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General Review

Peptide Hormone and Growth Factor Regulation of Nuclear Proto-oncogenes and Specific Functions in Adrenal Cells

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Among the large number of immediate early genes, nuclear proto-oncogenes of the Fos and Jun families, have been postulated to be involved in the long-term effects of several growth factors on cell differentiation and/or multiplication. Since adrenal cell differentiated functions appear to be regulated by specific hormones and growth factors, the effects of these factors on proto-oncogene mRNA levels were analysed in bovine adrenal fasciculata cells (BAC) in culture. Corticotropin (ACTH) and insulin-like growth factor I increased c-*fos* and *jun*-B mRNA, but had no effect on c-*jun* mRNA and these early changes were associated with a later increase in BAC specific function [ACTH receptors, cytochrome *P*450 17α) and 3β-hydroxysteroid dehydrogenase (3β-HSD)] and an enhanced steroidogenic responsiveness to both ACTH and angiotensin-II (A-II). On the other hand, A-II increased the three proto-oncogene (c-*fos*, c-*jun* and *jun*-B) mRNAs, induced a decrease of *P*450 17α and 3β-HSD and caused a marked homologous and heterologous (ACTH) densitization. Transforming growth factor β_1 which only increased *jun*-B mRNA, markedly reduced BAC differentiated functions and the steroidogenic responsiveness to both ACTH and A-II. Thus, it is postulated that the proto-oncoproteins encoded by the immediate early genes may play a role in the long-term effects of peptide hormones and growth factors on BAC differentiated functions.

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INTRODUCTION

Most nucleated eukaryotic cells constantly respond to a variety of factors, such as growth factor and peptide hormones, that bind to cell surface transmembrane receptors and which, in addition to acute effects, induce long-term transcription-dependent changes in phenotype. One mechanism which has been proposed to link ligand–receptor interaction with long-term effects is the induction of transcription regulatory proteins, which are encoded by "primary response genes" or "immediate early genes", the induction of which is independent of protein synthesis but requires only the post-transcriptional modification of pre-existing transcriptional factors [1]. Until recently, it was thought that the ligand-specific response in a cell type and the distinct responses to a same ligand among different cell types were related to restricted patterns of gene expression. However, it has now been clearly shown that a variety of ligand-induced long-term phenotypic changes, either ligand-specific for a given cell type, or cell-specific in response to a common ligand, are associated with an initial induction of many common genes. Therefore, the biological diversity in response to alternative inductive signals might be a consequence of varied combinatory utilizations of a common pool of primary response genes, rather than the result of the induction of transcripts specific to a particular ligand or cell type [1].

Among the very large numbers of immediate early genes, cellular nuclear proto-oncogenes, in particular members of the Fos and Jun families, appear to play a crucial role in linking the initial ligand–receptor interaction to the promoting effects on growth, development and/or differentiation of such ligands [1–3]. Nuclear proto-oncogenes share many common features such as

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rapid and transient expression in response to peptide hormones and growth factors, short half-life of mRNAs and proteins, and post-translational modification of proto-oncoproteins, mainly by serine phosphorylation.

Members of the Fos and Jun families of proto-oncoproteins interact at their leucine zipper to form homodimers or heterodimers [4, 5]. The Fos/Jun dimers bind a specific DNA sequence (the AP-1 site) found in the 5′ regulatory region of many genes including c-*fos* and c-*jun* [2]. Heterodimers composed of one member of the Fos family and one of the Jun family exhibit much higher affinity for AP-1 sites and more potent transcription regulating activity than homo- and heterodimers composed solely of members of the Jun family. These differing DNA binding affinities and transcriptional activation suggest a mechanism by which the relative concentration of Fos and Jun proto-oncoproteins may have regulatory effects [4, 6, 7].

