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REGULAR ARTICLE

Immunohistochemical Jun/Fos protein localization and DNA synthesis in rat adrenal cortex after treatment with ACTH or FGF2

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Abstract In vitro and in vivo studies have suggested that the expression of the early response genes for Jun and Fos proteins plays an important role in adrenal cell proliferation. In order to study the expression pattern of the activating protein-1 (AP-1) family of oncogenes in the adrenal gland, we have used immunohistochemistry to localize Jun and Fos protein expression in rat adrenal cortex infused in situ with adrenocorticotropic hormone (ACTH), fibroblast growth factor 2 (FGF2), or both. The expression of AP-1 factors has been found to be correlated with in vivo ACTH and FGF2 proliferation in rats treated with dexamethasone and bromodeoxyuridine (BrdU). Induction of c-Jun and c-Fos in the zona fasciculata and of FosB in the zona reticularis suggests that, after ACTH stimulation, these proteins are the main AP-1 components in these zones. In vivo, ACTH increases BrdU-positive cells in the zona fasciculata and zona reticularis suggesting that the composition of AP-1 complexes in these zones is correlated with proliferation. Patterns of Fos and Jun induction by FGF2 do not resemble those after ACTH induction. However, in isolation, neither affects the zona glomerulosa. In the zona fasciculata, and more so in the zona reticularis, FGF2 modulates responses to ACTH, reducing the numbers of Jun-positive cells, Fos-positive cells, and DNA synthesis. This indicates that FGF2 antagonizes ACTH, and that ACTH thus controls the trophic effect independently of exogenous FGF2. Our results implicate the AP-1 family of transcription factors in the regulation of cell progression and the control of ACTH-induced proliferation in the zona fasciculata and zona reticularis.

Keywords Adrenal cortex · Infusion · Corticotropin · Fibroblast growth factor 2 · Proto-oncogene proteins · Fos and Jun · DNA synthesis · Rat (Sprague Dawley, male)

Introduction

Adrenocorticotropic hormone (ACTH) is considered mitogenic because of the correlation between the levels of circulating ACTH and the size of the adrenal cortex (New 1998). In contrast, observations made in vitro indicate that ACTH is a weak mitogenic hormone in the Y-1 mouse adrenocortical tumor cell line (Lotfi et al. 1997; Mattos and Lotfi 2005) and an antimitogenic hormone in cell lines and primary cultures of mouse, rat, and human adrenocortical cells. These observations suggest that, in vivo, ACTH is an indirect mitogenic hormone (Hornsby 1985), possibly mediated by paracrine factors. However, little is known of the key regulatory genes involved in this process, and the way that they are regulated in the diverse cells that comprise the adrenal cortex to bring about adrenal cortex proliferation is poorly understood.

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Early events in the course of the ACTH effect have been correlated with changes, in vivo and in vitro, to the expression of early response genes (Imai et al. 1990; Clark et al. 1992; Kimura et al. 1993; LeHoux and Ducharme 1995; Lotfi et al. 1997; Lotfi and Armelin 2001). ACTH and fibroblast growth factor 2 (FGF2) have both been shown rapidly to induce the c-fos proto-oncogene in the Y-1 line (Kimura et al. 1993; Lotfi et al. 1997; Lotfi and Armelin 2001), in primary cultures (Viard et al. 1992; Mattos and Lotfi 2005), and in vivo (Yang et al. 1990; Imai et al. 1990), suggesting that these factors regulate the activity of the activating protein-1 (AP-1) transcription factor, which is composed of homodimers and heterodimers of the Jun and Fos family of proteins (Angel and Karin 1991). The AP-1 complex can, therefore, consist of many different combinations of heterodimers and homodimers, and this combination determines which genes are regulated by AP-1 (Eferl and Wagner 2003). Recent evidence indicates that the degree to which AP-1 determines cell fates depends on the relative abundance of AP-1 subunits, the composition of AP-1 dimers, the quality of the stimulus, the cell type, and the cellular environment (Hess et al. 2004).

The studies mentioned above have been reviewed by Zwermann et al. (2004), who have found that, following the wide variety of in vivo and in vitro techniques that have been used, the results regarding the growth effects of ACTH appear contradictory. Several signaling molecules promote a final biological response that depends on the cell type, the responding state, and the relationship of the cell with other tissue cells and signals in the environment. For these reasons, we have investigated the effect on adrenal cortex growth under conditions that maintain both the gland and the vasculature intact. We have also isolated the gland from the systemic circulation and stimulated the gland only under controlled conditions.

In this study, we have used a modified version of the in situ infusion system first developed by Sibley et al. (1981) and later modified by Hinson et al. (1985). In this system, the adrenal gland receives perfusion medium only, and its circulation is isolated from the rest of the body, which eliminates the influence of the kidneys and pituitary gland. The vasculature remains intact, and the structures of the different cell types and the relationships among them remain undisturbed. To determine whether ACTH and FGF2 regulate fos and jun gene activity, we have analyzed their effects on the expression and nuclear immunolocalization of c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 proteins by using an isolated infused gland. In addition, AP-1 composition has been correlated with the control of ACTH- or FGF2-induced proliferation and the regulation of cell progression, by measuring DNA synthesis in vivo with 12-h 5-bromo-2′-deoxyuridine (BrdU) incorporation in animals treated for 2 days with dexamethasone (Dex).

Materials and methods

In situ rat adrenal infusion

We used male Sprague-Dawley rats, weighing 200–250 g, bred at the Biomedical Sciences Institute of the University of São Paulo. The rats were maintained under a controlled 12-h light/dark cycle, with free access to normal rat chow and tap water. The Animal Experimentation Ethics Committee of the Biomedical Sciences Institute approved all protocols. Animals were anesthetized by using intraperitoneal injections of ketamine (5 mg/100 g body weight [BW]), acepromazine (0.2 mg/100 g BW), and xylazine (1 mg/100 g BW). The left adrenal gland was prepared for infusion by the method described in Sibley et al. (1981) with modifications. In brief, Dulbecco's modified Eagle's medium (DMEM) at 37°C was introduced, via a polypropylene cannula, into an isolated segment of the aorta near the celiac trunk. This cannula was linked to an infusion pump, which delivered the solution at a constant rate of 0.2 ml/min (Bouloux et al. 1986). After passing through the adrenal gland, the medium was drained off through an incision made in the renal vein. The circulation of the left adrenal gland was isolated from the rest of the circulation by clamping, in strict order, the following vessels: the dorsal aorta below the diaphragm; the renal artery and vein near the renal hilum; the aorta; and the inferior cava vein below the renal vein. The gland was thus totally isolated from the systemic circulation. After the vessels had been clamped, the animals were immediately killed by pneumothorax. A heating coil was employed to maintain the animal bed temperature at 37°C. In all experiments, prior to the introduction of any test substance, the glands were sequentially infused for 30 min with 10 IU/ml heparin sodium (Hoffmann-LaRoche, Basel, Switzerland) in DMEM followed by DMEM alone. As reported in Lotfi et al. (2000) and Lotfi and Armelin (2001), the concentration-response curves of c-Fos to in vitro ACTH and FGF2 are linearly correlated, maximal stimulation of c-Fos being achieved with 10^{-7} M ACTH and 10–20 ng/ml FGF2. Based on these observations, four or five animals were used in groups undergoing various treatments: 10^{-7} M $ACTH_{39}$ (Sigma, St. Louis, Mo., USA), 20 ng/ml bovine recombinant FGF2, ACTH (10^{-7} M) plus FGF2 (20 ng/ml), or DMEM alone for 2 h. All experiments were initiated between 8:00 a.m. and 10:00 a.m.

