal cortex is controlled by adrenocorticotropin hormone (ACTH) which is secreted by the pituitary gland in response to neuronal stimuli [2]. The key agent responsible for ACTH release is the corticotropin-releasing factor (CRF), a hormone produced in the hypothalamus and delivered to the adenohypophysis via the pituitary portal system [3]. In turn, binding of ACTH to a specific high affinity receptor in the adrenal cortex, induces steroidogenesis [4]. Circulating corticosteroids mediate many facets of the physiological response to stress and also negatively regulate ACTH production through a feedback action on the pituitary corticotrophs [5,6].

The cAMP signalling pathway plays a key role in the function of the neuroendocrine system. In the pituitary cAMP mediates hormonal secretion of ACTH [7,8]. Furthermore, ACTH receptors are positively coupled to adenylate cyclase. Thereby ACTH induces cAMP levels which stimulate steroid hormone production in the adrenal gland, as well as long-term trophic responses [4,9]. Changes in gene expression constitute crucial long-term adaptations mediated by cAMP signalling. cAMP responsive transcription factors are constituted by a family of closely related basic domain-leucine zipper (bZip) factors, both activators and repressors which bind to cAMP responsive promoter elements (CREs) located within the regulatory regions of cAMP-controlled genes [10]. Amongst these factors, the products of the CREM (CRE modulator) gene seem to play a preferential role in neuroendocrine tissues [10]. The CREM gene incorporates two alternative promoters. The first (P1) generates a family of transcripts which are the subject of extensive cell-specific alternative splicing and thereby encode both activators and repressors of transcription [11–13]. Notably, high levels of the activator CREMτ are expressed in postmeiotic maturing male germ cells where it regulates postmeiotic gene expression [12,14]. The second promoter (P2) lies near the 3′ end of the gene and encodes the repressor ICER (Inducible cAMP Early Repressor). ICER consists of the CREM bZip domain and functions as a powerful repressor [15,16]. The P2 promoter is strongly cAMP inducible by virtue of four tandemly repeated CRE elements [15]. It directs a rapid but transient induction of ICER expression which places CREM within the early response gene class [15]. ICER protein binds to and represses transcription directed by the CRE elements in the P2 promoter and thus constitutes a negative feedback autoregulatory loop [15]. ICER constitutes the most abundant CREM transcript in neuroendocrine tissues [16]. Recently we have shown that ICER is also inducible through activation of the Ras-dependent, nerve growth factor (NGF) signalling pathway [17].

Given the key role of the cAMP pathway in the function of the adrenal gland, we examined the expression of the cAMP responsive transcription factor CREM in this system. Here we show that in the adrenal glands of normal rats there is a significant variability in the level of CREM expression between individuals. CREM expression corresponds to the cAMP inducible repressor, ICER. We demonstrate that ICER expression in the adrenal gland is induced strongly following surgical stress. Hypophysectomisation completely eliminates the ICER induction following surgery as well as reducing the basal levels of expression. Injection of hypophysectomised rats with ACTH results in a rapid and transient induction of ICER mRNA in the cortical cells of the adrenal gland. We thus demonstrate an important example of the coupling of hormonal regulation to gene expression in the response to stress.

*Corresponding author. Fax: (33) (3) 88 65 32 46.
E-mail: paolosc@igbmc.u-strasbg.fr

[1]On leave from: Istituto di Patologia Generale, Università di Perugia, Policlinico Monteluce, 06100 Perugia, Italy.

Abbreviations: HPA, hypothalamo-pituitary-adrenal; cAMP, cyclic adenosine monophosphate; ICER, inducible cAMP early repressor; ACTH, adrenocorticotropin hormone; CRE, cAMP-responsive element; CREM, CRE modulator; NGF, nerve growth factor; bZip, basic domain-leucine zipper

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2. Materials and methods

2.1. Animals

All normal and hypophysectomised rats (200–250-g male Sprague-Dawley) were purchased from Iffa Credo (France). Hypophysectomised rats were sacrificed within 2–3 weeks following surgery. Rats were maintained under a standard LD 12:12 light/dark cycle and food and water were provided ad libitum. Five rats were housed per large cage and bedding was changed every 3–4 days. Surgery was performed in the morning, between 1 and 5 h after 'lights on' using ether anesthesia. Animals were partially hepatectomised (PH), according to the Higgins and Anderson procedure [18], removing approximately 70% of the liver mass. For sham operation (SO), the surgical procedure was identical except that the liver was displaced, manipulated and then replaced in the abdomen. Rats were then sacrificed by decapitation 30 min, 2 or 5 h after surgery. The adrenal glands were rapidly removed and frozen in liquid nitrogen for subsequent RNA extraction. Non-hepatectomised animals were used as negative controls.