In steroidogenic cells, the specific protein hormones induce two well-established types of responses: (a) acute steroid output, which does not require transcription, and (b) long-term trophic effects on cell differentiation. In addition, recent data indicate that growth factors, including insulin-like growth factor I (IGF-I) and transforming growth factor β (TGFβ), which have no or very small acute steroidogenic effects, can regulate positively or negatively the state of steroidogenic cell differentiation. In order to determine the role of nuclear proto-oncogenes in the long-term effects of peptide hormones and growth factors in steroidogenic cells, we have investigated the induction by these factors of nuclear proto-oncogenes of the Jun and Fos families using a well-defined steroidogenic model, bovine adrenal fasciculata-reticularis cells (BAC) in primary culture. These cellws contain specific membrane receptors for corticotropin (ACTH) [8], angiotensin-II (A-II) [9, 10], IGF-I [11] and TGFβ [12] and these factors regulate the expression of specific functions of BAC. Therefore, we have analysed the relations between the initial effects of these factors on proto-oncogenes and their long-term effects.

EFFECTS OF ACTH AND A-II ON BAC PROTO-ONCOGENE mRNAs

The time-course effects of both hormones on proto-oncogene mRNA are shown in Fig. 1. ACTH increased c-*fos* and *jun*-B mRNA but had no effect on c-*jun* mRNA, whereas A-II enhanced the mRNAs of the three proto-oncogenes. The accumulation of mRNA was transient, reaching maximal values between 1 and 2 h after hormonal stimulation and decreasing thereafter. The effect of both hormones on proto-oncogene mRNA accumulation was dose-dependent [13]. The concentrations of ACTH required to induce half-maximal and maximal proto-oncogene mRNA levels were about 10^{-11} and 10^{-9} M, respectively, whereas the corresponding A-II concentrations were about 10^{-9} and 10^{-7} M, respectively. These concentrations for both hormones are similar to those required to induce maximal and half-maximal cortisol secretion [13].

These results confirm and extend previous studies. *In vivo* studies in the rat have shown that ACTH treatment produced a rapid but transient increase in adrenal c-*fos* mRNA levels [14] and that either acute stress [15] or ACTH administration [16] enhanced the number of adrenal cells containing c-*fos*-like immunoreactive material. Moreover, ACTH also increased c-*fos* mRNA levels in Y-1 adrenocortical cells [17], cultured rat adrenal cells [18] and cultured bovine glomerulosa cells [19]. Similarly, the effect of A-II on proto-oncogene mRNA levels in BAC is consistent with a recent report, showing that A-II increases the three proto-oncogene mRNA levels in bovine adrenal glomerulosa cells [19].

The stimulatory action of ACTH on proto-oncogene mRNAs as well as in steroid production is mediated through protein kinase activation, since cAMP agonist mimicked the effects of ACTH [13, 18] and since a selective inhibitor of protein kinase A suppressed the effects of ACTH on rat adrenal cells [18].

Since BAC contain two A-II receptor subtypes AT1 and AT2 [10], we have investigated which receptor subtype was involved in the effects of A-II or BAC

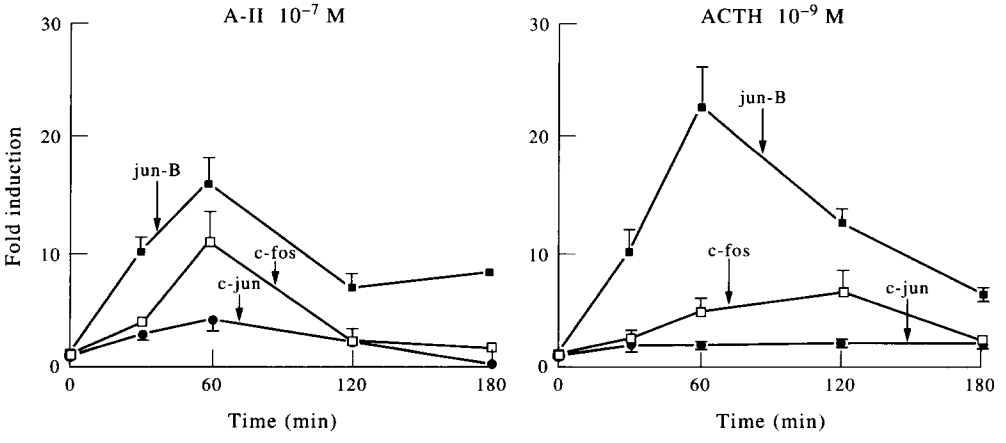

Fig. 1. Time-course of A-II (10^{-7} M) and ACTH (10^{-9} M) effects on BAC proto-oncogene mRNAs.

