Regulation of Aldosterone Synthase by Activator Transcription Factor/cAMP Response Element-Binding Protein Family Members

Edson F. Nogueira and William E. Rainey

Department of Physiology, Medical College of Georgia, Augusta, Georgia 30912

Aldosterone synthesis is regulated by angiotensin II (Ang II) and K^+ acting in the adrenal zona glomerulosa, in part through the regulation of aldosterone synthase (CYP11B2). Here, we analyzed the role of cAMP response element (CRE)-binding proteins (CREBs) in the regulation of CYP11B2. Expression analysis of activator transcription factor (ATF)/CREB family members, namely the ATF1 and ATF2, the CREB, and the CRE modulator, in H295R cells and normal human adrenal tissue was performed using quantitative real-time PCR. Ang II-induced phosphorylation of ATF/CREB members was analyzed by Western blot analysis, and their subsequent binding to the CYP11B2 promoter using chromatin immunoprecipitation assay. Aldosterone production and CYP11B2 expression were measured in small interfering RNA-transfected cells to knockdown the expression of ATF/CREB members. CYP11B2 promoter activity was measured in H295R cells cotransfected with NURR1 (NR4A2) alone or with constitutively active vectors for ATF/CREB members. Ang II induced phosphorylation of ATF1, ATF2, and CRE modulator in a time-dependent manner. Based on chromatin immunoprecipitation analysis, there was an increased association of these proteins with the CYP11B2 promoter after Ang II and K^+ treatment. Phosphorylated ATF/CREB members also bound the CYP11B2 promoter. Knockdown of ATF/CREB members reduced Ang II and K^+ induction of adrenal cell CYP11B2 mRNA expression and aldosterone production. The constitutively active ATF/CREB vectors increased the promoter activity of CYP11B2 and had a synergistic effect with NURR1. In summary, these results suggest that ATF/CREB and NGFI-B family members play a crucial role in the transcriptional regulation of CYP11B2 and adrenal cell capacity to produce aldosterone. **(*Endocrinology* 151: 1060–1070, 2010)**

Aldosterone biosynthesis in the adrenal zona glomerulosa is regulated mainly by angiotensin II (Ang II) and K^+ (1, 2). Binding of Ang II to its type 1 receptor stimulates a variety of signaling cascades, leading to induction of aldosterone synthase (CYP11B2) transcription, thereby increasing the capacity to produce aldosterone (3–7). The principal signaling pathways stimulated by type 1 Ang II receptor are the activation of protein kinase C isoforms and inositol trisphosphate/Ca^{2+} pathways (8). In addition, small increases in extracellular K^+ cause depolarization of the glomerulosa cell membrane, leading to increased intracellular Ca^{2+} (9, 10). Ang II and K^+ effects culminate with synthesis of new transcription factors, such as the neuronal growth factor-induced clone B (NGFI-B) family members that have been reported to regulate CYP11B2 expression (11–13). The NGFI-B response element (NBRE) within the CYP11B2 promoter has been shown to regulate transcription by its binding to members of the NGFI-B family (NGFI-B, NURR1, and NOR1) (14). Ang II may also induce posttranslational modifications of transcription factors already present in the cell, such as phosphorylation of cAMP response element (CRE)-binding proteins (CREBs) (15).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0977 Received August 19, 2009. Accepted December 8, 2009.
First Published Online January 22, 2010

Abbreviations: Ang II, Angiotensin II; AP-1, activation protein-1; ATF, activator transcription factor; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE-binding protein; CREm, mutated CRE; CREM, CRE modulator; Ct, threshold cycle; NBRE, NGFI-B response element; NBREm, mutated NBRE; NGFI-B, neuronal growth factor-induced clone B; qPCR, quantitative PCR; pGL3b, pGL3 basic; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory; TBS-T, Tris-buffered saline/0.01% Tween 20; WT, wild type.

The study of the 5′-flanking region of CYP11B2 has demonstrated the presence of a sequence that resembles a CRE at position −74/−64 (16). Activator transcription factor (ATF)/CREB family members are well described CRE-binding transcription factors, and their activity is implicated in the transcriptional regulation of several genes (17–20). Previous studies have associated Ang II with activation of CREBs by phosphorylation in several tissues (5, 15, 21–28), but not in adrenal cells. However, the relative role of ATF1, ATF2, CREB, and CRE modulator (CREM) in the regulation of CYP11B2 expression and aldosterone production has not been defined.

In the present investigation, we identified and characterized the role of ATF/CREB family members in the transcriptional regulation of CYP11B2. We showed that Ang II time-dependently induced phosphorylation of these transcription factors in adrenocortical cells and demonstrated that their binding to the human CYP11B2 promoter was induced by Ang II and K^+. In addition, knockdown of these transcription factors decreased Ang II- and K^+-induced expression of CYP11B2 and aldosterone production. Moreover, combined knockdown of ATF/CREB family members and NURR1 caused a further decrease in CYP11B2 expression. Interestingly, cotransfection of constitutively active ATF/CREB members with NURR1 (NGFI-B family member) had a synergistic effect on CYP11B2 promoter activity. Taken together, these lines of evidence suggest an essential role for the NGFI-B members and the ATF/CREB family in the control of aldosterone production by adrenocortical cells.

Materials and Methods

Subjects and tissues

Ten normal human adult adrenals were obtained through the Cooperative Human Tissue Network (Philadelphia, PA) and CLONTECH (Mountain View, CA). These samples came from patients who each underwent adrenalectomy, secondary to renalectomy due to renal carcinoma or at the time of autopsy. The use of these tissues was approved by the Institutional Review Boards of the Medical College of Georgia (Augusta, GA) and informed consent was obtained from every patient. Total RNA isolated from these samples was used for quantitative PCR (qPCR) analysis as previously described (29).

Cell culture and treatments

H295R human adrenocortical tumor cells were cultured in DME/Ham's F12 medium (Invitrogen, Grand Island, NY) and supplemented with 2.5% Ultroser G (Pall Life Sciences, Cergy, Saint-Christophe, France), 1% penicillin/streptomycin (Invitrogen), 0.01% gentamycin (Invitrogen), and 1% ITS™ + Premix (BD Biosciences, Bedford, MA). Cells were maintained in a 37 C humidified atmosphere (5% CO_2).

For analysis of ATF/CREB family member expression, H295R cells were subcultured onto 12-well culture dishes (Corning Costar, Corning, NY) at a density of 4×10^5 cells/well. Cells were incubated with 10 nM Ang II or 18 mM K^+ for various time points (15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, and 24 h). Total RNA was isolated, reverse transcribed, and used for qPCR analysis.

Adrenal cell plasmid transfection

H295R cells were subcultured onto 24-well culture dishes (Corning Costar) at a density of 2×10^5 cells/well for subsequent transfection using Transfast reagent (Promega Corp.) following the manufacturer's protocols. Cells were transfected with pGL3 basic (pGL3b) expression vectors containing a wild-type (WT) CYP11B2/luciferase reporter (1521 bp), or with CRE, NBRE, or CRE + NBRE mutants of this insert. The details regarding these vectors have been previously described by Bassett *et al.* (16, 30). The cells were allowed to recover for 24 h posttransfection, after which they were treated with 10 nM Ang II or 18 mM K^+ for 6 h.