2.2. ACTH injections

Both normal and hypophysectomised rats were injected intraperitoneally with 0.9% saline carrier solution (saline) or ACTH (Sigma) at a dose of 5 IU/100 g body weight. Animals were sacrificed by decapitation 1 or 2 h after injection and the adrenal glands were rapidly dissected and stored in liquid nitrogen prior to RNA extraction.

2.3. RNA analysis

RNA was isolated using an acid-phenol extraction method [19]. RNase protection analysis using the riboprobe p75 was performed as previously described [16]. Primer extension analysis of the ICER transcript was performed using the oligonucleotide 5′-CAGTTTCATCTCCAGTTACAGCCATGTTGG-3′ as primer and a standard protocol [20]. 10 μg of total adrenal gland RNA was included in each reaction and four different annealing temperatures were tested as described in the figure legends. As a size marker, a set of dideoxy sequencing reactions primed with the same primer was run in parallel.

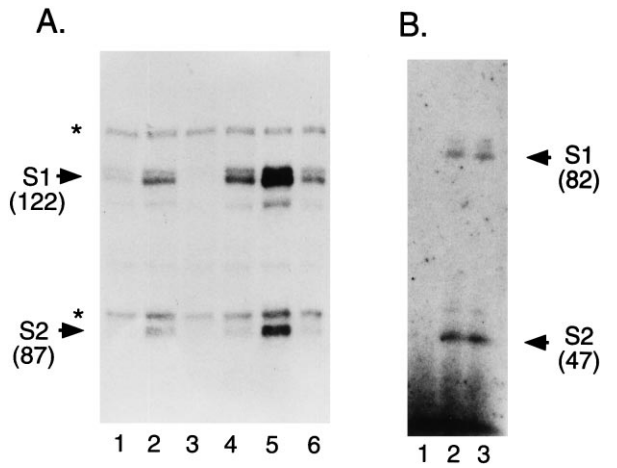

Fig. 1. ICER mRNA expression in the rat adrenal gland. Panel A: RNase protection analysis of CREM expression in the adrenal gland using the riboprobe p75. 10-μg aliquots of adrenal gland total RNA were prepared from individual rats. Arrowheads indicate fragments protected by ICER transcripts initiated from the S1 and S2 transcription start sites within the P2 promoter. The size of the protected fragments (nucleotides) is indicated in parentheses. Lane numbers 1–6 denote individual rats. Asterisks indicate non-specific, autoprotected fragments. Panel B: Primer extension analysis of P2 promoter-derived ICER transcripts. A ^{32}P-end-labelled 30-mer antisense oligonucleotide was hybridized with 10-μg aliquots of adrenal gland total RNA at 37°C, 45°C and 55°C (lanes 1, 2 and 3, respectively) and then cDNA was synthesised by AMV reverse transcriptase. Arrowheads denote cDNA products extending to the S1 and S2 transcription start sites. The size of the products (nucleotides) is shown in parentheses.

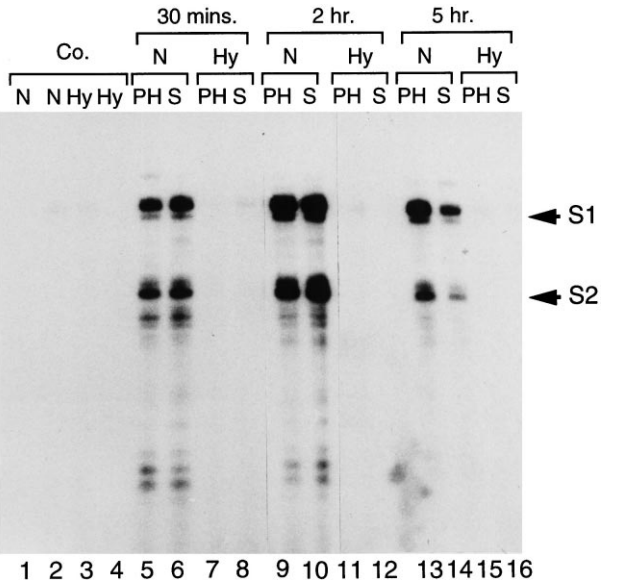

Fig. 2. ICER expression is induced by surgical injury. RNase protection analysis of ICER mRNA expression in total adrenal RNA samples prepared from normal (N) and hypophysectomised (Hy) rats which were subjected either to partial hepatectomy (PH) or a sham operation (S). Animals were sacrificed 30 min, 2 h or 5 h following surgery. Two groups of both normal and hypophysectomised rats which were not manipulated were used as controls (Co). Each sample represents a pool of 6 adrenal glands prepared from 5 individual rats. S1 and S2 denote the protected fragments obtained with the p75 riboprobe which correspond to the ICER. Both sham operation and partial hepatectomy result in a rapid and transient induction of the ICER transcript in normal rats. This induction is completely abolished in hypophysectomised animals.