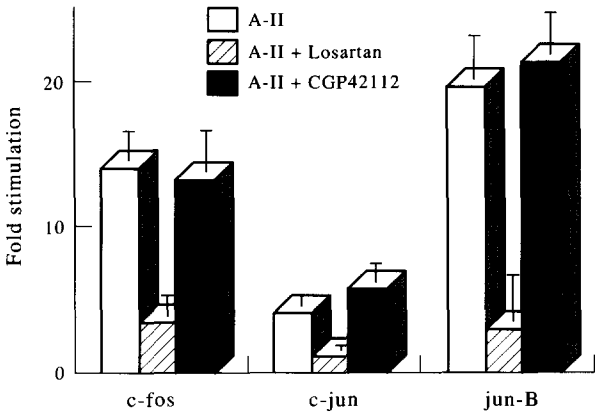

Fig. 2. Effects of A-II and its antagonists on c-*fos*, c-*jun* and *jun*-B mRNA levels. BAC were treated with A-II 10^{-8} M in the absence (□) or presence of Losartan 10^{-5} M (▨) or CGP 42112 5×10^{-8} M (■). Proto-oncogene mRNA levels were evaluated 1 h later by Northern blot. The results are mean ± SEM of three independent experiments.

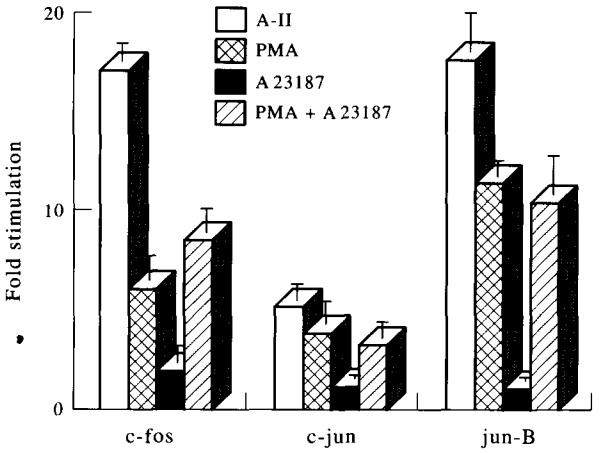

Fig. 3. Effects of A-II, phorbol ester PMA, and calcium ionophore A28187 on proto-oncogene mRNA levels in BAC. Cells were treated with A-II 10^{-7} M, PMA 10^{-7} M, or A23187 10^{-7} M. The proto-oncogene mRNA levels were evaluated 1 h later by Northern blot. The results are mean ± SEM of 3 to 9 experiments.

proto-oncogene mRNA. Treatment of BAC with either Losartan, a specific AT1 antagonist or CGP 42112, a specific ligand of AT2 receptor, did not change proto-oncogene mRNA levels [20]. However, Losartan blocked almost completely the stimulatory effects of A-II on the three proto-oncogene mRNAs, whereas CGP 42112, at 5×10^{-8} M, a concentration at which this compound occupied most of the AT2 receptors [21], had no effect on the stimulatory action of A-II (Fig. 2). These results indicate therefore that the effects of A-II on proto-oncogene mRNAs were mediated exclusively by the AT1 receptor subtype.

The steroidogenic effects of A-II on BAC are mediated exclusively by AT1 receptor subtype [21], through activation of the two branches of the phosphoinositide pathway, protein kinase C and Ca^{2+}/calmodulin [13]. Therefore, we have investigated whether these two branches are also required to induce the expression of proto-oncogenes in BAC. Two approaches were used. First, we compared the stimulatory effects of A-II on proto-oncogene mRNA with those produced by an activator of either protein kinase C (phorbol ester PMA) or Ca^{2+}/calmodulin (calcium ionophore A23187) or both. PMA alone was able to increase proto-oncogene mRNA levels, but its effects were significantly lower than those induced by A-II (Fig. 3). In contrast, A23192 either alone or together with PMA, had no stimulatory action on proto-oncogene mRNAs.