In vivo treatments and BrdU incorporation

The Animal Experimentation Ethics Committee of the Biomedical Sciences Institute approved all protocols. Male Sprague-Dawley rats, weighing approximately 200 g, were divided into five groups (n=4–5) for treatment with Dex

(decadron-dexamethasone disodium phosphate; Aché Laboratórios Farmacêuticos, Campinas, SP, Brazil): Dex+, Dex+ACTH, Dex+FGF2, Dex+ACTH+FGF2, and control (Dex−). Dex was administered intraperitoneally at a dose of 50 mg/100 g BW once a day at 9:00 a.m. for 2 days (Dex+). Control rats received identical injections of saline only (Dex−). Study group rats subsequently received single intraperitoneal injections of 10^{-7} M $ACTH_{39}$ (Dex+ACTH), 20 ng/ml bovine recombinant FGF2 (Dex+FGF2), ACTH (10^{-7} M) plus 20 ng/ml FGF2 (Dex+ACTH+FGF2), or saline (Dex+). At 12 h after the initial injections, all animals received single injections of 100 mg/100 g BW BrdU (Sigma), based on the protocols of in vitro BrdU incorporation described in Lotfi et al. (1997) and Mattos and Lotfi (2005). At 24 h after the initial injections, the rats were killed by decapitation. This protocol permitted the reagents to promote the transition of cells from the G1 to the S phase of the cell cycle and allowed a 12-h BrdU incorporation, resulting in the accumulation of S-phase cells. Adrenal glands were removed, fixed for 8 h in 10% formaldehyde, and embedded in paraffin. Circulating plasma ACTH was measured by using an immunochemiluminescent assay (Immulite; Diagnostic Products, Los Angeles, Calif., USA) of trunk blood samples collected at the time of sacrifice.

Histological analysis: light microscopy

Left adrenal glands were removed, fixed in 4% paraformaldehyde in a solution of 0.1 M phosphate buffer (PBS; pH 7.4) for 8 h at room temperature, and then immersed in PBS plus 6% sucrose for 12 h. Fixed adrenal tissue was dehydrated and then embedded in paraffin. Transverse sections (3–5 μm) were cut and mounted on gelatin-coated glass slides. After deparaffinization (24 h at 60°C), sections were rehydrated in graded ethanol and sterile Milli-Q water and stained with hematoxylin and eosin (H&E) in order to analyze the integrity of the infused adrenal gland. Prior to the immunohistochemical assay, the H&E-stained sections were examined by light microscopy in order to evaluate (1) the integrity of the capsule and (2) the cell zones and the vascular architecture of the capillaries and of the central adrenal vein.

Immunohistochemistry for Fos and Jun proteins

After being fixed and deparaffinized, the sections were rehydrated in graded ethanol, sterile Milli-Q water, and PBS, followed by PBS with 0.5% hydrogen peroxide. Sections were incubated, overnight at 4°C in a humidified container, with anti-Fra-1, anti-Fra-2, anti-FosB, anti-c-Jun, anti-JunB, and anti-JunD antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) diluted 1:100 in PBS, or with an antibody against c-Fos (Oncogene Science, Cambridge, Mass., USA) diluted 1:800 in 0.1 M PBS plus 1 mg/ml bovine serum albumin, and then rinsed with PBS. The immune complexes were subsequently detected by immunoperoxidase staining with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif., USA) and diaminobenzidine (Sigma Fast; Sigma Aldrich, St. Louis, Mo., USA). Sections were washed in Milli-Q water, stained with Harris's hematoxylin, and differentiated with a saturated solution of lithium carbonate. After dehydration, sections were mounted with Entellan (Merck, Darmstadt, Germany). Adrenal sections incubated in nonimmune primary sera yielded negative results (not shown).

Immunostaining for BrdU

After being fixed and deparaffinized, the sections were rehydrated in graded ethanol, sterile Milli-Q water, and PBS, followed by methanol plus 1% hydrogen peroxide, and then incubated with a monoclonal anti-mouse-BrdU antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Immune complexes were detected as described above. Adrenal sections incubated in pre-absorbed primary antibody with 100 mg/ml BrdU or nonimmune primary sera were negative (not shown). Jejunal sections were used as positive controls.

Immunoreactive cell counts

The effect of the various treatments on the expression of Fos and Jun proteins in the adrenal cortex was quantified by counting the number of immunostained cortical cells per unit area of adrenal cortex. Four or five infused glands were used in each treatment group. For each infused adrenal gland, three sections containing the medulla were randomly selected. In each section, five to ten test fields of 0.75 mm^2 each were sampled randomly in the three zones of the adrenal cortex and examined with a 40× objective attached to a Nikon microscope equipped with a video camera. Images were digitalized by using Image-Pro Plus, and all immunoreactive nuclear profiles, regardless of the intensity of the immunostaining, were counted. For the quantification of BrdU incorporation, ten to fifteen areas (with 90–100 nuclei/area) in each section were sampled randomly in the three zones of the adrenal cortex by using the same equipment and image program as those described above. For each zone of each adrenal gland, the BrdU index was defined as the number of BrdU-positive nuclei, expressed as a percentage of the total number of nuclei.

Statistical analysis

Data obtained from each zone were averaged for each experimental or control infused gland or rat, and standard

deviations were calculated. After an analysis of variance (ANOVA), statistical comparisons were made by using the Tukey-Kramer Multiple Comparisons Test. Values of $P \leq 0.05$ were considered statistically significant.

Results

Immunoreactivity to early response genes in rat adrenal glands under basal conditions

When no stimulation was applied (under basal conditions), the zonal distribution of immunoreactivity to Fos and Jun (Table 1) was easily detected in the cortex cell nuclei of saline-infused adrenal glands. In addition, the numbers of c-Jun-positive nuclei were significantly higher in the zona glomerulosa (ZG; 70.5±4.2%) than in the zona reticularis (ZR; 62.6±2.4%, ZG vs. ZR $P<0.05$) and zona fasciculata (ZF; 62.3±2.2%, ZG vs. ZF $P<0.05$). No JunB-positive nuclei were detected in the ZF (Fig. 1c), although 52.2±9.3% were detected in the ZG, and 36.9±3.1% were detected in the ZR. In contrast, the numbers of JunD-positive nuclei were comparable in all three zones (ZG: 34.0±2.4%; ZF: 43.3±7.8%; ZR: 45.6±3.2%).

