

# Angiotensin-II-induced expression of proto-oncogene (*c-fos*, *jun-B* and *c-jun*) mRNA in bovine adrenocortical fasciculata cells (BAC) is mediated by AT-1 receptors

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We have shown previously that angiotensin-II (A-II) controls proto-oncogene (*c-fos*, *jun-B* and *c-jun*) mRNA accumulation in bovine adrenal fasciculata cells (BAC). Since BAC contain both subtypes (AT-1 and AT-2) of the A-II receptor, we have investigated which subtype was involved in the effect of A-II on proto-oncogene mRNA by using a selective antagonist for AT-1 (DUP 753) and for AT-2 (CGP 42112A). DUP 753, but not CGP 42112A, inhibited the stimulatory effect of A-II on proto-oncogene mRNA, with  $ID_{50}$ s of  $4 \times 10^{-7}$  M,  $7 \times 10^{-7}$  M and  $2 \times 10^{-6}$  M for *c-fos*, *jun-B* and *c-jun*, respectively. Neither of the two antagonists by themselves had a direct effect on proto-oncogene mRNA. As the A-II AT-1 receptors are coupled to the phospholipase C system in BAC, we have investigated whether the A-II effects on the proto-oncogenes were mediated by protein kinase C (PKC) or by  $Ca^{2+}$  calmodulin. First, activation of PKC by the phorbol ester, PMA, increased the level of the three proto-oncogene mRNAs, whereas calcium ionophore had no effect. Second, staurosporine, a specific inhibitor of PKC, reduced the stimulatory action of A-II on proto-oncogene mRNA by 80–90%, whereas trifluoroperazine, an inhibitor of calmodulin, had no significant effect. These results demonstrate that the effects of A-II on proto-oncogene mRNA are mediated by AT1 receptor subtypes, mainly through activation of the PKC pathway.

Proto-oncogene: *c-fos*; *c-jun*; *jun-B*; Adrenal; Angiotensin-II

## 1. INTRODUCTION

Angiotensin-II (A-II) is a pleiotropic hormone which regulates a variety of responses including adrenal and renal function, blood pressure, pituitary function and fluid homeostasis [1]. In addition, A-II has multiple actions in the central nervous system [2]. A-II receptors are widely distributed throughout many tissues, but pharmacological and functional studies have suggested the existence of A-II receptor subtypes [1–3]. Recently binding studies with new A-II antagonists have revealed the existence of two distinct receptor subtypes, termed AT-1 and AT-2 [4,5], the first of which has recently been cloned [6,7].

Bovine adrenal fasciculata cells (BAC) contain both subtypes of A-II receptors [8], but all the acute effects of A-II on these cells are mediated by AT-1 receptors, which, as in other cell systems, are coupled to phosphoinositide breakdown. Recently, it has been shown that A-II increases the levels of three nuclear proto-oncogene mRNA levels in BAC [9]. However, the A-II receptor subtype involved in this effect of the hormone on early gene expression is unknown. Therefore in the present study we have investigated which subtype of A-II

receptors is involved in these effects of A-II on early gene expression.

## 2. MATERIALS AND METHODS

Synthetic angiotensin-II (A-II) was obtained from Bachem (Bubendorf, Switzerland); Staurosporine from Boehringer (Mannheim, Germany); insulin, transferrin, 4 $\beta$ -phorbol 12-myristate-13-acetate (PMA), ionophore A23187, trifluoroperazine dihydrochloride (TFP) from Sigma Chemicals Co. (St. Louis, MO); Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) in powder form, nystatin, penicillin/streptomycin, trypsin/EDTA, fetal calf serum from Gibco (Paris, France). Mouse *c-jun* and *jun-B* cDNAs were kindly provided by Dr. I. Verma (Salk Institute, San Diego, CA) [5] and Dr. D. Nathans (Johns Hopkins, Baltimore, MD) [6], respectively. Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA was a gift from Dr. J.M. Blanchard (Montpellier, France) [7]. *c-fos* was purchased from Oncor (Gaithersburg, MD). The non-peptide antagonist DUP753, was provided by Dr. R.D. Smith (Dupont Merck Pharmaceutical Co., Wilmington, DE) and the peptide antagonist CGP 42112A was a gift from Dr. M. De Gasparo (Ciba-Geigy, Basel, Switzerland).