For cotransfection experiments, 1 μg of the pGL3b plasmid containing a luciferase reporter gene linked to the promoter region of WT CYP11B2 was used for each transfection experiment. The pGL3b-CYP11B2 vectors were individually cotransfected with 0.1 μg of vectors expressing constitutively active ATF/CREB members (pCMX-VP16-ATF1, pCMX-VP16-ATF2, pCMX-VP16-CREB, and pCMX-VP16-CREM) in the presence or absence of 0.1 μg of pCMV-XL5 vector for NURR1. All experiments were repeated a minimum of five times. We also tested the effects of these constitutively active vectors on a somatostatin reporter vector (in pGL3), which contains a well described CRE in its promoter region.

In addition, we also tested the role of the CRE and NBRE elements present in the promoter region of CYP11B2 by cotransfecting intact or mutant (CRE, NBRE, and CRE + NBRE mutants) CYP11B2 reporter vectors with NURR1 or constitutively active ATF2 into H295R cells.

Constitutively active vectors for CREB and ATF1, and their control vector pCMX-VP16, were generously donated by Yoshikuni Nagamine (Friedrich Miescher-Institute, Basel, Switzerland). Constitutively active ATF2 and its control were kindly donated by Jae Hun Cheong (Pusan National University, Busan, Korea), and the constitutively active CREM was donated by Richard Goodman (Vollum Institute, Portland, OR).

Luciferase-mediated bioluminescence assay

Cell lysates from transfected H295R cells were loaded in a 20 μl/well volume followed by immediate addition of 50 μl of dual luciferase assay reagent (Promega Corp.) following the manufacturer's instructions. The microplate was read using the FLUOstar Optima bioluminometer (BMG Labtech, Durham, NC). The empty vector containing pCMX-VP16 was used as control, and renilla luciferase was used to normalize the measurements of firefly luciferase activity.

RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted using the RNeasy Plus kit (QIAGEN, Valencia, CA) according to protocols from the manufacturer. Concentration and purity of the RNA were checked spectroscopically using Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Bio-

systems, Foster City, CA). Primers for ATF1 (catalog no. HS00909673_m1), ATF2 (catalog no. HS00153779_m1), CREB (catalog no. HS00231713_m1), and CREM (catalog no. HS01590456_m1) were purchased from Applied Biosystems. CYP11B2 transcript expression was monitored using specific primer/probe sets as previously described (13). Quantitative PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) following the reaction parameters recommended by the manufacturer, using 20 μl of total volume consisting of Fast Universal PCR Master Mix (Applied Biosystems), primers/probes mix, and cDNA. Quantification of 18S levels (Applied Biosystems) was used to normalize samples. Negative controls contained water instead of cDNA.

In all experiments, the relative gene expression was calculated by the $\Delta\Delta Ct$ method. Briefly, the resultant threshold cycles (Ct) were normalized to a calibrator; in each case, the calibrator chosen was the basal sample. Final results were expressed as *n*-fold difference in gene expression relative to 18s rRNA and calibrator as follows: $n\text{-fold} = 2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ calibrator})}$, where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the 18s rRNA gene from the average Ct value of the transcript under investigation for each sample.

Protein assay and Western blot analysis

H295R cells were treated with 10 nM Ang II or 18 mM K^+ during the following periods: 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, and 24 h. Cells were lysed in 1× sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, 2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue) following recommendations from Cell Signaling Technology (Danvers, MA). Twenty microliters of cell lysate were run in a 10% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane. After transfer, the membranes were blocked for 1 h at room temperature with a 5% BSA solution in a 1× Tris-buffered saline/0.01% Tween 20 (TBS-T) solution. Then membranes were incubated for 1 h at room temperature with primary antibodies against phosphorylated-ATF1/CREM and phosphorylated-ATF2 obtained from Cell Signaling Technology, both at 1:10,000 dilutions in 5% BSA/TBS-T. For normalization, membranes were incubated with primary antibodies against total ATF1, total ATF2, total CREB, and total CREM purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Membranes were washed (3 × 5 min washes) with TBS-T before incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution in 5% BSA/TBS-T) obtained from Santa Cruz Biotechnology. The membranes were washed with TBS-T, and immunoreactive bands were visualized using the ECL Western Blotting Substrate from Pierce Thermo Scientific (Rockford, IL). Specificity of the antibodies against unphosphorylated (total) was confirmed by the specific knockdown of each ATF/CREB family member in cells transfected with the specific small interfering RNA (siRNA).

Where appropriate, the protein content of the samples was determined using the bicinchoninic acid protein assay kit (Pierce Thermo Scientific).

EMSA

EMSAs were carried out as previously described (16). Double-stranded oligonucleotides (25 pmol) were labeled with 30 μCi of [γ^{-32}P] ATP and 10 U of T_4 polynucleotide kinase at 37 C for 30 min. For CYP11B2 the following CRE sequence was used: 5′-CCG GTT CTC CCA TGA CGT GAT ATG TTT CGT AC-3′. Nuclear extract and each radiolabeled probe (40,000 dpm) were incubated at room temperature for 20 min in 20 μl of reaction mixture [20 mM HEPES (pH 8.0), 80 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mg/ml BSA, and 0.025 mg/ml poly(deoxyinosine-deoxycytosine) as nonspecific competitor]. For competition analysis, reaction mixtures containing various amounts of nonradiolabeled oligonucleotide were added simultaneously with probe. The resulting DNA/protein complexes were separated from free probe by electrophoresis using a 4% high-ionic-strength native polyacrylamide gel with 1× Tris-glycine running buffer (2). The gel was dried and visualized after autoradiography at −70 C for 24 h. Nuclear extracts from cultured H295R cells were prepared as previously described (9). Human CREB, ATF1, ATF-2, and CREM were prepared using a Promega *in vitro* transcription/translation system.

Chromatin immunoprecipitation (ChIP) assay

H295R cells were plated in 100-mm dishes at a density of 5 million cells per dish, followed by incubation with Ang II or K^+ for 1 h. ChIP was performed using EZ-ChIP (Upstate, Charlottesville, VA) following the manufacturer's protocols. Antibodies against ATF1, ATF2, and CREM were purchased from Santa Cruz Biotechnologies. DNA was diluted into 20 μl of nuclease-free water, and 5 μl was used for each PCR of 32 cycles. The PCR primer sequences used for CYP11B2 promoter were: forward, 5′-ACCTTCCACCAGCATGGACC-3′; reverse, 5′-GAGCAGGTTCCTGGGTGAGA-3′. Products were then run on 4% agarose gel for detection of amplification of the CYP11B2 promoter region.

Adrenal cell siRNA electroporation

H295R cells were electroporated using the Amaxa System (Gaithersburg, MD) and transfected with siRNA for ATF1, ATF2, CREB, CREM, NURR1, or control siRNA, all purchased from Santa Cruz Biotechnology. Transfected cells were plated at a density of 2 million cells per well onto six-well dishes and then allowed to recover for 48 h followed by incubation with 10 nM Ang II or 18 mM K^+ for 6 h. The effects of decreasing ATF/CREB family member expression (alone or in combination with NURR1) were evaluated by comparison of CYP11B2 mRNA expression and aldosterone production to control cells. Experiments were repeated a minimum of three times.

Aldosterone measurement

Aldosterone content of the experimental medium was determined with aldosterone standards prepared in low-serum medium using aldosterone RIA (Siemens, Los Angeles, CA). The results of the aldosterone assay were normalized to cellular protein and expressed as pmol aldosterone per mg cell protein.

Statistical analysis

All values were expressed as a mean ± SEM. One-way ANOVA was used to compare groups. *P* values lower than 0.05 were considered statistically significant.