In the second approach, we used the specific inhibitors of each branch of the phosphoinositide pathway. Staurosporine has been shown to be a potent inhibitor of protein kinase C (PKC) in many cell systems, including BAC [22], whereas trifluoroperazine (TFP) is an inhibitor of calmodulin in several cell types [23] including BAC [22]. As shown in Fig. 4, staurosporine inhibited the stimulatory action of A-II on proto-oncogene mRNA levels by 80–90%. On the other hand, TFP either alone or together with PMA produced a small and not statistically significant inhibition of the effects of A-II on proto-oncogene expression. The low inhibitory action of TFP might be due to its ability to slightly inhibit PKC at high concentrations [23]. Thus, both sets of experimental approaches indicate that the stimulatory action of A-II on proto-oncogene mRNA levels is mainly mediated through the action of PKC.

EFFECTS OF GROWTH FACTORS ON PROTO-ONCOGENE mRNA LEVELS IN BAC

A property of most growth factors is their ability to induce proto-oncogene expression in many cell types. This early expression of proto-oncogenes has been

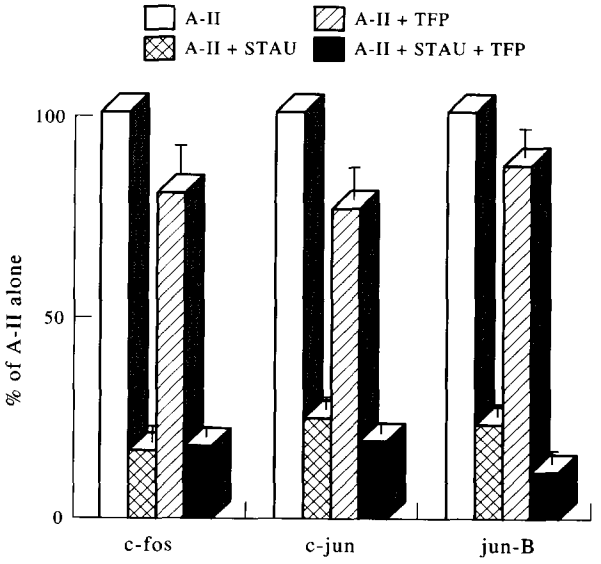

Fig. 4. Effects of A-II (10^{-7} M) in the absence or presence of Staurosporine (Stau) (10^{-6} M), trifluoroperazine (TFP) (10^{-5} M) or both on proto-oncogene mRNA levels in BAC. The results are the mean ± SEM of 3 to 5 experiments.

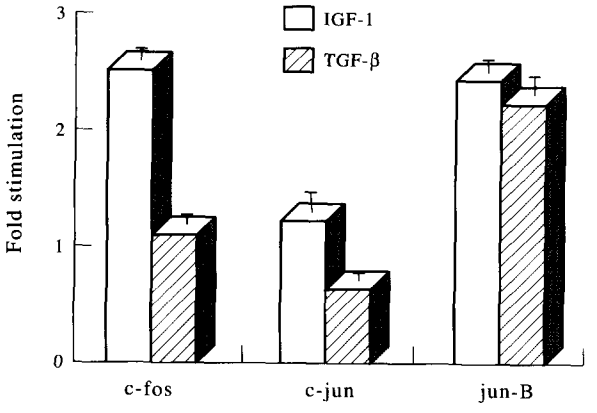

Fig. 5. Effects of IGF-I (7.10^{-9} M) and TGFβ1 (4×10^{-11} M) on proto-oncogene mRNA levels in BAC.

thought to be required for the later mitogenic effect of growth factors [2]. In addition to their mitogenic effect, many growth factors have pleiotropic actions and are able to regulate cell differentiation positively or negatively [2]. Many groups have shown that differentiated functions of adrenal cells can be regulated by growth factors including IGF-I [11, 24–26], and TGFβ [12, 27–31]. Therefore, we have investigated the effects of these factors on proto-oncogene mRNA in BAC (Fig. 5). IGF-I significantly increased c-*fos* and *jun*-B but not c-*jun*, whereas TGFβ increased *jun*-B, decreased c-*jun*, and had no significant effect on c-*fos*. These observations are consistent with the results reported previously, showing that in all cell types studied, TGFβ induced the expression of *jun*-B, whereas it had little effect on c-*jun* and c-*fos* [32–24].