### 2.1. Cell isolation and culture

BAC were prepared by sequential treatment of adrenal cortical slices with trypsin (0.19%) as previously described [8,9]. The cells were cultured in a chemically defined medium, Ham's F12/DMEM (1:1), containing transferrin (10  $\mu$ g/ml), insulin (5  $\mu$ g/ml), vitamin C ( $10^{-4}$  M) and antibiotics. The experiments were carried out on cells cultured for three days. The A-II antagonists and the inhibitors of protein kinase C and calmodulin were added 15 min before addition of the stimulatory agents, and continued for 1 h.

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### 2.2. Cortisol determination and measurement of the accumulation of [<sup>3</sup>H]inositol phosphates

Cortisol and [<sup>3</sup>H]inositol phosphate accumulation were carried out as previously described [8,9].

### 2.3. RNA isolation and Northern or slot blot analysis

For each treatment, the culture medium was removed from 25 cm<sup>2</sup> flasks, cells were harvested in guanidine thiocyanate, and total RNA was extracted according to the method of Chomczynski and Sacchi [10]. For Northern blot analysis, total cytoplasmic RNA (10 μg) was subjected to electrophoresis through 1% agarose gels and transferred under vacuum to Hybond-N nylon membrane (Amersham, UK). For each sample analyzed by slot blot, increasing amounts of total cytoplasmic RNA (1, 2, 4, and 6 μg) were transferred directly to Hybond-N nylon membrane using a multiwell filtration manifold (BRL). Membranes with bound RNA were irradiated for 2 min by ultraviolet light and were baked at 80°C to cross-link the RNA to the filters. Hybridization with complementary DNA <sup>32</sup>P-labelled probes and the analysis of blots were carried out as previously described [9].

Analysis of each Northern blot was realized in duplicate. The densitometric values for the proto-oncogene mRNA levels were normalized to those for GAPDH to control for quantity of RNA transferred to the blot.

### 2.4. Statistics

Data are reported as the mean ± S.E.M. for a minimum of three different cell preparations. Statistical significance was determined with Student's *t*-test. The null hypothesis was rejected when *P* < 0.05 was obtained.

## 3. RESULTS AND DISCUSSION

In the first series of experiments, the effects of A-II, either alone or together with its antagonists, on inositol phosphate accumulation, cortisol production and proto-oncogene mRNA were studied (Table I). Neither the AT-1 antagonist, DUP 753, nor the AT-2 antagonist, CGP 42112A, had any agonistic action, whereas A-II (Sar<sup>1</sup>,Thr<sup>8</sup>), an antagonist of A-II, had a small stimulatory effect on cortisol production. As expected, this peptide almost completely blocked the stimulatory effects of A-II on BAC. DUP 753 at 10<sup>-5</sup> M, a concen-