TABLE 1. Expression analysis of ATF/CREB family members in adrenal cells and tissue

Gene	H295R (average Ct value ± SE)	Normal adrenal (average Ct value ± SE)
ATF1	24.95 ± 0.05	27.21 ± 0.14
ATF2	23.80 ± 0.03	25.58 ± 0.26
CREB	35.35 ± 0.03	27.13 ± 0.21
CREM	30.35 ± 0.03	32.85 ± 0.20

mRNA expression analysis of ATF/CREB members in H295R cells *vs.* normal human adrenal tissue. Values represent Ct measurements detected by qPCR. A minimum of 10 samples was used for analysis.

Results

Expression analysis of ATF/CREB family members in the adrenal and H295R cells

Comparative analysis of ATF1, ATF2, CREB, and CREM transcript levels in H295R adrenocortical cells and adrenal tissue has not been previously reported. Here, the mRNA expression of these transcription factors was compared by qPCR. In qPCR analysis, lower Ct values indicate higher amplification efficiency, and a difference of three cycles corresponds to approximately 10-fold difference between samples. As seen in Table 1, the Ct values for ATF1, ATF2, and CREM were lower in H295R cells in comparison with normal adrenals, suggesting that H295R cells have higher mRNA expression of these transcription factors. On the other hand, the Ct values for CREB were about eight cycles higher in H295R cells in comparison with normal adrenals, suggesting that the expression of this gene is approximately 300-fold higher in human adrenals. Importantly, the 18s values used to normalize the samples did not vary by more than one and a half cycles between the samples. RNA quality was also shown to be similar using glyceraldehyde 3-phosphate dehydrogenase and cyclophilin A as normalization genes (data not shown). The low expression of CREB in H295R cells was confirmed by protein analysis of total and/or phosphorylated CREB (data not shown) and has been previously reported in other studies (31, 32, 48).

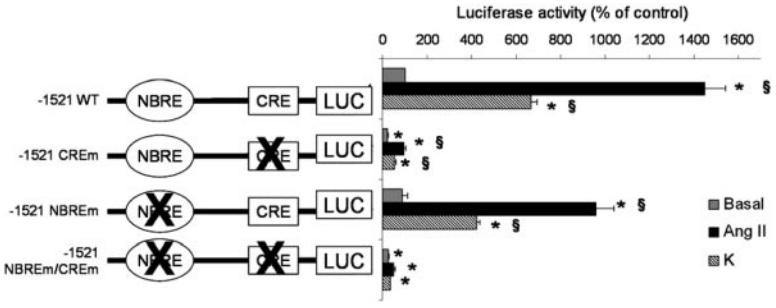

FIG. 1. Relative role of NBRE and CRE DNA sequences in the regulation of CYP11B2 promoter activity. Analysis of the role of *cis*-elements present in the human CYP11B2 promoter. H295R transfected with vectors for WT CYP11B2 promoter linked to a luciferase reporter (−1521 wt), CRE mutated sequence (−1521 CREm), NBRE mutated sequence (−1521 NBREm), or CRE + NBRE mutated sequence (−1521 CREm/NBREm). Luciferase activity was compared in transfected cells treated with Ang II or K^+. Data are expressed as a percentage of the basal for each individual vector. *, $P < 0.05$ (mutant *vs.* WT). §, $P < 0.05$ (basal *vs.* treatment with Ang II or K^+). Results represent the mean ± SEM from three independent experiments. $P < 0.05$ was considered significant.

Ang II and K^+ regulation of CYP11B2 promoter activity

Incubation of adrenocortical cells with Ang II leads to increased expression of CYP11B2 (33). The presence of CRE and NBRE domains in the CYP11B2 gene promoter was previously described (5, 30). We have previously reported the induction of NGFI-B family members by Ang II treatment (12) and their role in regulating the expression of CYP11B2 in H295R cells (13). In Fig. 1, we show that Ang II and K^+ increased CYP11B2 promoter activity approximately by 14- and 6-fold, respectively. Responsiveness of the CYP11B2 promoter to Ang II and K^+ decreased by approximately 70% when the CRE domain was mutated [−1521 mutated CRE (CREm)], whereas a 40% decrease (to both, Ang II or K^+) occurred when the NBRE site was mutated [−1521 mutated NBRE (NBREm)] (Fig. 1). A double mutation (−1521 CREm/NBREm) in the CYP11B2 promoter abolished the induction of CYP11B2 by Ang II and K^+.

Ang II-induced phosphorylation of ATF/CREB members

The ATF/CREB family of transcription factors are activated by phosphorylation (34, 35). We used Western blot analysis to detect phosphorylated levels of ATF1, ATF2, and CREM after incubation of H295R cells with Ang II. This is the first examination of Ang II-induced phosphorylation of ATF1, ATF2, and CREM in adrenal cells. As shown in Fig. 2, A–C, phosphorylated levels of these three transcription factors reached their highest values at 15 min and decreased in the following time points. Moreover, it is interesting to note that phosphorylation of ATF2 (Fig. 2B) persisted longer than of the other two transcription factors. The expression of total ATF/CREB family members in H295R cells was not affected by treatment with Ang II in our studies (Fig. 2, A–C).

Ang II- and K^+-induced association of ATF/CREB members with the CYP11B2 promoter

We have previously shown that the highly similar CYP11B1 and CYP11B2 CRE can bind *in vitro* prepared ATF1, ATF2, and CREB (16, 48). EMSA analysis in Fig. 3A confirms these findings and shows CRE binding to *in vitro* prepared CREM. Using H295R nuclear ex-

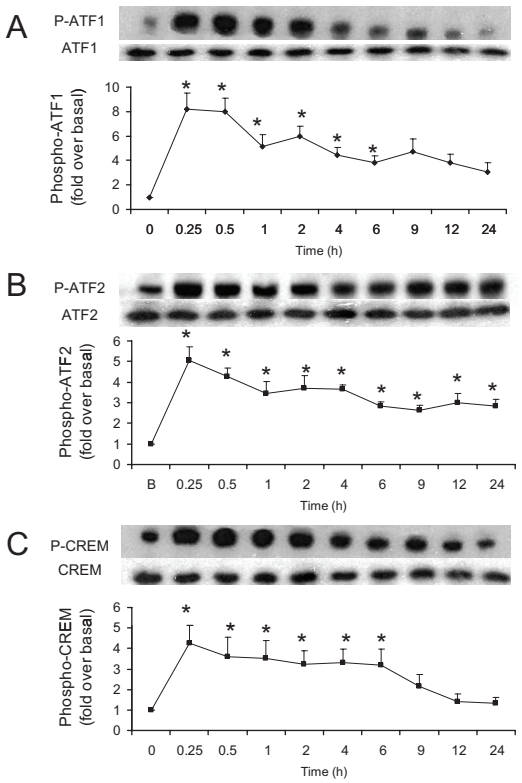

FIG. 2. Ang II-induced phosphorylation of ATF/CREB family members in human adrenocortical cells. H295R cells were treated for 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, and 24 h with 10 nM Ang II. Western blot analysis revealed time-dependent phosphorylation of ATF1 (panel A), ATF2 (panel B), and CREM (panel C). Western blot analysis of total protein for each individual transcription factor was used to normalize our results. Results represent the mean ± SEM for five independent experiments.

tract, there were three complexes formed. One complex corresponded to ATF2, and a minor band was observed at the position seen for *in vitro* prepared CREM. It should be noted that the *in vitro* prepared ATF1 migrated near but not at the exact location as a major band observed in the H295R nuclear extract. This could be due to H295R cellular posttranslational modifications in ATF1 that are not carried out in the *in vitro* translation or this complex may correspond to another transcription factor. No EMSA band was observed using the H295R nuclear lysates that corresponded to *in vitro* prepared CREB.