EFFECTS OF ACTH, A-II, IGF-I AND TGFβ ON BAC SPECIFIC FUNCTIONS AND STEROIDOGENIC RESPONSIVENESS

The main specific function of BAC is to produce cortisol in response to hormonal stimulation. This steroidogenic responsiveness depends upon the state of cell differentiation, in particular the number of membrane bound receptors for the specific hormone, the coupling of these receptors to intracellular effectors and the activity of the enzymes involved in the steroidogenic pathway. Figure 6 shows the effect of a 2-day pre-treatment with several factors on the steroidogenic responsiveness to both ACTH and A-II. Clearly, pre-treatment with either ACTH or IGF-I enhanced the steroidogenic responsiveness to both ACTH and A-II, whereas pre-treatment with A-II or TGFβ blunted the response to both hormones. These changes in the steroidogenic responsiveness are associated, and probably are dependent with marked changes in the expression of specific function of BAC. Thus, ACTH is one of the few peptide hormones that increases rather than desensitizes the responsiveness of its target cells. This is not only due to its positive action on steroidogenic enzyme gene expression [35], but also to its stimulatory effects on ACTH receptor mRNA and binding sites ([8], A. Penhoat, C. Jaillard and J. M. Saez, submitted) and on the α_s subunit of G protein [36]. In contrast, A-II strongly reduced its own receptors ([19], R. Ouali and J. M. Saez, unpublished data) and *P*450 17α and 3β-hydroxysteroid dehydrogenase (3β-HSD) [30, 37]. IGF-I and TGFβ had also opposite effects on BAC differentiated function. Thus, IGF-I increases ACTH and A-II receptor mRNA and binding sites [11 and unpublished data] as well as the mRNAs, proteins and activities of *P*450 17α and 3β-HSD ([37, 38], unpublished data), whereas TGFβ has the opposite effects (27–31], D. Langlois, R. Ouali, M. C. Berthelon and J. M. Saez, submitted).

DISCUSSION

The effects of peptide hormones and growth factors on BAC proto-oncogene and specific functions that have been discussed are summarized in Table 1. Although there was not a clear-cut relation between proto-oncogene and expression of differentiated functions, three patterns of response were observed.

(1) Increased cAMP production induced by ACTH or cAMP derivatives [13, 18] caused an increase in c-*fos* and *jun*-B levels and thereafter an increase in $ACTH_R$, *P*450 17α and 3β-HSD mRNA levels. This would suggest that the regulation of these three genes are cAMP-dependent. Whether the 5′-flanking region of bovine $ACTH_R$ and 3β-HSD genes contains consensus CRE *cis*-elements is unknown since this region has not been analysed yet. In bovine *P*450 17α gene, the 5′-flanking region contains cAMP-responsive sequences but no consensus CREs have been identified [38]. Moreover, the cAMP stimulatory effects on *P*450

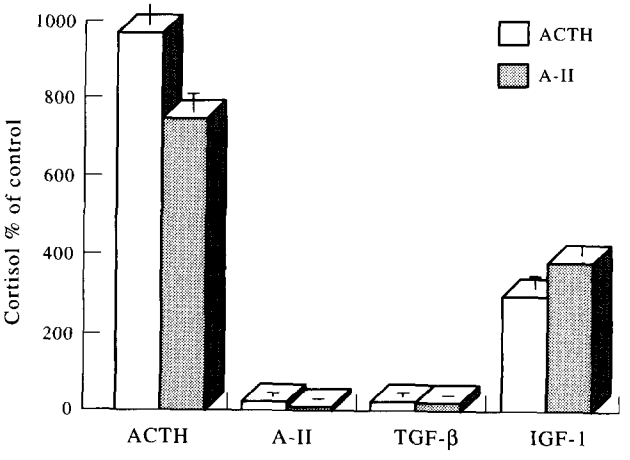

Fig. 6. Effects of a 2 day pre-treatment with ACTH (10^{-8} M, A-II (10^{-7} M), TGFβ (4×10^{-11} M) and IGF-I (7×10^{-9} M) on BAC steroidogenic responsiveness. At the end of the experimental period, the medium was removed and cells were incubated in the presence of either ACTH (10^{-8} M) or A-II (10^{-7} M). After 2 h the medium was removed and the cortisol measured (mean ± SEM of 4 to 6 experiments).

