Volume 313, number 1, 43–46 FEBS 11756 November 1992

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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Received 22 September 1992

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 4×10^{-7} M, 7×10^{-7} M and 2×10^{-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.

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2. MATERIALS AND METHODS

Synthetic angiotensin-II (A-II) was obtained from Bachem (Bubendorf, Switzerland); Staurosporine from Boehringer (Mannheim, Germany); insulin, transferrin, 4β-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 μg/ml), insulin (5 μg/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.

2.2. *Cortisol determination and measurement of the accumulation of [^{3}H]inositol phosphates*

Cortisol and [^{3}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^2 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 ^{32}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^1,Thr^8), 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^{-5} M, a concentration 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^{-8} 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).

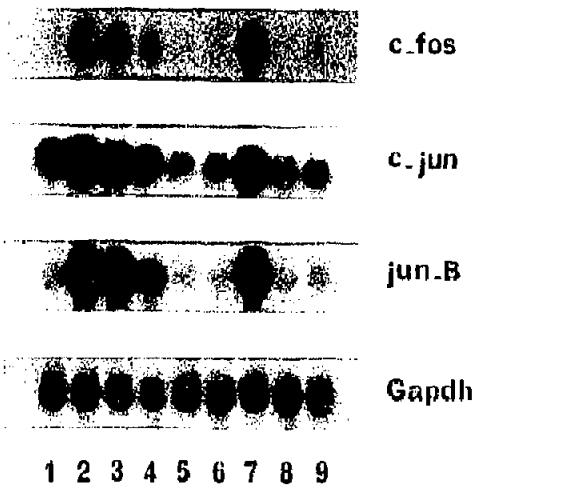

Fig. 1. Effects of A-II and/or its antagonists on proto-oncogene mRNA levels. Representative Northern blots. 1, Control; 2, A-II (10^{-8} M); 3, A-II + DUP 753 (10^{-7} M); 4, A-II + DUP 753 (10^{-6} M); 5, A-II + DUP 753 (10^{-5} M); 6, A-II + DUP 753 (10^{-4} M); 7, A-II + CGP (5×10^{-8} M); 8, A-II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

The inhibitory effects of DUP 753 on A-II-induced proto-oncogene mRNA levels were dose-dependent, with ID_{50}s of $3.3 \pm 0.8 \times 10^{-7}$ M, $7.3 \pm 1.8 \times 10^{-7}$ M and $2 \pm 0.4 \times 10^{-6}$ 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^{-8} M			
	Control	A-II (Sar^1,Thr^8) (10^{-8} M)	DUP 753 (10^{-5} M)	CGP 42112A (5×10^{-8} M)	Control	A-II (Sar^1,Thr^8) (10^{-8} M)	DUP 753 (10^{-5} M)	CGP 42112A (5×10^{-8} M)
InsP	1	1.1 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	7 ± 0.4[a]	1.2 ± 0.2[b]	1.3 ± 0.1[b]	6.4 ± 0.3[a]
Cortisol	1	2.4 ± 0.3[a]	1.2 ± 0.4	0.7 ± 0.1	117 ± 11[a]	2.5 ± 0.4[b]	12 ± 1[a,b]	118 ± 13[a]
jun-B	1	1.1 ± 0.3	1.3 ± 0.2	0.8 ± 0.05	24 ± 2[a]	1.3 ± 0.4[b]	5 ± 2[b]	31 ± 7[a]
c-*jun*	1	1.2 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	4.1 ± 0.5[a]	1.4 ± 0.3[b]	0.9 ± 0.2[b]	5.4 ± 1.2[a]
c-*fos*	1	1.5 ± 0.1	1.0 ± 0.3	1.2 ± 0.2	14 ± 2[a]	2 ± 0.3[b]	3.4 ± 1.3[b]	13 ± 3[a]

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.

[a] $P < 0.05$ vs. control without A-II of the same row.

[b] $P < 0.01$ 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_{50} 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-oncogene mRNA levels is mainly mediated through activation of PKC.

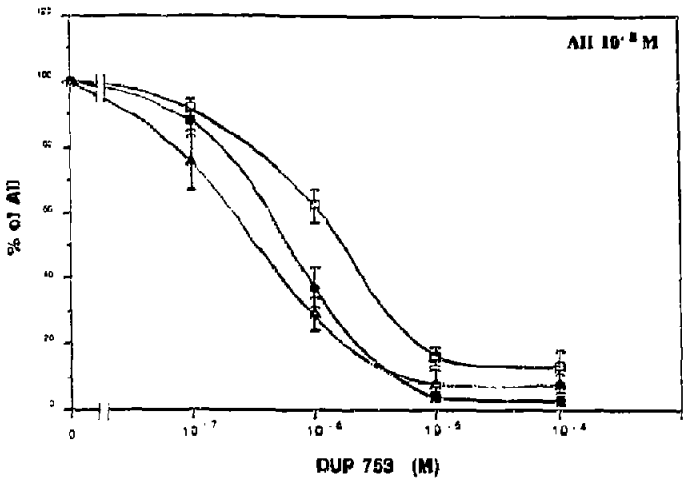

Fig. 2. Inhibitory effects of increasing concentrations of DUP 753 on the A-II-induced increase in c-*fos* (△), c-*jun* (□) and *jun*-B (●) mRNA levels. The results are the mean ± S.E.M. of three different experiments.

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	*jun*-B	c-*jun*	c-*fos*
A-II (10^{-7} M)	84 ± 12	17.4 ± 1.1	5.0 ± 0.8	16.9 ± 1.7
PMA (10^{-7} M)	28 ± 4[a]	11.1 ± 9.9[a]	3.8 ± 1.2	5.8 ± 2.2[a]
A23187 (10^{-7} M)	18 ± 3[a]	0.7 ± 0.1[a]	0.9 ± 0.05[a]	1.7 ± 1.0[a]
PMA + ionophore	51 ± 4[b]	10.2 ± 2.0[a]	2.9 ± 0.6	8.5 ± 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 ± S.E.M. of 3–10 experiments.

[a] $P < 0.05$ 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		
	jun-B	c-*jun*	c-*fos*
A-II (10^{-7} M)	100	100	100
Control	5.7 ± 2.1	24.0 ± 6.5	10.0 ± 2.5
A-II + staurosporine (10^{-6} M)	23.0 ± 1.1[a]	24.0 ± 3.0[a]	15.0 ± 4.0[a]
A-II + TFP (10^{-5} M)	86.0 ± 15	76.0 ± 8.3	80.0 ± 23.5
A-II + staurosporine + TFP	10.0 ± 1.5[b,c]	19.5 ± 0.5[c]	18.0 ± 2.0[c]

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.

[a] $P < 0.01$ compared to A-II alone.

[b] $P < 0.05$ compared to A-II + staurosporine.

[c] $P < 0.05$ 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.

Acknowledgements: This study was supported by an INSERM–Merck, Sharp & Dohme grant and by the Ligue Nationale contre le Cancer. The authors thank Drs. R.D. Smith and C. Sweet for providing DUP 753, and Dr. M. De Gasparo for providing CGP 42112A. We also thank Dr. A.M. Clark for critical reading of the manuscript and Ms. J. Bois for her expert secretarial assistance. I.V. is a recipient of a fellowship from Association de Recherche contre le Cancer (ARC). R.O. is a recipient of a fellowship from INSERM–Merck, Sharp & Dohme.

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