To extend the observations seen in EMSA, we carried out ChIP analysis. ChIP analysis of ATF/CREB binding to the promoter region of CYP11B2 has not been previously reported. ChIP assays were used to test whether ATF1/CREB members are recruited to the endogenous CYP11B2 gene promoter in H295R cells. The cross-linked, sheared chromatin preparations were subjected to immunoprecipitation with antibodies against total ATF1, ATF2, and CREM. The precipitated DNA was analyzed by PCR amplification of the CYP11B2 promoter containing the CRE site. Use of antirabbit IgG (as a negative control) failed to precipitate the CYP11B2 promoter. Here, we report for the first time that both aldosterone secretagogues, Ang II and K^+, induced association of ATF1, ATF2, and CREM with the CYP11B2 promoter (Fig. 3, B–D). Moreover, phosphorylated ATF1/CREM (Fig. 3E) and ATF2 (Fig. 3F) also bound to the segment of the human CYP11B2 promoter containing the CRE site. Due to low expression levels, CREB was excluded from our analysis of protein phosphorylation and ChIP analysis. The results from ChIP experiments strongly support a physiological role of ATF/CREB family members in Ang II and K^+ induction of the human CYP11B2 gene transcription.

Effects of ATF/CREB member knockdown on the expression of CYP11B2 and aldosterone production

We confirmed the importance of ATF/CREB members in H295R cells by decreasing their expression via transfection with siRNA for ATF1, ATF2, and CREM. H295R cells transfected with scrambled siRNA were used as controls. After recovery, cells were incubated with Ang II or K^+ for 6 h, followed by qPCR analysis of CYP11B2 expression and analysis of aldosterone production by RIA. Transfection of siRNA for each independent transcription factor reduced the expression of the specific transcription factor and did not interfere with the expression of the other ATF/CREB members being studied. That is, transfection of H295R cells with siRNA for ATF1, ATF2, and CREM reduced the protein expression of these transcription factors by approximately 65% (Fig. 4, A–C). Knockdown of the ATF1, ATF2, and CREM decreased the Ang II-induced CYP11B2 mRNA expression respectively by 35, 50, and 50% (Fig. 4D). The induction of CYP11B2 by K^+ was decreased by 40, 50, and 70% after transfection with siATF1, siATF2, and siCREM, respectively (Fig. 4D). Ang II-induced aldosterone production was reduced by 45, 35, and 40% in siATF1, siATF2, and siCREM transfected cells, respectively (Fig. 4E). Finally, K^+-induced aldosterone production was reduced by 20, 30, and 40% in siATF1, siATF2, and siCREM transfected cells, respectively (Fig. 4E). Although siATF1 transfection caused a 35% decrease in CYP11B2 mRNA expression, the consequent 20% reduction in aldosterone production by K^+-stimulated cells was not deemed statistically significant. These results suggests that ATF1, ATF2, and CREM can each play a role in Ang II and K^+ medicated CYP11B2 expression and aldosterone production.

Transfection of H295R cells with siRNA for NURR1 alone or with siRNA for ATF/CREB family members effectively reduced the mRNA levels for NURR1 (Fig. 5A). Transfection of H295R cells with siNURR1 reduced the induction of CYP11B2 transcription by Ang II and K^+ by

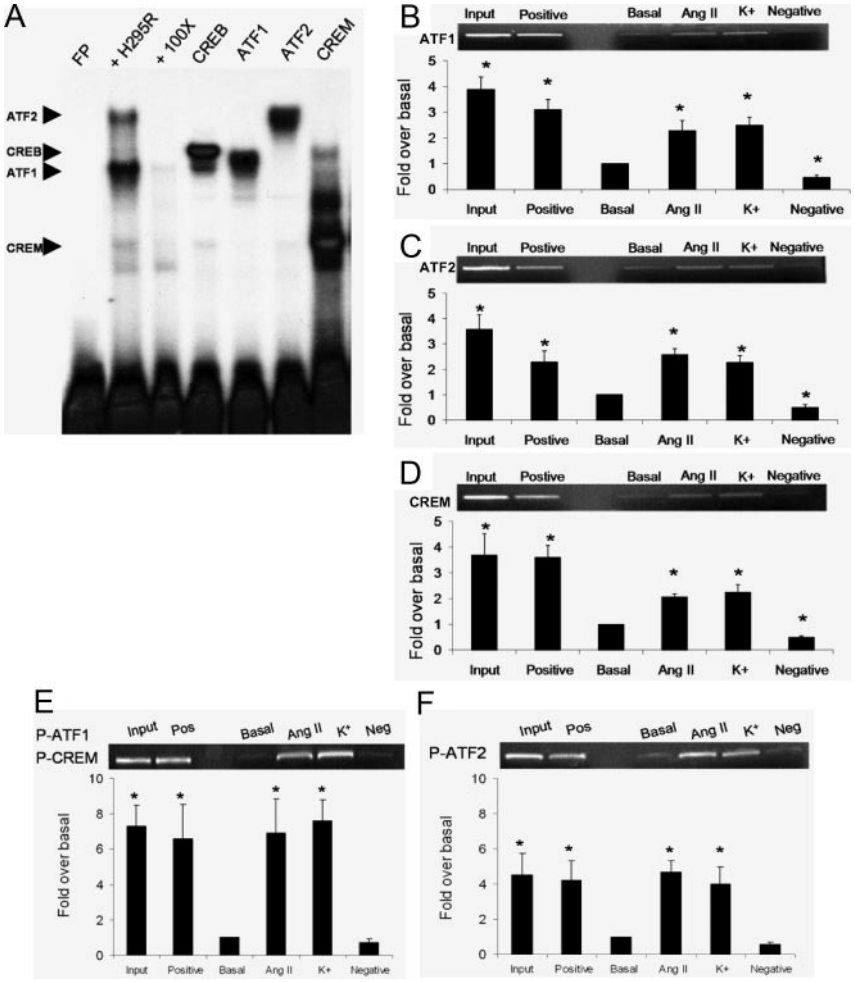

FIG. 3. Analysis of ATF/CREB protein recruitment to the human CYP11B2 promoter. EMSA was performed using ^{32}P-labeled oligonucleotide probes containing the CRE site from sequence of human CYP11B2 gene (panel A). *Lane 1*, Radiolabeled probe alone (FP, free probe); *lane 2*, labeled probe incubated with nuclear extract from H295R cells; *lane 3*, nonradiolabeled self-competitor oligonucleotides; *lanes 4–6*, labeled probe incubated with *in vitro* prepared CREB, ATF1, and ATF2. ChIP was used to investigate the induction of binding of ATF/CREB members to the segment of human CYP11B2 containing the CRE site (panels B–D). Incubation of H295R cells for 1 h with Ang II and K^+ induced binding of ATF1, ATF2, and CREM to the CYP11B2 promoter. Recruitment of phosphorylated ATF2 and ATF1/CREM (cross-reacting antibody against serine 133) to the promoter region of CYP11B2 is shown in panels E and F. Antiacetylated histone antibody was used for the positive controls, antirabbit IgG was added to negative controls, and 1% of the supernatant (chromatin in dilution buffer) was used for the input. *, $P < 0.05$ (compared with basal). Results represent the mean ± SEM for three or more independent experiments. $P < 0.05$ was considered significant.

approximately 50%, whereas in cotransfection with siATF1, siATF2, and siCREM, it had an additive effect on diminishing the expression of CYP11B2 mRNA expression by H295R cells (Fig. 5, B–D).