Fos proteins exhibited more variability. c-Fos-positive nuclei were detected in 28.7±0.3% of cells in the ZG and in 67.3±3.0% of cells in the ZR but were undetectable in the ZF (Fig. 2a). In contrast, FosB-positive nuclei were detected in similar numbers in the ZG and ZF (70.6±1.1% and 65.9±3.2%, respectively), compared with 34.7±2.3% in the ZR (a significant difference; $P<0.001$). Under basal conditions, the numbers of Fra-1-positive nuclei varied significantly among zones. No Fra-1-positive nuclei were detected in the ZR, compared with 23.1±1.5% in the ZG (a statistically significant difference; $P<0.001$). The difference between the number of Fra-1-positive nuclei in the ZG and that observed in the ZF (71.1±0.7%) was also significant ($P<0.001$). In summary, the highest numbers of c-Jun-positive nuclei were detected under basal conditions, regardless of the zone analyzed, whereas the numbers of FosB/Fra-2-positive, FosB/Fra-1-positive, and c-Fos/Fra-2-positive nuclei were highest in the ZG, ZF, and ZR, respectively. Moreover, under basal conditions, no immunoreactivity for JunB (Fig. 1c) and c-Fos (Fig. 2a) was seen in the ZF, whereas no Fra-1 immunoreactivity was present in the ZR.

Induction of c-Jun/c-Fos proteins in zona fasciculata of rat adrenal cortex after infusion of ACTH

Following ACTH infusion, a moderate increase over the baseline number of c-Jun-positive cells (Fig. 3a) was seen in the ZF (10.9±1.8%; $P<0.001$; Fig. 1b). However, in the same zone, JunB immunoreactivity was unaltered from to the baseline value (Fig. 3b), viz., no JunB-positive nuclei were detected, whereas that of JunD (Fig. 3c) was completely inhibited (Fig. 1f). The behavior of JunB and JunD indicated that c-Jun protein induction was most significantly altered in the ZF. In the ZG, ACTH infusion had no effect on the numbers of Jun-positive cells, whereas in the ZR, it resulted in lower numbers of JunB- and JunD-positive nuclei, which were decreased by 36.9±3.1% (Fig. 3b) and 17.9±0.6% (Fig. 3c), respectively, in comparison to baseline values. In response to ACTH infusion, the expression of Jun proteins remained the same in the ZG, whereas JunB and JunD were significantly inhibited in the ZR and ZF. No c-Fos-positive nuclei were found in the ZF under baseline conditions (Fig. 2a), but ACTH infusion provoked c-Fos expression (Fig. 2b), and the number of c-Fos-positive cells observed in this zone (72.9±1.4%; Fig. 4a) was significantly higher than the baseline value ($P<0.001$). When the same treatment was used, the number of c-Fos-positive nuclei in the ZG increased by 50.2±1.4% over the baseline value ($P<0.001$). In contrast, ACTH infusion had no effect on the number of c-Fos-positive cells in the ZR in comparison with baseline

Table 1 Zonal immunoreactivity of Fos and Jun proteins under basal conditions (*ZG* zona glomerulosa, *ZF* zona fasciculata, *ZR* zona reticularis, *NS* not significant as shown by ANOVA and the Tukey-Kramer multiple comparisons test). Data are expressed as the mean±SD

Protein	Immunoreactive nuclei in % (fold over the lower numbers of nuclei immunoreactive to proteins of the Fos or Jun protein families)			*P*-value		
	ZG	ZF	ZR	ZG vs. ZF	ZG vs. ZR	ZF vs. ZR
c-Jun	70.5±4.2 (2.1)	62.3±2.2 (1.4)	62.6±2.4 (1.7)	0.05	0.05	NS
JunB	52.2±9.3 (1.5)	0	36.9±3.1 (1)	0.001	0.05	0.001
JunD	34.0±2.4 (1)	43.3±7.8 (1)	45.6±3.2 (1.2)	NS	NS	NS
c-Fos	28.7±0.3 (1.2)	0	67.3±3.0 (1.9)	0.001	0.001	0.001
FosB	70.6±1.1 (3.1)	65.9±3.2 (1.1)	34.7±2.3 (1)	NS	0.001	0.001
Fra-1	23.1±1.5 (1)	71.1±0.7 (1.2)	0	0.001	0.001	0.001
Fra-2	62.8±1.0 (2.7)	61.8±0.8 (1)	71.5±1.2 (2.1)	NS	0.001	0.001

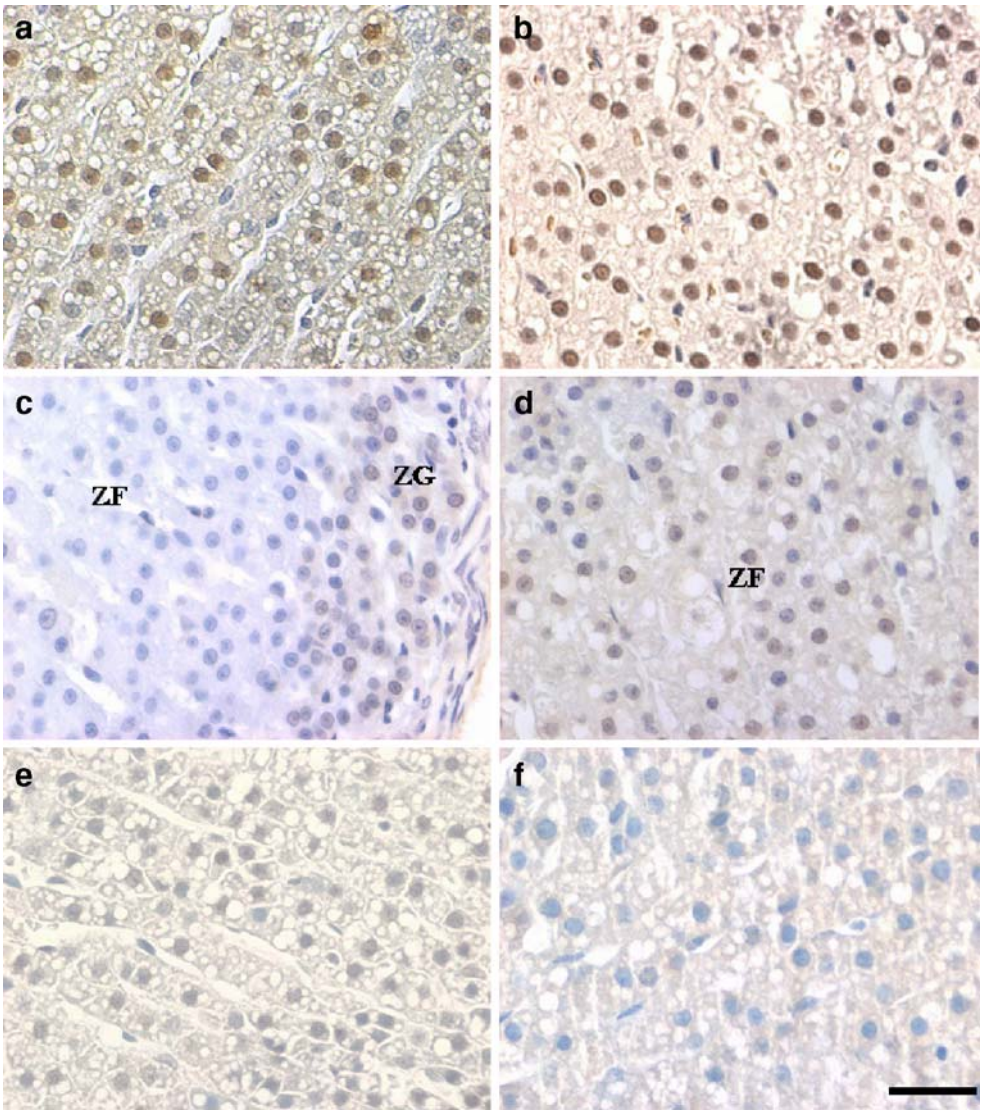

Fig. 1 Photomicrographs of immunoreactivity to Jun proteins in adrenal glands infused with ACTH (10^{-7} M), FGF2 (20 ng/ml), or DMEM (controls). **a**, **b** c-Jun immunohistostaining in the zona fasciculata (**a** control, **b** ACTH-infused). **c**, **d** JunB immunohistostaining in the zona glomerulosa (*ZG*) and zona fasciculata (*ZF*; **c** control, **d** FGF2-infused). **e**, **f** JunD immunohistostaining in the ZF (**e** control, **f** ACTH-infused). *Bar* 50 μm