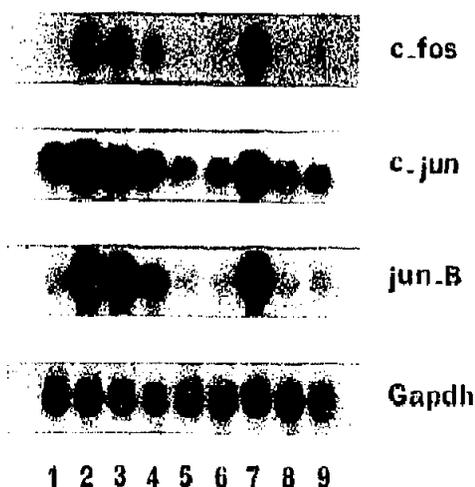


Fig. 1. Effects of A-II and/or its antagonists on proto-oncogene mRNA levels. Representative Northern blots. 1, Control; 2, A-II (10<sup>-8</sup> M); 3, A-II + DUP 753 (10<sup>-7</sup> M); 4, A-II + DUP 753 (10<sup>-6</sup> M); 5, A-II + DUP 753 (10<sup>-5</sup> M); 6, A-II + DUP 753 (10<sup>-4</sup> M); 7, A-II + CGP (5 × 10<sup>-8</sup> M); 8, A-II (Sar<sup>1</sup>,Thr<sup>8</sup>) (10<sup>-7</sup> M); 9, A-II + A-II (Sar<sup>1</sup>,Thr<sup>8</sup>) (10<sup>-7</sup> M).

tration which occupies most of the AT-1 receptors, blocked the effects of A-II on inositol phosphate accumulation, cortisol production and *jun-B*, *c-fos* and *c-jun* mRNA levels by 80–90%. In contrast, CGP 42112A at 5 × 10<sup>-8</sup> M, a concentration at which this compound occupied most of the AT-2 receptors [8], had no effect on the stimulatory actions of A-II. These results, therefore, suggest that the effects of A-II on proto-oncogene mRNA were mediated by the AT-1 receptor subtype (Table I and Fig. 1).

The inhibitory effects of DUP 753 on A-II-induced proto-oncogene mRNA levels were dose-dependent, with ID<sub>50</sub>s of 3.3 ± 0.8 × 10<sup>-7</sup> M, 7.3 ± 1.8 × 10<sup>-7</sup> M and 2 ± 0.4 × 10<sup>-6</sup> M (*n* = 4) for *c-fos*, *jun-B* and *c-jun*, respectively (Fig. 1). Due to the large variation in val-

Table I  
Effects of A-II and/or its antagonists on BAC

	Fold stimulation over control without A-II							
	- A-II			+ A-II 10 <sup>-8</sup> M				
	Control	A-II (Sar <sup>1</sup> ,Thr <sup>8</sup> ) (10 <sup>-5</sup> M)	DUP 753 (10 <sup>-5</sup> M)	CGP 42112A (5 × 10 <sup>-8</sup> M)	Control	A-II (Sar <sup>1</sup> ,Thr <sup>8</sup> ) (10 <sup>-5</sup> M)	DUP 753 (10 <sup>-5</sup> M)	CGP 42112A (5 × 10 <sup>-8</sup> M)
InsP	1	1.1 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	7 ± 0.4 <sup>a</sup>	1.2 ± 0.2 <sup>b</sup>	1.3 ± 0.1 <sup>b</sup>	6.4 ± 0.3 <sup>a</sup>
Cortisol	1	2.4 ± 0.3 <sup>a</sup>	1.2 ± 0.4	0.7 ± 0.1	117 ± 11 <sup>a</sup>	2.5 ± 0.4 <sup>b</sup>	12 ± 1 <sup>a,b</sup>	118 ± 13 <sup>a</sup>
<i>jun-B</i>	1	1.1 ± 0.3	1.3 ± 0.2	0.8 ± 0.05	24 ± 2 <sup>a</sup>	1.3 ± 0.4 <sup>b</sup>	5 ± 2 <sup>b</sup>	31 ± 7 <sup>a</sup>
<i>c-jun</i>	1	1.2 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	4.1 ± 0.5 <sup>a</sup>	1.4 ± 0.3 <sup>b</sup>	0.9 ± 0.2 <sup>b</sup>	5.4 ± 1.2 <sup>a</sup>
<i>c-fos</i>	1	1.5 ± 0.1	1.0 ± 0.3	1.2 ± 0.2	14 ± 2 <sup>a</sup>	2 ± 0.3 <sup>b</sup>	3.4 ± 1.3 <sup>b</sup>	13 ± 3 <sup>a</sup>

On the third day of culture, cells were treated with the indicated effector. The antagonists were added 15 min before A-II. Inositol phosphate (InsP) accumulation was measured 30 min after addition of A-II, whereas cortisol production and proto-oncogene mRNA accumulation were evaluated 30 min later. The results are given as mean ± S.E.M. of at least three different cell preparations. The proto-oncogene mRNAs were evaluated by Northern blot analysis.