Effects of ATF/CREB transcription factors and NURR1 on the promoter activity of CYP11B2

ATF/CREB members are known to be activated by phosphorylation (15). Constitutively active forms for ATF/CREB members are able to bind to CRE sites independent of agonist-induced posttranslational modifications (36–38). Transfection of H295R cells with constitutively active vectors for ATF1, ATF2, CREB, and CREM significantly increased basal promoter activity of CYP11B2 by 2.5-, 4.5-, 3.5-, and 2.0-fold, respectively (Fig. 6A). In addition, our results showed a significant effect of the constitutively active CREB on the CYP11B2 promoter activity, suggesting that this transcription factor could also regulate CYP11B2 gene in normal adrenal.

We have previously reported the regulation of CYP11B2 promoter activity by NURR1 in H295R cells. Here, we also investigated the role of NURR1 in cotransfection experiments with constitutively active vectors for the ATF/CREB family members. Interestingly, cotransfection of NURR1 had a synergistic effect on CYP11B2 promoter activity (Fig. 6A). NURR1 cotransfection increased the effect of ATF1, ATF2, CREB, and CREM by 4.5-, 3-, 3.5-, and 3-fold, respectively.

As seen in Fig. 6B, NURR1 and constitutively active ATF2 caused a significant induction of CYP11B2 promoter activity. However, when the CRE was mutated, the effect of ATF2 was abolished, whereas no effect was observed on the induction by NURR1 (comparison takes into consideration the fact that the CREm vector has lower basal activity). Mutation of the NBRE abolished the stimulation by NURR1 on the CYP11B2 promoter, and interestingly, this mutation also reduced the effects of ATF2. Double mutation (CRE/NBREm) in the CYP11B2 reporter vectors abolished its induction by NURR1 and ATF2. These data confirmed the importance of NGFI-B family and the participation of ATF/CREB members in the regulation of the human CYP11B2 gene.

Discussion

One of the chronic actions of Ang II is its ability to increase the biosynthesis of aldosterone in the adrenal cortex (39). The expression of CYP11B2 in the zona glomerulosa of the adrenal gland determines the capacity of the adrenal

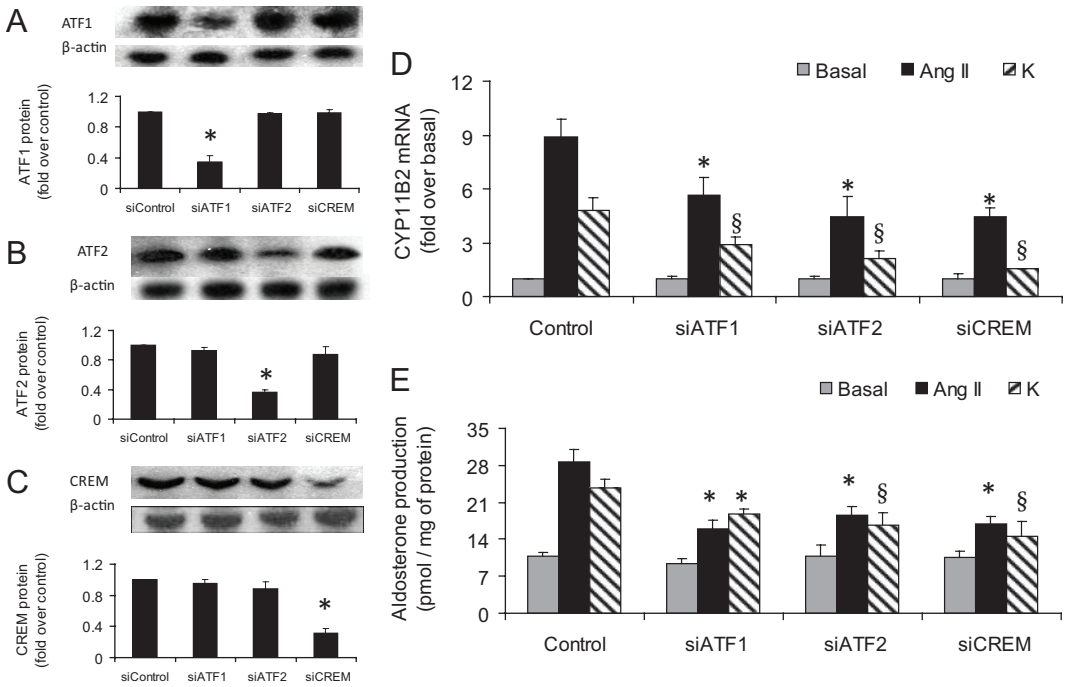

FIG. 4. Effects of the knockdown of ATF/CREB transcription factors on CYP11B2 mRNA expression and aldosterone production. H295R cells transfected with siRNA for ATF1, ATF2, and CREM, were incubated with Ang II or K^+. Western blot analysis was used to confirm the knockdown of these proteins in transfected cells (panels A–C). Real-time qPCR was used to measure expression of CYP11B2 (panel D). Aldosterone levels in the cell culture medium were determined by RIA, and values were normalized to the amount of protein in each well (panel E). *, $P < 0.05$ in siATFs/CREM *vs.* control (panels A–C) and siATF/CREB in Control *vs.* Ang II-treated conditions (panels D and E). §, $P < 0.05$ in siATF/CREM *vs.* control in Control and K^+-treated cells (panels D and E). Results represent the mean ± SEM for three or more independent experiments. $P < 0.05$ was considered significant.

cortex to produce aldosterone (33). The regulation of CYP11B2 by Ang II at the transcriptional level occurs through the rapid induction of protein synthesis as well as posttranslational modification of transcription factors already present in the cell (33). We recently demonstrated the Ang II rapid induction of NGFI-B family members and their role in CYP11B2 expression in adrenocortical cells (13, 30, 40). Moreover, CYP11B2 expression does not seem to be solely regulated by these transcription factors. In addition to the NBRE site, our laboratory has previously reported the presence of a CRE domain in the 5′ promoter region of the CYP11B2 gene (16). Sequence analysis of the CRE shows that this site is highly conserved between species and is important for bovine, rodent (mouse, rat, and hamster), and human CYP11B2 gene activity (41–46). Our transfection study using mutant vectors for these two sites (separately and in combination) confirmed the importance of these two sites in the regulation of the human CYP11B2 gene by Ang II and K^+.

Interestingly, our results regarding the expression of ATF1, ATF2, and CREM revealed that these transcripts are more highly expressed in H295R cells than in normal adrenals. On the other hand, CREB transcripts were considerably lower in H295R cells than in normal adrenal tissue. These results confirmed and extended previously reported data describing the overexpression of CREM to compensate for CREB deficiency in H295R cells (47). Wang *et al.* (48) have previously shown the expression of high levels of ATF1 and ATF2 in nuclear extracts of H295R cells, whereas the expression of CREB varied among the strains of H295R tested (decreasing with time in long-term culture). Here, we show that not only CREM, but also ATF1 and ATF2, are more highly expressed in H295R cells and therefore could account for a compensatory mechanism to activate genes with CRE-dependent transcription. A role for CREM in the regulation of steroidogenesis has been previously studied (25, 49, 50). In these studies, CREM was shown to regulate the expression of the steroidogenic acute regulatory (StAR) protein, which is described as the early regulatory step in aldosterone production (51). Importantly, StAR is also induced by Ang II and K^+ in adrenocortical cells (6), and therefore, activation of ATF/CREB members is likely involved in the expression of this protein and, by extension, the acute regulation of aldosterone production.