values (Fig. 4a). Similarly, the number of Fra-1-positive cells was 50.0±1.6% higher than the baseline value in the ZG (Fig. 4c), compared with the ZR, in which the numbers of Fra-1- and FosB-positive cells (Fig. 4b) increased significantly (54.3±1.9% and 43.5±0.9%, respectively; $P<0.001$). In the ZF, the induction of Fra-2-positive cells was significantly lower ($P<0.05$) than that seen for c-Fos protein, whereas in the ZG and ZR, it decreased by 9.2±0.5% and 7.3±2.3%, respectively, in the ZG and ZR in comparison with baseline levels. Thus, ACTH infusion induced significant accumulation of c-Fos-positive cells in the ZF. In the ZG, the ACTH-induced accumulation of c-Fos-positive cells was equal to that of Fra-1-positive cells. Similarly, in the ZR, the induction of FosB-positive nuclei was comparable with that of Fra-1-positive nuclei. After a 2-h infusion with ACTH (10^{-7} M), the early response pattern of gene expression was more specific in the ZF, suggesting that the AP-1 complex was composed of c-Jun and c-Fos.

Induction of JunB, but not c-Fos, expression in zona fasciculata of rat adrenal cortex after FGF2 infusion

Similar to the results obtained with ACTH infusion, infusion of FGF2 into the adrenal cortex did not alter the numbers of ZG nuclei that were immunoreactive for Jun proteins. In addition, FGF2 infusion increased the number of c-Jun-positive nuclei in the ZF by an amount (10.9±2.2%) equal to that induced by ACTH infusion (Fig. 3a). In contrast, FGF2 infusion increased the number of c-Jun-positive cells in the ZR by 9.9±2.3% over the baseline value ($P<0.001$), and the number of JunB-positive cells was also higher (Fig. 3b), albeit to different degrees, in the ZF (Fig. 1d) and the ZR (56.3±9.5% and 26.3±4.9%, respectively; $P<0.001$). This increase in the number of JunB-positive cells in the ZF after FGF2 treatment was significant and contrasted with the lack of an effect of ACTH infusion on JunB in that zone. Both treatments inhibited JunD expression (Fig. 3c) in the ZF (Fig. 1f).

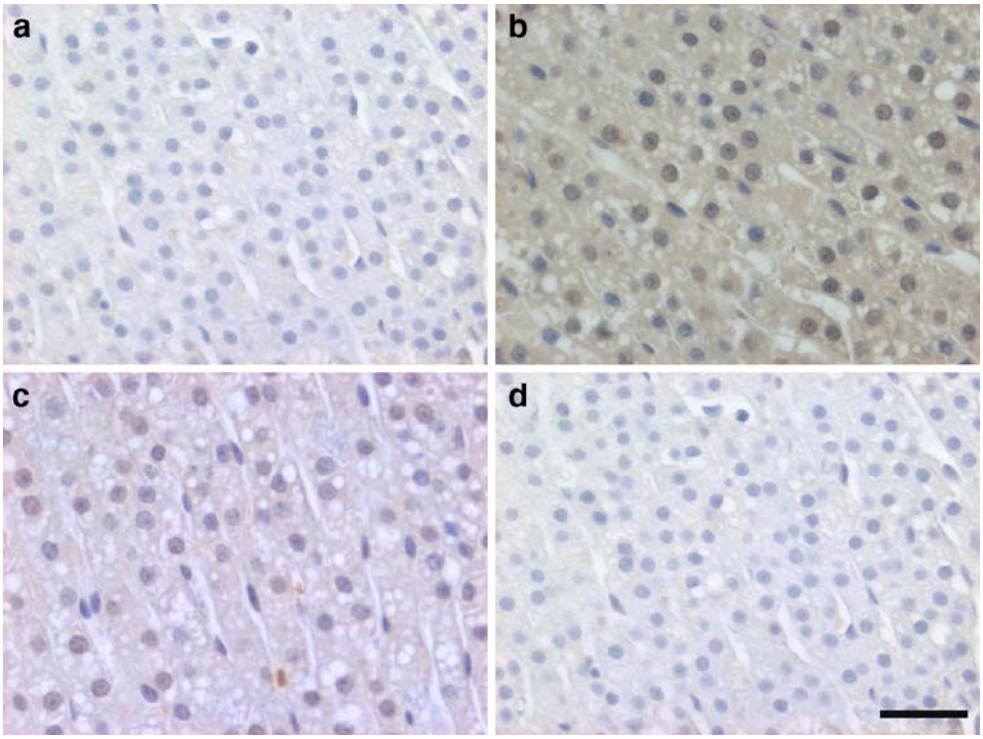

Fig. 2 Photomicrographs of Fos protein immunoreactivity in adrenal glands infused with ACTH (10^{-7} M), FGF2 (20 ng/ml), or DMEM (controls). **a**, **b** c-Fos immunohistostaining in the zona fasciculata. **a** Control. **b** ACTH-infused. **c**, **d** FosB immunohistostaining in the zona fasciculata. **c** Control. **d** ACTH-infused. *Bar* 50 μm

Differences in Fos protein expression among the adrenal cortex zones were more pronounced after FGF2 infusion than those seen after infusion of ACTH (Fig. 4). In the ZG, FGF2 infusion induced significant ($P<0.001$) increases in the numbers of c-Fos-positive cells (42.7±1.2%) and Fra-1-positive cells (36.9±1.5%), whereas it inhibited FosB protein expression in this zone. In the ZR, the same treatment increased the numbers of FosB- and Fra-1-positive cells (36.1±3.7% and 57.4±1.6%, respectively). In the ZF, FGF2 infusion induced a modest but significant ($P<0.01$) increase in the number of Fra-2-positive cells (9.6±4.7%). However, in the same zone, the number of Fra-1-positive cells was lower, and FosB protein inhibition was greater (Fig. 2d). In addition, FGF2 infusion inhibited the expression of FosB protein in the ZG and ZF, whereas it led to an increase in the number of FosB-positive cells in the ZR.