Table 1. Effects of several factors on BAC proto-oncogenes and differentiated functions

	c-*fos*	c-*jun*	*jun*-B	$ACTH_R$	$A\text{-}II_R$	*P*450 17α	3β-HSD
ACTH	↗	—	↗	⇗	↘	⇗	⇗
A-II	↗	↗	↗	↗	⇘	↘	↘
TGF-β	—	—	↗	↘	↘	↘	↘
IGF-I	↗	—	↗	↗	↗	↗	↗

Abbreviations used: ACTH, corticotropin; $ACTH_R$, ACTH recptor; A-II, angiotensin II; $A\text{-}II_R$, A-II receptor type AT1; TGFβ, transforming growth factor β1; IGF-I, insulin-like growth factor I; *P*450 17α, cytochrome *P*450 17α hydroxylase; 3β-HSD, 3β-hydroxysteriod dehydrogenase 5-en–4-en-isomerase.

17α and 3β-HSD are blocked by cycloheximide [39, 40]. These findings suggest that the expression of these genes is probably not mediated through one of the members of CREB/ATF family, but the exact nature of these transcriptional factors is unknown. A similar pattern of response was observed following stimulation with IGF-I, but its effect on proto-oncogene as well as on the BAC specific function were lower than those produced by ACTH or cAMP.

(2) Activation of PKC by PMA or A-II enhanced the expression of the three proto-oncogene and this was associated with a decrease in the mRNA levels of A-II receptor, *P*450 17α and 3β-HSD. The 5′-flanking region of bovine A-II receptor (AT1) and 3β-HSD has not yet been analysed and that of *P*450 17α does not contain consensus AP-1 binding sites [41]. However, it is known that the proto-oncoprotein homodimer (Jun) and heterodimer (Jun/Fos) can bind to sequences other than the classical AP-1 binding site and that Fos/c-Jun heterodimer can repress the expression of several genes [2].

(3) TGFβ caused an increase in *jun*-B, a decrease in c-*jun* and later on a decrease in the mRNA levels of the main genes encoding BAC differentiated functions. Although the effect of TGFβ on *jun*-B expression has been observed in other cell types [32, 33], how this preferential stimulation can be related to its pleitropic effects is unknown. A TGFβ inhibitory element (TIE) has been described in the 5′-flanking region of several genes, the expression of which is inhibited by TGFβ [32]. TIE binds a protein complex, induced in TGFβ-treated cells, that contains Fos and probably Jun-B [43]. Although the TIE sequence (GnnTTGGtGa) and the AP-1 sequence (TGAg/cTCA) are different, AP-1 sequence can compete for binding to TIE by the TGFβ inducible nuclear protein [42, 43].

In another steroidogenic cell type, Leydig cell, peptide hormones and growth factors also regulate proto-oncogene expression [44, 45] and later on, differentiated functions. Thus, the different regulation of proto-oncogene expression, the multiple combinatory formation of homodimers and heterodimers between Fos and Jun [2, 3] and between Jun and some members of the CREB/ATF family [46] and the different transcriptional activity of heterodimers containing c-Jun or Jun-B [7, 47, 48], illustrate the possibilities for subtle gene regulation by these proto-oncoproteins. However, to clarify the role of these proto-oncoproteins in the long-term effect of peptide hormones and growth factors on steroidogenic cell differentiated function, it would be necessary to complete the studies on proto-oncogene expression by other approaches, in particular by transfection experiments with wild or mutated proto-oncogenes alone or in combination, and by using antisense oligodeoxynucleotides [49].

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