Here, we describe for the first time that ATF/CREB transcription factors are time-dependently phosphorylated in response to Ang II. This suggests that these agonist can posttranslationally regulate the activity of ATF1, ATF2, and CREM as has been shown previously for Ang II in nonadrenal models (15, 35). We have previously reported that nuclear extract from H295R produces three

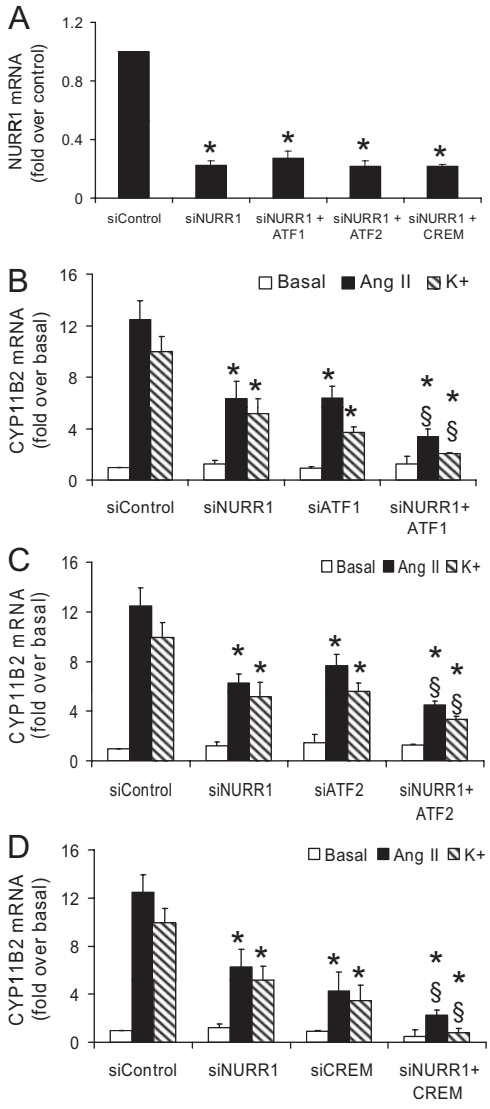

FIG. 5. Effects of the knockdown of NURR1 alone and in combination with ATF/CREM transcription factors on CYP11B2 expression. H295R cells transfected with siRNA for NURR1, ATF1, ATF2, and CREM, were incubated with Ang II or K^+. Real-time PCR analysis was used to confirm the knockdown of NURR1 (panel A) and to measure its effect on CYP11B2 mRNA expression in transfected cells (panel B–D). *, $P < 0.05$ (siRNA transfected cells *vs.* control); §, $P < 0.05$ (combined siNURR1 + siATF/CREB *vs.* control siNURR1 or siATF/CREB alone). Results represent the mean ± SEM for three independent experiments. $P < 0.05$ was considered significant.

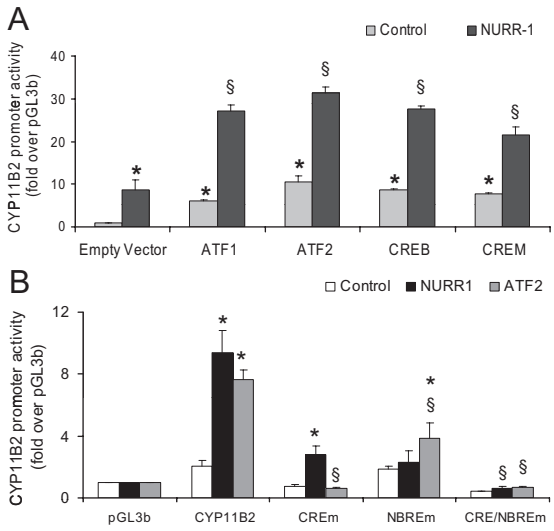

FIG. 6. Effects of constitutively active ATF/CREB family members and NURR1 on the promoter activity of CYP11B2. Results are expressed as fold increase over control. In panel A, *gray bars* represent CYP11B2 cotransfected with empty vector or with each ATF/CREB family member. *Black bars* represent CYP11B2 cotransfected with empty vector or each ATF/CREB family member in the presence of NURR1. *, $P < 0.05$ (ATF/CREB members *vs.* empty vector); §, $P < 0.05$ (ATF/CREB member + NURR1 *vs.* NURR1 alone). In panel B, *white bars* represent the basal promoter activity of intact and mutant CYP11B2, *black bars* represent such promoter activity in the presence of constitutively active ATF2, and *the gray bars* represent CYP11B2 (intact and mutant) promoter activity in the presence of NURR1. *, $P < 0.05$ (basal *vs.* ATF2 or NURR1); §, $P < 0.05$ (mutant *vs.* intact vector). The results represent the mean ± SEM for four independent experiments. $P < 0.05$ was considered significant.

protein complexes in EMSA using the CYP11B2 CRE. Using supershift studies, complex 1 is defined as ATF2. Here, using *in vitro* prepared CREM, we show that complex 3 is likely CREM. The nature of the complex 2 is not clear because it migrates close but not exactly with ATF1. In support for a role of these factors in the regulation of CYP11B2, ChIP analysis indicated binding of ATF1, ATF2, and CREM to the CRE region of the CYP11B2 promoter. In addition, ChIP suggests that both Ang II and K^+ induced recruitment of these transcription factors to the CYP11B2 CRE region. ChIP analysis using antibodies for phoshorylated ATF1/CREM and ATF2 indicated a greater fold increase in CRE binding after Ang II or K^+ treatment. These findings can be interpreted to indicate that either the phospho-antibodies were better for the ChIP assay or that the phorphorylated transcription factors preferentially increase binding after hormonal treatment. Thus, the increased expression of CYP11B2 in the adrenal cortex that is associated with Ang II treatment correlates with activation and binding of ATF/CREB proteins to the promoter region of the human CYP11B2 gene.

The knockdown studies revealed the role of ATF/CREB members and NURR1 in the regulation of CYP11B2 and aldosterone production induced by Ang II and K^+. Due to the relatively low expression of CREB in the H295R cell, its endogenous role was not accessed by siRNA knockdown. However, CREM, ATF1, and ATF2 each appear to be activated and have a potential role in CYP11B2 transcription. This concept was confirmed by our studies with constitutively active expression vectors which demonstrated that ATF1, ATF2, CREM, and also CREB could regulate CYP11B2 promoter activity. Taken together, this data suggests that, *in vivo*, these four members of the ATF/CREB family participate in the regulation of CYP11B2

expression/aldosterone production. Our phosphorylation studies suggested that, in comparison with ATF1 and CREM, ATF2 remains phosphorylated longer after Ang II treatment and could account for a more chronic role for ATF2 in the regulation of aldosterone synthase expression. However, our experiments using ChIP, siRNA, and transfection of constitutively active vectors suggested that the four ATF/CREB members studied here may similarly participate in such regulation.