Antagonistic effect on Jun and Fos expression in rat adrenal cortex after infusion of ACTH-FGF2 combination

In comparison with baseline values, the combined infusion of ACTH and FGF2 in rat adrenal glands resulted in a significantly higher number of JunD-positive nuclei (Fig. 3c) only in the ZG (19.7±1.7%; $P<0.001$), a reduction in the number of c-Jun-positive nuclei, and no change in JunB immunoreactivity. Infusion of the ACTH-FGF2 combination induced increases in the numbers of c-Fos-positive cells in the ZG (35.6±1.2%) and in the ZF (63.3±3.6%). However, as can be seen in Fig. 4a, these levels of c-Fos expression, although higher than the baseline values, were still significantly lower than those seen when ACTH and FGF2 treatments were applied separately (ZG: $P<0.001$ for ACTH vs. ACTH+FGF2, $P<0.01$ for FGF2 vs. ACTH+FGF2; ZF: $P<0.001$ for ACTH vs. ACTH+FGF2, $P<0.001$ for FGF2 vs. ACTH+FGF2). Therefore, FGF2 antagonized c-Fos protein/ACTH activity in the ZG and ZF. In all zones, the numbers of nuclei that were immunoreactive to other members of the Fos family were either reduced or were unaltered (Fig. 4).

Induction of S-phase entry in zona fasciculata and zona reticularis of rat adrenal cortex after in vivo ACTH treatment

We found that, irrespective of whether tissue was treated with ACTH, FGF2, or the combination of the two, no significant increase occurred in BrdU incorporation within the ZG. However, after ACTH treatment, positive cells were located in the capsule (Fig. 5). In contrast, ACTH treatment produced significant ($P<0.01$) increases in the percentage of nuclei labeled for BrdU (Fig. 6a) in the ZF (two-fold increase) and ZR (2.5-fold increase). After treatment with FGF2, BrdU uptake was significantly ($P<0.05$) greater only in the ZR (2.2-fold increase). The combination of ACTH and FGF2 had an antagonistic effect, blocking S-phase entry in the ZF and ZR. Indeed, both zones responded to the ACTH-FGF2 combination by a decrease in cell cycle progression.

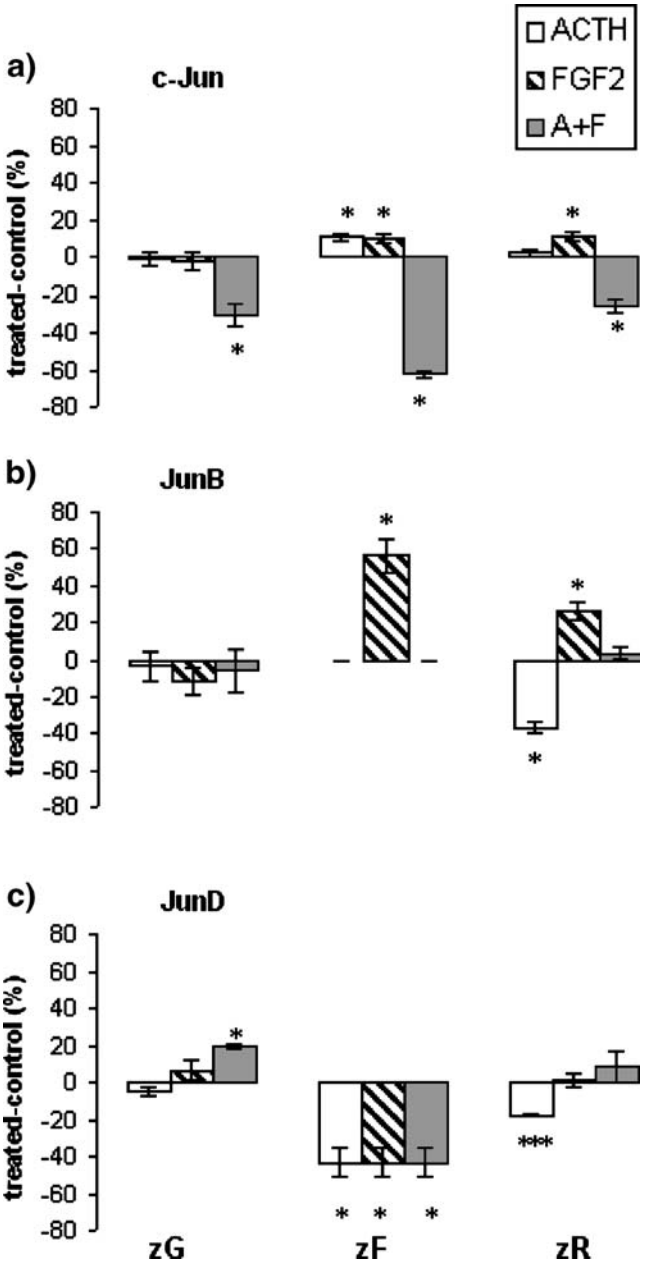

Fig. 3 Effect of infusion with ACTH (10^{-7} M), FGF2 (20 ng/ml), and the ACTH-FGF2 combination (*A+F*) on immunoreactivity for c-Jun, JunB, and JunD proteins. Values are given as the percentage of immunoreactive nuclei counted in treated infused adrenal sections minus the percentage of immunoreactive nuclei counted in control infused adrenal sections. The differences between basal and postinfusion values for Jun proteins were analyzed by ANOVA and the Tukey-Kramer multiple comparisons test. *$P<0.001$, ***$P<0.05$ compared with baseline values (*zG* zona glomerulosa, *zF* zona fasciculata, *zR* zona reticularis)