<sup>a</sup> *P* < 0.05 vs. control without A-II of the same row.

<sup>b</sup> *P* < 0.01 vs. A-II alone of the same row.

ues, the  $ID_{50}$ s for *c-fos* and *jun-B* are not statistically different, but that of *c-jun* was higher. However, all these values are in range of the  $ID_{30}$  to inhibit [ $^{125}$ I]A-II binding ( $6 \pm 1.4 \times 10^{-7}$  M) and the  $IC_{50}$  to inhibit A-II-induced inositol phosphate accumulation ( $1.7 \pm 1 \times 10^{-6}$  M) [8].

Activation of the two branches of the phosphoinositide pathway, protein kinase C and calmodulin, are required for full expression of the steroidogenic effects of A-II on both adrenal glomerulosa cells [11] and BAC [12] (Table II). We have investigated whether these two branches are also required to induce the expression of proto-oncogenes in BAC. Two approaches were used. In the first, we compared the stimulatory effects of A-II on proto-oncogene mRNA levels with those induced by activators of protein kinase C (phorbol ester PMA) or  $Ca^{2+}$ /calmodulin (calcium ionophore A23187) or both (Table II). PMA alone was able to increase both cortisol secretion and proto-oncogene mRNA levels, but its effects were significantly lower than those induced by A-II. In contrast, A23187, 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 in many cell systems [13], including BAC [14], whereas trifluoroperazine (TFP) is an inhibitor of calmodulin in several cell types [15], including BAC [14]. As shown in Table III, staurosporine inhibited the stimulatory action of A-II on proto-oncogene mRNA levels by 80–90%. TFP produced a small, although not statistically significant, inhibition of the effects of A-II on proto-oncogene expression. A combination of both inhibitors had only small effects on *jun-B* mRNA when compared to the effect of staurosporine. The low inhibitory action of TFP might be due to its ability to slightly inhibit PKC at high concentrations [15]. Thus, both sets of experimental approaches indicate that the stimulatory action of A-II on proto-onco-

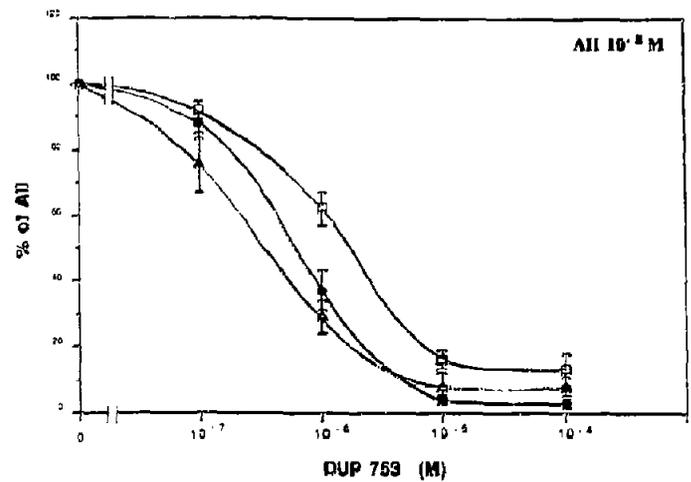


Fig. 2. Inhibitory effects of increasing concentrations of DUP 753 on the A-II-induced increase in *c-fos* ( $\Delta$ ), *c-jun* ( $\square$ ) and *jun-B* ( $\bullet$ ) mRNA levels. The results are the mean  $\pm$  S.E.M. of three different experiments.

gene mRNA levels is mainly mediated through activation of PKC.