Previous studies from our laboratory have reported the importance of NGFI-B family members in the regulation of CYP11B2 transcription (13, 30, 52, 53). Interestingly, NURR1 acted synergistically with members of the ATF/CREB family to increase CYP11B2 expression. In addition, mutation of the NBRE site not only abolished the effect of NURR1 on CYP11B2 promoter activity, but it also caused a partial decrease in the effect of ATF2, a fact that also suggests synergy between these two family of transcription factors. These results confirm the importance of both sites, the CRE and the NBRE, suggested by our study involving transfection with the mutated CYP11B2 promoter vectors. The synergism and protein-protein interaction of NBRE and ATF/CREB members have been previously described in the transcription of the propiomelanocortin gene (54). In addition, it is important to note that the transcription of NGFI-B family members is also regulated by CREBs (55–58). Therefore, activation of ATF/CREB members may regulate aldosterone production directly by binding of these transcription factors to the promoter of StAR and CYP11B2 gene and also indirectly through activation of NGFI-B transcription. Members of the ATF/CREB family and of the activation protein-1 (AP-1) are bZip proteins that recognize similar DNA sequences, and certain ATF/CREB factors are described to interact with members of the AP-1 complex, including JUN and FOS (59–61). It is worth noting that members of the AP-1 complex were also described to be directly regulated by Ang II in our comparative study of adrenocortical cells (12).

In summary, we present evidence that the regulation of aldosterone production by Ang II and K^+ rely on the presence of key regulatory domains in the promoter region of the human CYP11B2 gene. Our study brings together two mechanisms of Ang II- and K^+-induced regulation of CYP11B2: the induction of NGFI-B family expression and the activation of ATF/CREB members. The interaction of CREB/ATF family members with other transcription factor(s) and/or coactivator(s) or corepressor(s) on these *cis*-elements of the CYP11B2 promoter is a topic for future studies on the regulation of adrenal zonation and steroidogenesis.

Acknowledgments

We thank Daniel Gerry for technical assistance and review of the manuscript.

Address all correspondence and requests for reprints to: William E. Rainey, Ph.D., Department of Physiology, Medical College of Georgia, 1120 15th Street, CA Building, Room 3094, Augusta, Georgia 30912. E-mail: wrainey@mail.mcg.edu.

This work was supported by National Institutes of Health Grant DK43140 (to W.E.R.).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Quinn SJ, Williams GH** 1988 Regulation of aldosterone secretion. Annu Rev Physiol 50:409–426
2. **Müller J** 1995 Aldosterone: the minority hormone of the adrenal cortex. Steroids 60:2–9
3. **Shibata H, Ogishima T, Mitani F, Suzuki H, Murakami M, Saruta T, Ishimura Y** 1991 Regulation of aldosterone synthase cytochrome P-450 in rat adrenals by angiotensin II and potassium. Endocrinology 128:2534–2539
4. **Adler GK, Chen R, Menachery AI, Braley LM, Williams GH** 1993 Sodium restriction increases aldosterone biosynthesis by increasing late pathway, but not early pathway, messenger ribonucleic acid levels and enzyme activity in normotensive rats. Endocrinology 133: 2235–2240
5. **Clyne CD, Zhang Y, Slutsker L, Mathis JM, White PC, Rainey WE** 1997 Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements. Mol Endocrinol 11:638–649
6. **Clark BJ, Pezzi V, Stocco DM, Rainey WE** 1995 The steroidogenic acute regulatory protein is induced by angiotensin II and K^+ in H295R adrenocortical cells. Mol Cell Endocrinol 115:215–219
7. **Kim YC, Ariyoshi N, Artemenko I, Elliott ME, Bhattacharyya KK, Jefcoate CR** 1997 Control of cholesterol access to cytochrome P450scc in rat adrenal cells mediated by regulation of the steroidogenic acute regulatory protein. Steroids 62:10–20
8. **Hunyady L, Catt KJ** 2006 Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. Mol Endocrinol 20:953–970
9. **Lotshaw DP** 1997 Effects of K^+ channel blockers on K^+ channels, membrane potential, and aldosterone secretion in rat adrenal zona glomerulosa cells. Endocrinology 138:4167–4175
10. **Chen XL, Bayliss DA, Fern RJ, Barrett PQ** 1999 A role for T-type Ca^{2+} channels in the synergistic control of aldosterone production by ANG II and K^+. Am J Physiol 276:F674–F683
11. **Romero DG, Rilli S, Plonczynski MW, Yanes LL, Zhou MY, Gomez-Sanchez EP, Gomez-Sanchez CE** 2007 Adrenal transcription regulatory genes modulated by angiotensin II and their role in steroidogenesis. Physiol Genomics 30:26–34
12. **Nogueira EF, Vargas CA, Otis M, Gallo-Payet N, Bollag WB, Rainey WE** 2007 Angiotensin-II acute regulation of rapid response genes in human, bovine, and rat adrenocortical cells. J Mol Endocrinol 39:365–374
13. **Nogueira EF, Xing Y, Morris CA, Rainey WE** 2009 Role of angiotensin II-induced rapid response genes in the regulation of enzymes needed for aldosterone synthesis. J Mol Endocrinol 42:319–330
14. **Wilson TE, Fahrner TJ, Johnston M, Milbrandt J** 1991 Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science 252:1296–1300
15. **Cammarota M, Bevilaqua LR, Dunkley PR, Rostas JA** 2001 Angiotensin II promotes the phosphorylation of cyclic AMP-responsive