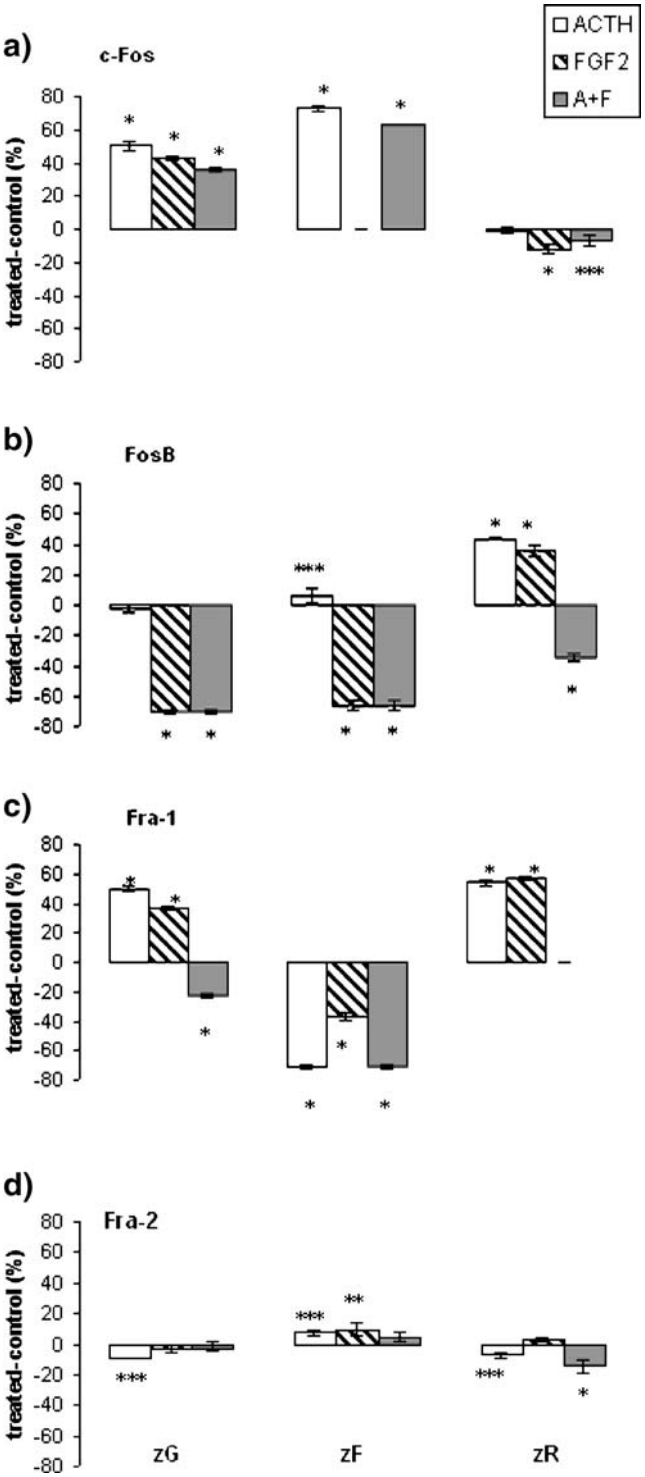

Fig. 4 Effect of infusion with ACTH (10^{-7} M), FGF2 (20 ng/ml) and the ACTH-FGF2 combination (*A+F*) on immunoreactivity for c-Fos, FosB, Fra-1, and Fra-2 proteins. Values are given as the percentage of immunoreactive nuclei counted in treated infused adrenal sections minus the percentage of immunoreactive nuclei counted in control infused adrenal sections. The differences between basal and post-infusion values for Fos proteins were analyzed by ANOVA and the Tukey-Kramer multiple comparisons test. *$P<0.001$, **$P<0.01$, ***$P<0.05$ compared with baseline values (*zG* zona glomerulosa, *zF* zona fasciculata, *zR* zona reticularis)

As seen in Fig. 6b, plasma ACTH levels were significantly lower ($P<0.023$) in the Dex+ group (12.8± 1.4 pg/ml) than in the Dex− group (45.6±3.4 pg/ml), providing evidence of a Dex-induced decrease in endogenous ACTH. Administration of Dex for 2 days caused a

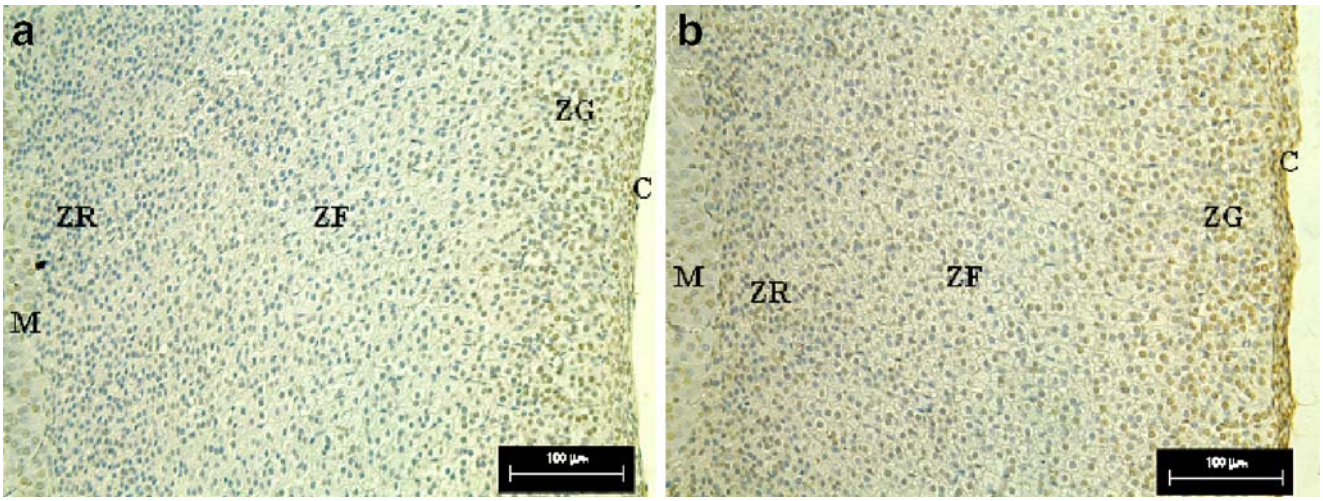

Fig. 5 Distribution of BrdU-labeled cells in the adrenal cortex of animals treated for 2 days with dexamethasone (50 mg/100 g BW; **a**) and after treatment with dexamethasone+ACTH (10^{-7} M; **b**). BrdU (100 mg/100 g BW) was injected (i.p.) 12 h before animals were killed (*brown nuclei* BrdU incorporated into nuclei in S-phase cells). Sections were also stained with Harris's hematoxylin and differentiated with a saturated solution of lithium carbonate (*ZG* zona glomerulosa, *ZF* zona fasciculata, *ZR* zona reticularis, *C* capsule, *M* medulla). *Bar* 100 µm

significant ($P<0.05$) decrease in the numbers of BrdU-positive stained nuclei in the ZF (Fig. 6c), whereas no significant alterations were observed in the ZG or ZR. These data suggested that the pituitary gland control, evident in the ZF, was limited in the ZG and ZR.

Correlations between in situ expression of AP-1 proteins and in vivo regulation of cell proliferation after ACTH treatment

The presumed composition of AP-1 factors and the increase in BrdU incorporation after treatment with ACTH, FGF2,