3. Results

The cAMP signalling pathway plays a key role in the function of the HPA axis [10]. Fundamental to long-term adaptations made by this axis in response to environmental stimuli are changes in gene expression. We have previously documented elevated expression of the cAMP-inducible transcription repressor, ICER in the adrenal gland [16]. Furthermore, we have implicated ICER as playing an important role in the regulation of gene expression in many neuroendocrine tissues [21–23]. We therefore chose to examine the expression of ICER in the adrenal gland in more detail. Initially, we sacrificed several male rats of identical age at the same time points and then analysed the level of ICER expression by RNase protection assay in individual adrenal glands. Our results revealed a striking inter-animal variability in the levels of ICER expression (Fig. 1A). Furthermore, the size of the protected fragment obtained using the riboprobe p75 confirmed that the major CREM product in this system was ICER (Fig. 1A) [15,16]. This was further verified by primer extension analysis using an oligonucleotide primer complementary to the 3′ end of the first ICER-specific exon [15] (Fig. 1B). Expression of ICER mRNAs at both the S1 and S2 transcription start sites of the CREM P2 promoter were detected (Fig. 1A,B) [15].

Given the importance of cAMP in regulating adrenal gland function during stress and the cAMP-inducibility of the CREM P2 promoter, we speculated that the observed differences in ICER expression between individual rats might well reflect variability in the levels of stress. In order to directly assess the effect of stress on the expression of ICER in the

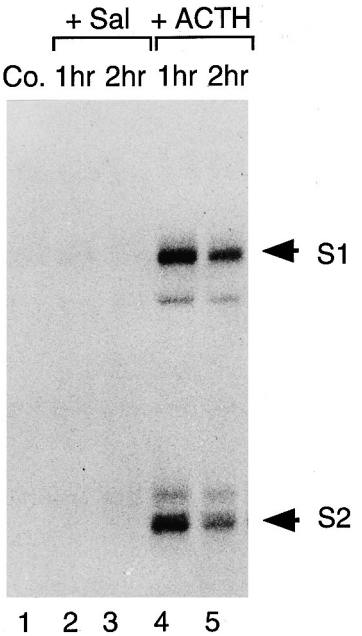

Fig. 3. ACTH injection induces ICER expression in the adrenal gland. Groups of 5 hypophysectomised rats were injected with either physiological saline solution (+Sal) or ACTH at 5 IU per 100 g body weight (+ACTH) and then sacrificed 1 or 2 h following injection. Adrenal gland RNA was prepared and assayed by RNase protection assay using the p75 riboprobe. S1 and S2 denote the ICER protected fragments which are strongly induced upon injection of ACTH.

adrenal gland we chose to subject rats to an acute stress. Surgery has been well documented to induce a characteristic acute stress response in rats [3]. As part of a parallel study on liver regeneration [24] we performed partial hepatectomy (PH) on normal rats where 70% of the hepatic mass was surgically removed [18]. We also performed a sham operation (S) where the body cavity was opened and the hepatic tissue was displaced but not removed. In both cases, the rats were subjected to ether anesthesia. Subsequently the animals were allowed to recover and then sacrificed at various time points following the operation. Adrenal glands were dissected from 6 rats per point and then pooled together so as to compensate for inter-animal variability. RNA extracted from the pooled glands was assayed for ICER expression by RNase protection analysis (Fig. 2) [15]. We demonstrate a strong induction of the ICER transcript in the 30 min to 2 h following operation (Fig. 2; compare lanes 5 and 9 with lanes 1 and 2). Induced levels were reduced by 5 h after PH (lane 13). An equivalent pattern of induction was detected following sham operation (compare lanes 6, 10 and 14 with lanes 1 and 2), confirming that this observed increase results from the stress of the surgical procedure rather than the removal of the hepatic mass.