Although the acute effects of A-II on both steroid production and proto-oncogene mRNA levels are mediated by the AT-1 receptor subtype, the effects are unrelated since inhibitors of protein synthesis block the stimulation of steroidogenesis by A-II but potentiate its effects on proto-oncogene mRNA [9]. In addition to these acute effects, A-II has a long-term inhibitory effect on the differentiated functions of BAC by inducing a down-regulation of its own receptors [16,17]. This is, in part, related to a decrease in the mRNA for the AT-1 receptor subtype (R. Ouali and J.M. Saez, unpublished results), and a decrease in the expression of genes encoding for steroidogenic enzymes [17–19]. How this long-term action of A-II is related to its effects on proto-oncogene expression is unknown. However, ACTH, which also increases *c-fos* and *jun-B* but not *c-jun* mRNA levels, has a long-term positive effect on the differentiated functions of BAC [17,20,21]. Since *c-jun*

Table II

Comparative effects of A-II, the phorbol ester, PMA, and the calcium ionophore, A23187, on cortisol production and proto-oncogene mRNA levels in BAC

	Fold stimulation over control			
	Cortisol	<i>jun-B</i>	<i>c-jun</i>	<i>c-fos</i>
A-II ( $10^{-7}$ M)	$84 \pm 12$	$17.4 \pm 1.1$	$5.0 \pm 0.8$	$16.9 \pm 1.7$
PMA ( $10^{-7}$ M)	$28 \pm 4^a$	$11.1 \pm 0.9^a$	$3.8 \pm 1.2$	$5.8 \pm 2.2^a$
A23187 ( $10^{-7}$ M)	$18 \pm 3^a$	$0.7 \pm 0.1^a$	$0.9 \pm 0.05^a$	$1.7 \pm 1.0^a$
PMA + ionophore	$51 \pm 4^b$	$10.2 \pm 2.0^a$	$2.9 \pm 0.6$	$8.5 \pm 1.1^a$

Cells, on the third day of culture, were incubated with the indicated effectors. After 1 h, the cortisol in the medium was measured and total RNA was extracted. The levels of proto-oncogene mRNA were determined by slot blot analysis as indicated in Materials and Methods. The results are given as the mean  $\pm$  S.E.M. of 3–10 experiments.

<sup>a</sup>  $P < 0.05$  compared to A-II alone.

Table III

Inhibition of A-II-induced proto-oncogene expression by protein kinase C and/or calmodulin inhibitor

	% of A-II alone		
	<i>jun-B</i>	<i>c-jun</i>	<i>c-fos</i>
A-II (10 <sup>-7</sup> M)	100	100	100
Control	5.7 ± 2.1	24.0 ± 6.5	10.0 ± 2.5
A-II + staurosporine (10 <sup>-6</sup> M)	23.0 ± 1.1 <sup>a</sup>	24.0 ± 3.0 <sup>a</sup>	15.0 ± 4.0 <sup>a</sup>
A-II + TFP (10 <sup>-5</sup> M)	86.0 ± 15	76.0 ± 8.3	80.0 ± 23.5
A-II + staurosporine + TFP	10.0 ± 1.5 <sup>b,c</sup>	19.5 ± 0.5 <sup>c</sup>	18.0 ± 2.0 <sup>c</sup>

On the third day of culture, BAC were incubated with the indicated effectors for 1 h. The proto-oncogene mRNAs were quantified by slot blot analysis as described in Materials and Methods. The results are given as the mean ± S.E.M. of 3–6 experiments.

<sup>a</sup> *P* < 0.01 compared to A-II alone.

<sup>b</sup> *P* < 0.05 compared to A-II + staurosporine.

<sup>c</sup> *P* < 0.05 compared to A-II + TFP.

and *jun-B* have different activation domains and differ in their biological properties in several cell types [22,23], it has been postulated that the homodimer, *c-jun*, and/or the heterodimer, *c-jun/c-fos*, may be responsible for the long-term negative effects of A-II on differentiated function of BAC. Further studies, in particular those using transfection experiments with normal or mutated proto-oncogenes, are required to determine the role of early proto-oncogene expression on the long-term pleiotropic effects of A-II on cell differentiation.

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