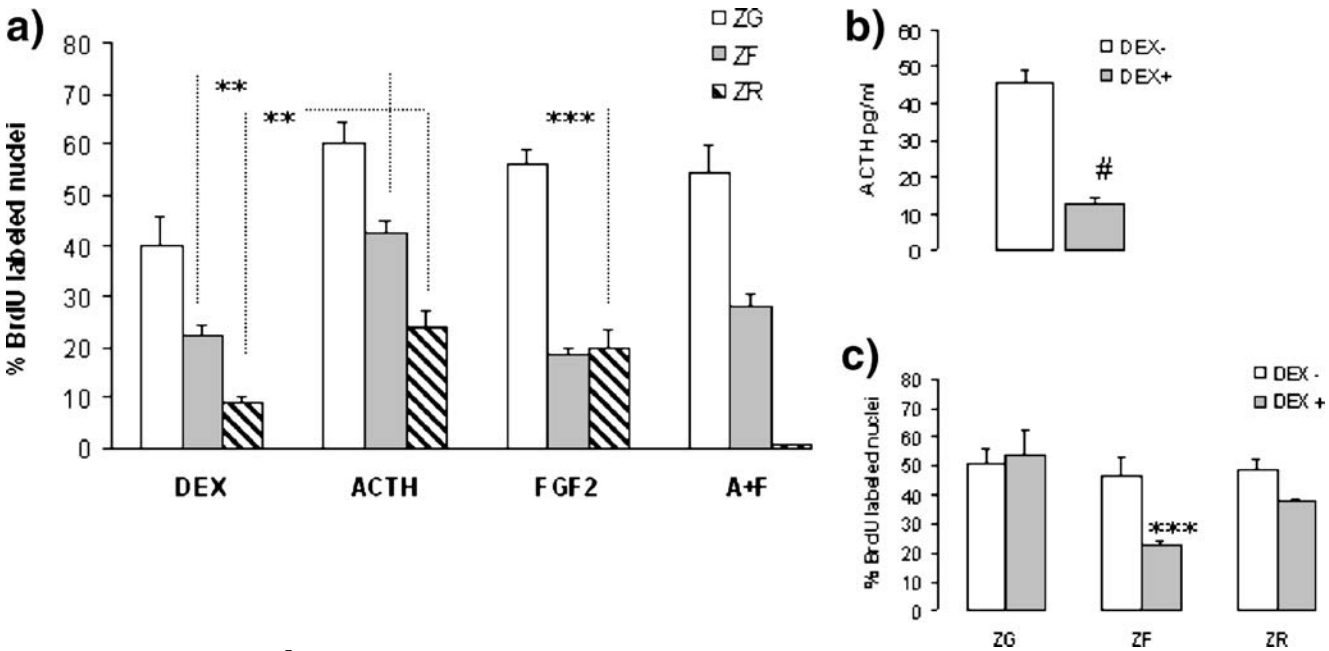

Fig. 6 **a** Effect of ACTH (10^{-7} M), FGF2 (20 ng/ml), and the ACTH-FGF2 combination (*A+F*) on the percentage of BrdU-positive nuclei in the various zones of the adrenal cortex in animals treated with dexamethasone (*Dex*). Values are given as the number of BrdU-positive nuclei expressed as a percentage of the total number of nuclei. **b** Effect of dexamethasone (*Dex*) on the ACTH plasma concentration in animals treated with dexamethasone (*Dex+*) and nontreated animals (*Dex−*). Values are given in picogram per milliliter. **c** Effect of dexamethasone (*Dex*) on the percentage of BrdU-positive nuclei in animals treated with dexamethasone (*Dex+*) and nontreated animals (*Dex−*). The differences between DEX and treated values for BrdU incorporation were analyzed by ANOVA and the Tukey-Kramer multiple comparisons tTest. $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$, $^{\#}P<0.023$ (*ZG* zona glomerulosa, *ZF* zona fasciculata, *ZR* zona reticularis)

and the ACTH-FGF2 combination is shown in Table 2. As can be seen, after ACTH infusion, the pattern of Jun and Fos protein expression was not altered in the ZG, and the administration of ACTH in vivo did not alter the percentage of BrdU-positive cells in that zone. Indeed, cell proliferation in the ZG appeared to be unaffected by any of the treatments, reinforcing the widely-held notion that the pituitary has limited control over glomerulosa function. In contrast, a significantly greater number of BrdU-positive cells was identified in the ZF and ZR in ACTH-treated rats when the probable composition of AP-1 was, respectively, c-Jun/c-Fos and c-Jun/c-Fos/FosB/Fra-1. Notably, in these rats, we observed inhibition or no expression of Jun B (in the ZF) and JunD (in the ZR). Similarly, in the ZR, FGF2 administration significantly increased DNA synthesis and c-Jun/FosB expression, regardless of JunB expression. In the ZF, FGF2 treatment resulted in strong expression of JunB and no change in BrdU incorporation. Interestingly, when rats were infused with the combination of ACTH and FGF2, the antagonistic effect observed in the expression of Jun and Fos proteins was also observed in the number of BrdU-positive nuclei in the ZF and ZR, most evidently in the ZR. After treatment with the ACTH-FGF2 combination, marked inhibition of DNA synthesis was detected in the ZR, whereas, in the same zone, the expression of JunB and JunD proteins increased.

Discussion

In this study, we have examined the effects of ACTH and FGF2 on the expression of Jun and Fos proteins in the organ that is the physiological target of ACTH: the adrenal gland. In addition, we have examined the correlations between AP-1 factors and the progression of the cell cycle by investigating the effects that ACTH and FGF2 have on the G1 to S-phase cell cycle transition of adrenal cells in an in vivo rat model. We have used the modified in situ infusion system developed by Sibley et al. (1981) and Hinson et al. (1985) to study the expression pattern of AP-1 family members. The observation made by other authors (Imai et al. 1990; Yang et al. 1990; LeHoux and Ducharme 1995), viz., that ACTH induces the c-fos proto-oncogene, has inspired us to investigate whether ACTH also modulates the AP-1 transcription factor.

The results presented here show that ACTH and FGF2 regulate the expression of nuclear Jun and Fos proteins in isolated infused rat adrenal glands, which display differential patterns of Fos and Jun protein induction. Nevertheless, various aspects must be taken into consideration when interpreting our results. In the in situ method of infusion, unlike the in vivo method, the gland receives only the medium flowing from the pump. Thus, no blood or other substances are provided by the hypothalamus, pituitary gland, or renin-angiotensin system of the animal. Indeed, after being killed, the dead animals are retained for the sole purpose of supporting the adrenal gland and its vasculature. During the initial phase of infusion, the gland quickly clears as blood is eliminated from the system. As described by Hinson et al. (1985), although the gland is exposed to endogenous stimulation in the initial period, this exposure is eliminated within 15–20 min, thereby reducing the level of steroid output. Under basal conditions, several different members of the AP-1 immediate response gene family are detectable in the adrenal gland. The adrenal gland is unusual because, in the untreated state, it expresses diverse AP-1 members (Pennypacker et al. 1992), and baseline AP-1 activity is higher in the cortex than in the medulla (Pelto-Huikko et al. 1991). This higher AP-1 activity in the adrenal cortex under baseline conditions is apparently unrelated to adrenal glucocorticoid production, since AP-1 activity is repressed by glucocorticoids (Smith et al. 1996; Karin and Chang 2001). Under basal conditions, we have found that all Jun and Fos proteins are expressed in the ZG, whereas JunB and c-Fos proteins are expressed in the ZF, and Fra-1 protein is undetectable in the ZR. These results are in partial agreement with those reported by Pelto-Huikko et al. (1991); their in situ hybridization data have revealed that, in untreated animals, low levels of c-fos mRNA occur in the ZF of the adrenal cortex, whereas the

Table 2 Correlation between the putative composition of AP-1 proteins and BrdU incorporation (*ZG* zona glomerulosa, *ZF* zona fasciculata, *ZR* zona reticularis, *JUN* Jun family proteins, *FOS* Fos family proteins). The supposed AP-1 composition associated with a significant increase in DNA synthesis is given in *bold*

Treatments	ZG		ZF		ZR	
	JUN	FOS	JUN	FOS	JUN	FOS
Basal conditions	c-Jun	FosB	c-Jun/JunD	Fra-1	c-Jun/JunD	Fra-1
ACTH	c-Jun	FosB	**c-Jun**	**c-Fos**	**c-Jun**	**c-Fos/FosB/Fra1**
FGF2	c-Jun	c-Fos/Fra-2	c-Jun/JunB	Fra-2	**c-Jun**	**FosB**
A+F	JunD	c-Fos/Fra-2	–	–	JunB/JunD	–