The pituitary gland is a crucial player in the regulation and maintenance of the adrenal cortex function via the secretion of ACTH. Indeed, hypophysectomisation ultimately leads to atrophy of the cortex, but this can be reversed by the injection of ACTH [25], suggesting that ACTH may regulate adrenal cortex proliferation [26]. ACTH acts by binding to high affinity receptors which are positively coupled to adenylate cyclase. Thus ACTH release from the corticotrophs of the pituitary leads to increase in cAMP levels in the adrenal cortex. We therefore tested whether hypophysectomisation might effect the induction of ICER expression in response to surgical stress. Hypophysectomised rats (Hy) were subjected to partial hepatectomy (PH) or a sham operation (S) and then sacrificed as previously described for the normal rats (Fig. 2). ICER induction was completely abolished in the hypophysectomised rats during both partial hepatectomy and sham operation, demonstrating that the pituitary gland is crucial in directing the stress-induced ICER expression (compare lanes 7, 8, 11, 12, 15 and 16 with lanes 3 and 4). Finally we tested whether ACTH might constitute a signal from the pituitary gland which directs ICER expression in the adrenal gland. Groups of hypophysectomised rats were injected intraperitoneally with ACTH or with the physiological saline carrier solution, and then sacrificed one or two hours following injection (Fig. 3). RNase protection analysis demonstrated a strong induction of ICER transcript in the adrenal gland at both 1 and 2 h following injection (lanes 4 and 5) while saline injected controls showed no change in the basal expression (lanes 2 and 3).

4. Discussion

We document for the first time the expression of the cAMP-inducible transcriptional repressor, ICER in the adrenal gland. In normal rats, levels of the CREM transcripts are extremely variable, most likely as a result of differences in the levels of stress experienced by the individual animals (Fig. 1). Consistently, the stress associated with surgical operation results in a very rapid and strong induction of the ICER transcript (Fig. 2). Significantly, this induction is completely abolished by prior hypophysectomisation of the rats (Fig. 2). Injection of hypophysectomised rats with ACTH results in a strong upregulation of ICER expression (Fig. 3). In agreement with these findings we have demonstrated by in situ hybridisation that ICER expression seems restricted to the adrenal gland cortex, the principal site of action of ACTH (in preparation). This suggests a potential role for ICER in directing changes in gene expression in response to ACTH in the adrenal cortex.

Previously, inducibility of ICER expression both by cAMP and NGF has been described in the pheochromocytoma cell line PC12 [15,17]. This cell line is derived from cells of the adrenal medulla. Furthermore, the adrenal medulla is innervated by sympathetic nerve fibres and is regulated by adrenergic signals in response to stress [27]. ICER has already been shown to be an important regulatory target of adrenergic signals in the pineal gland [16]. Our findings suggest that ICER induction in response to surgical stress seems limited to the adrenal cortex. This suggests that either ICER is not inducible in primary medulla cells or the conditions of our experimental analysis were not sufficient to drive an induction of ICER in these cells.

It would be of great interest to identify gene regulatory targets of the induced ICER repressor. Activation of the cAMP signalling pathway stimulates steroidogenesis through the transcriptional induction of the genes encoding the enzymes involved in glucocorticoid synthesis [28,29]. It is tempting to speculate that ICER might be involved in attenuating this induced expression. In this way, ICER might be a player in directing more long-term adaptations to stress. We have previously documented how induction of ICER expression by cAMP might contribute to a refractory phase during which

additional cAMP stimuli fail to elicit a full transcriptional response [30,31]. In this previous work we studied the effect of induced ICER protein on the ICER promoter itself, and implicated the autoregulatory negative feedback loop in conferring refractory ICER inducibility [30]. However, it is possible that by the same mechanism other gene targets for ICER repression might also be refractory to cAMP induction in the wake of an initial induction [30]. An interesting implication of this hypothesis would be that the transcriptional response of the adrenal gland to repetitive stress might be defined either by the frequency or by the duration of previous stress episodes.

Hypophysectomisation ultimately leads to atrophy of the adrenal cortex while chronic ACTH stimulation leads to hypertrophy [25]. This suggests that ACTH may play a role in the regulation of proliferation of adrenal cortex cells [26]. In this respect it is interesting to note that CREM has been previously implicated in the regulation of cell proliferation [32,33]. Specifically, in the case of the pituitary corticotroph cell line, AtT20, it has been hypothesised that by repressing the expression of the cyclin A promoter, ICER regulates the passage of the cells through the G2/M phase of the cell cycle [32]. It is, however, too premature to link the absence of induced ICER expression in the hypophysectomised animals to the atrophy of the adrenal cortex. More detailed information on the role of ICER in the adrenal gland should be obtained by studying adrenal function in mice which carry a targeted mutation in the CREM gene [14]. The results which we present here represent a crucial first step in the characterisation of the regulation of this transcription factor. Furthermore, our findings should shed more light on the regulation of gene expression in the adrenal gland and during the adaptation to stress.

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