levels of c-Jun mRNA are high in the cortical layers of ZF and ZR. In contrast, no detectable labeling of c-fos or c-jun mRNAs is seen in the ZG in untreated animals (Pelto-Huikko et al. (1991). In this context, our results are in closer agreement with those reported by LeHoux and Ducharme (1995) who have shown that, in controls, c-jun mRNA is abundant in both the ZG and in the ZF-ZR, whereas c-fos mRNA is barely detected. In addition, in the Y1 tumor adrenocortical cell line, which closely mimics ZF cells of the normal adrenal cortex (Schimmer 1979, 1981), and in primary rat adrenal cells, the baseline expression of c-fos is negligible both at the mRNA level and at the protein level (Kimura et al. 1993; Mattos and Lotfi 2005). Infusion of ACTH differentially regulates the induction of Jun and Fos proteins in the ZF and ZR, which could lead to a variety of proteins forming the AP-1 complex. The reduction of the JunD-AP-1 and JunB-AP-1 subunits in the ZF and the strong c-Fos induction in the same layer suggest that, after ACTH infusion, c-Jun and c-Fos proteins are the main components of the AP-1 complex in this zone. The limiting components that trigger proliferation seem to be the Jun proteins, with Fos proteins having little effect (Shaulian and Karin 2001). The c-Jun protein is a positive regulator of proliferation and induces positive regulators of the progression of the cell cycle, although in vivo evidence exists that JunD and JunB are negative regulators of cell proliferation (Szremska et al. 2003; Meixner et al. 2004). Only JunB antagonizes c-Jun (Passegué and Wagner 2000). Therefore, the abundance of Jun proteins, under basal conditions, in all zones of the adrenal gland suggests their involvement in maintaining the integrity of the gland. In addition, the change in the c-Jun/c-Fos-containing AP-1 complex in the ZF, together with the lack of expression of JunB or JunD proteins in that zone after ACTH infusion indicates that proliferation occurs in this zone after ACTH stimulation. Following ACTH infusion, c-Jun protein is probably the main component of the AP-1 complex in the ZR. Indeed, in rats treated with Dex+ACTH, DNA synthesis increases significantly in the ZF and ZR, suggesting that the AP-1 modification is related to cell-cycle progression and proliferation. The Fos counterpart of AP-1 can be either FosB or Fra-1. Like c-Fos, FosB is related to the cell-cycle progression, whereas Fra-1 seems to be a positive regulator of apoptosis in photoreceptor cells (Hess et al. 2004). However, the in vivo role of FosB and Fra-1 remains unclear.

In the ZG, ACTH infusion differentially regulates the induction of Fos proteins, specifically c-Fos and Fra-1, but does not regulate Jun proteins. Nevertheless, c-Jun-positive cells have been found at high percentages in this zone. The abundance of c-Jun proteins found in the ZG, taken together with the notion that, in the outer part of the gland, mitotic activity is greater mainly in this zone (Vinson 2003), suggests that c-Jun proteins are involved in maintaining the integrity of the gland, independently of ACTH. Our results with regard to the in vivo BrdU uptake in the ZG reinforce the concept of this independence and the difference between the glomerulosa and inner zone function in the rat (Vinson 2003). In addition, lower levels of circulating ACTH are well known to result in adrenal atrophy of the ZF and ZR but not of the ZG (Thomas et al. 2004), in agreement with our results showing a significant reduction in DNA synthesis in the ZF after the administration of Dex.

The patterns of Fos and Jun induction by FGF2 treatment do not resemble those induced by ACTH, except in the ZG in which neither ACTH nor FGF2 regulates Jun protein expression or in vivo DNA synthesis. Alternatively, FGF2 increases JunB in the ZF, acting as a negative regulator of proliferation that might antagonize c-Jun expression. Nevertheless, the FGF2-induced increase in JunB expression in the ZR is not sufficient to antagonize c-Jun expression or to block the progression of the cell cycle. The induction of Fos proteins by FGF2 in the ZG resembles that induced by ACTH, except for the finding that FGF2 inhibits FosB expression. FGF2 infusion does not induce c-Fos expression in the ZF and ZR, although it has been shown to do so in the Y1 cell line and in primary rat adrenal cells (Lotfi et al. 1997; Lotfi and Armelin 2001; Mattos and Lotfi 2005). Therefore, the patterns of Fos/Jun expression and in vivo DNA synthesis seen in the adrenal cells of the ZF and ZR in response to FGF2 are unexpected and intriguing. In addition, chronic infusion of FGF2 in adult male rats submitted to adrenal autografts produce no significant morphological changes in the grafts when compared with saline-treated controls (Vendeira et al. 1999).

To test the hypothesis that the trophic effect of ACTH is modulated by paracrine factors (such as FGF2) that act through the induction of Jun/Fos proteins and DNA synthesis, we have also used a combination of ACTH and FGF2. Indeed, after infusion of the ACTH-FGF2 combination, the response of Jun/Fos proteins and in vivo BrdU incorporation to ACTH is apparently modulated by the FGF2, since the numbers of immunoreactive cells are reduced in both experimental models utilized. Our results suggest that FGF2 antagonizes ACTH, supporting the idea that ACTH controls the trophic effect regardless of exogenous FGF2. Moreover, a recent study suggests that ACTH controls the adrenal cortex through a mechanism that causes an anti-apoptotic effect on endocrine cells and an indirect vascular endothelial growth-factor-mediated action on endothelial cells (Thomas et al. 2004).

Our studies of BrdU uptake indicate that the effects of ACTH are reflected in the inner cortex, which increases in size in every condition associated with ACTH elevation, such as Cushing's syndrome or repeated ACTH injection

(Dallman 1984). Most authors describe the mitogenic region of the adrenal cortex as being situated in the outer cortical layers, in the subcapsular area of the ZG (Zajicek et al. 1986; Engeland et al. 1995; McNicol et al. 1997), although some have reported that it also encompasses the capsular stem cells (Pignatelli et al. 2002). This has been questioned by Mitani et al. (1999, 2003) who have attributed the proliferative region and a stem cell zone to an area intermediate between the ZG and ZF. Other features that have come to light suggest that the glomerulosa, excluding its inner zones, is the site of formation of a multitude of factors that can act in a paracrine/autocrine fashion (Vinson 2003). Further support comes from the glomerulosa localization of a characterized protease, viz., the adrenal secretory serine protease, which is postulated to act on pro-γ-melanocyte-stimulating hormone to yield a mitogenic component contributing to adrenal cell proliferation (Bicknell 2003). Our results regarding AP-1 expression and BrdU uptake reinforce the notion that the ZG is relatively ACTH-independent and that factors, such as angiotensin II or other paracrine components, probably maintain the glomerulosa (Vinson 2003). In contrast, we have identified BrdU-positive cells in the capsule after ACTH treatment, in agreement with the findings of Pignatelli et al. (2002).

Our findings with regard to BrdU incorporation and AP-1 protein expression indicate that neither the zonal theory nor the migration theory (Vinson 2003) can fully explain adrenocortical cytogenesis. In the present study, we have shown the continuous distribution of BrdU-positive cells after Dex administration across the cortex, thereby lending support to the migration theory. Nevertheless, we have demonstrated that, at least when used at a pharmacological dosage, a single administration of ACTH induces, in the ZF and ZR, an increase in DNA synthesis and in the expression of members of the Jun and Fos protein family that are associated with cell cycle progression. Our results confirm that ACTH induces proliferation in specific zones (ZF and ZR), supporting the zonal theory.

In summary, we have shown that ACTH infusion of the adrenal gland modulates Jun and Fos proteins. Our results regarding BrdU incorporation implicate the AP-1 family of transcription factors in the regulation of cell cycle progression and control in ACTH-induced proliferation. However, further studies are needed in order to clarify the role that these proto-oncogenes play in the mechanism of action of ACTH and to correlate them with the expression of other cell-cycle promoters, such as cyclin D and E, and of cell cycle inhibitors, such as Rb, p16, and p21.

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