element binding protein (CREB) at Ser133 through an ERK1/2-dependent mechanism. J Neurochem 79:1122–1128
16. **Bassett MH, Zhang Y, White PC, Rainey WE** 2000 Regulation of human CYP11B2 and CYP11B1: comparing the role of the common CRE/Ad1 element. Endocr Res 26:941–951
17. **Thomson DM, Herway ST, Fillmore N, Kim H, Brown JD, Barrow JR, Winder WW** 2008 AMP-activated protein kinase phosphorylates transcription factors of the CREB family. J Appl Physiol 104: 429–438
18. **Mayr B, Montminy M** 2001 Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599–609
19. **De Cesare D, Sassone-Corsi P** 2000 Transcriptional regulation by cyclic AMP-responsive factors. Prog Nucleic Acids Res Mol Biol 64:343–369
20. **Gonzalez GA, Montminy MR** 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680
21. **Goraya TY, Kessler SP, Stanton P, Hanson RW, Sen GC** 1995 The cyclic AMP response elements of the genes for angiotensin converting enzyme and phosphoenolpyruvate carboxykinase (GTP) can mediate transcriptional activation by CREM τ and CREM α. J Biol Chem 270:19078–19085
22. **Rose P, Bond J, Tighe S, Toth MJ, Wellman TL, Briso de Montiano EM, Lewinter MM, Lounsbury KM** 2008 Genes overexpressed in cerebral arteries following salt-induced hypertensive disease are regulated by angiotensin II, JunB, and CREB. Am J Physiol Heart Circ Physiol 294:H1075–H1085
23. **Chan SH, Wang LL, Tseng HL, Chan JY** 2007 Upregulation of AT1 receptor gene on activation of protein kinase Cβ/nicotinamide adenine dinucleotide diphosphate oxidase/ERK1/2/c-fos signaling cascade mediates long-term pressor effect of angiotensin II in rostral ventrolateral medulla. J Hypertens 25:1845–1861
24. **Pham H, Chong B, Vincenti R, Slice LW** 2008 Ang II and EGF synergistically induce COX-2 expression via CREB in intestinal epithelial cells. J Cell Physiol 214:96–109
25. **Sugawara T, Sakuragi N, Minakami H** 2006 CREM confers cAMP responsiveness in human steroidogenic acute regulatory protein expression in NCI-H295R cells rather than SF-1/Ad4BP. J Endocrinol 191:327–337
26. **Wang X, Murphy TJ** 2000 The inducible cAMP early repressor ICERIIγ inhibits CREB and AP-1 transcription but not AT1 receptor gene expression in vascular smooth muscle cells. Mol Cell Biochem 212:111–119
27. **Sano M, Fukuda K, Sato T, Kawaguchi H, Suematsu M, Matsuda S, Koyasu S, Matsui H, Yamauchi-Takihara K, Harada M, Saito Y, Ogawa S** 2001 ERK and p38 MAPK, but not NF-κB, are critically involved in reactive oxygen species-mediated induction of IL-6 by angiotensin II in cardiac fibroblasts. Circ Res 89:661–669
28. **Murasawa S, Matsubara H, Mori Y, Masaki H, Tsutsumi Y, Shibasaki Y, Kitabayashi I, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Iba S, Iwasaka T** 2000 Angiotensin II initiates tyrosine kinase Pyk2-dependent signalings leading to activation of Rac1-mediated c-Jun NH2-terminal kinase. J Biol Chem 275:26856–26863
29. **Ye P, Mariniello B, Mantero F, Shibata H, Rainey WE** 2007 G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism. J Endocrinol 195:39–48
30. **Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE** 2004 The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. Mol Endocrinol 18:279–290
31. **Rosenberg D, Groussin L, Jullian E, Perlemoine K, Medjane S, Louvel A, Bertagna X, Bertherat J** 2003 Transcription factor 3′,5′-cyclic adenosine 5′-monophosphate-responsive element-binding protein (CREB) is decreased during human adrenal cortex tumorigenesis and fetal development. J Clin Endocrinol Metab 88:3958–3965
32. **Rosenberg D, Groussin L, Jullian E, Perlemoine K, Bertagna X, Bertherat J** 2002 Role of the PKA-regulated transcription factor CREB in development and tumorigenesis of endocrine tissues. Ann NY Acad Sci 968:65–74
33. **Bassett MH, White PC, Rainey WE** 2004 The regulation of aldosterone synthase expression. Mol Cell Endocrinol 217:67–74
34. **Gu J, Wen Y, Mison A, Nadler JL** 2003 12-lipoxygenase pathway increases aldosterone production, 3′,5′-cyclic adenosine monophosphate response element-binding protein phosphorylation, and p38 mitogen-activated protein kinase activation in H295R human adrenocortical cells. Endocrinology 144:534–543
35. **Haus-Seuffert P, Meisterernst M** 2000 Mechanisms of transcriptional activation of cAMP-responsive element-binding protein CREB. Mol Cell Biochem 212:5–9
36. **Lee MY, Jung CH, Lee K, Choi YH, Hong S, Cheong J** 2002 Activating transcription factor-2 mediates transcriptional regulation of gluconeogenic gene PEPCK by retinoic acid. Diabetes 51:3400–3407
37. **Soubt MK, Marksitzer R, Menoud PA, Nagamine Y** 1998 Role of tissue-specific transcription factor LFB3 in a cyclic AMP-responsive enhancer of the urokinase-type plasminogen activator gene in LLC-PK1 cells. Mol Cell Biol 18:4698–4706
38. **Fass DM, Craig JC, Impey S, Goodman RH** 2001 Cooperative mechanism of transcriptional activation by a cyclic AMP-response element modulator α mutant containing a motif for constitutive binding to CREB-binding protein. J Biol Chem 276:2992–2997
39. **Williams GH** 2005 Aldosterone biosynthesis, regulation, and classical mechanism of action. Heart Fail Rev 10:7–13
40. **Nogueira EF, Bollag WB, Rainey WE** 2009 Angiotensin II regulation of adrenocortical gene transcription. Mol Cell Endocrinol 302: 230–236
41. **Rainey WE** 1999 Adrenal zonation: clues from 11β-hydroxylase and aldosterone synthase. Mol Cell Endocrinol 151:151–160
42. **Kirita S, Hashimoto T, Kitajima M, Honda S, Morohashi K, Omura T** 1990 Structural analysis of multiple bovine P450(11 β) genes and their promoter activities. J Biol Chem 108:1030–1041
43. **Honda S, Morohashi K, Omura T** 1990 Novel cAMP regulatory elements in the promoter region of bovine P450(11 β) gene. J Biochem 108:1042–1049
44. **Mouw AR, Rice DA, Meade JC, Chua SC, White PC, Schimmer BP, Parker KL** 1989 Structural and functional analysis of the promoter region of the gene encoding mouse steroid 11 β-hydroxylase. J Biol Chem 264:1305–1309
45. **Mukai K, Imai M, Shimada H, Ishimura Y** 1993 Isolation and characterization of rat CYP11B genes involved in late steps of mineralo- and glucocorticoid syntheses. J Biol Chem 268:9130–9137
46. **Coulombe N, Lefebvre A, Lehoux JG** 1997 Characterization of the hamster CYP11B2 gene encoding adrenal cytochrome P450 aldosterone synthase. DNA Cell Biol 16:993–1002
47. **Groussin L, Massias JF, Bertagna X, Bertherat J** 2000 Loss of expression of the ubiquitous transcription factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREMτ in the human adrenocortical cancer cell line H295R. J Clin Endocrinol Metab 85:345–354
48. **Wang XL, Bassett M, Zhang Y, Yin S, Clyne C, White PC, Rainey WE** 2000 Transcriptional regulation of human 11β-hydroxylase (hCYP11B1). Endocrinology 141:3587–3594
49. **Krug AW, Vleugels K, Schinner S, Lamounier-Zepter V, Ziegler CG, Bornstein SR, Ehrhart-Bornstein M** 2007 Human adipocytes induce an ERK1/2 MAP kinases-mediated upregulation of steroidogenic acute regulatory protein (StAR) and an angiotensin II-sensitization in human adrenocortical cells. Int J Obes 31:1605–1616
50. **Martin LJ, Tremblay JJ** 2009 The nuclear receptors NUR77 and SF1 play additive roles with c-JUN through distinct elements on the mouse Star promoter. J Mol Endocrinol 42:119–129
51. **Miller WL** 2008 Steroidogenic enzymes. Endocr Dev 13:1–18
52. **Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE** 2004 The NGFIB family of nuclear receptors regulates adrenal aldosterone production. Mol Endocrinol 18(2):279–290
53. **Bassett MH, White PC, Rainey WE** 2004 A role for the NGFI-B

family in adrenal zonation and adrenocortical disease. Endocr Res 30:567–574

54. **Mynard V, Latchoumanin O, Guignat L, Devin-Leclerc J, Bertagna X, Barré B, Fagart J, Coqueret O, Catelli MG** 2004 Synergistic signaling by corticotropin-releasing hormone and leukemia inhibitory factor bridged by phosphorylated 3′,5′-cyclic adenosine monophosphate response element binding protein at the Nur response element (NurRE)-signal transducers and activators of transcription (STAT) element of the proopiomelanocortin promoter. Mol Endocrinol 18:2997–3010
55. **McEvoy AN, Bresnihan B, Fitzgerald O, Murphy EP** 2002 Corticotropin-releasing hormone signaling in synovial tissue vascular endothelium is mediated through the cAMP/CREB pathway. Ann NY Acad Sci 966:119–130
56. **Chen X, Zachar V, Chang C, Ebbesen P, Liu X** 1998 Differential expression of Nur77 family members in human T-lymphotropic virus type 1-infected cells: transactivation of the TR3/nur77 gene by Tax protein. J Virol 72:6902–6906
57. **Saucedo-Cardenas O, Kardon R, Ediger TR, Lydon JP, Conneely OM** 1997 Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1. Gene 187:135–139
58. **Darragh J, Soloaga A, Beardmore VA, Wingate AD, Wiggin GR, Peggie M, Arthur JS** 2005 MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling. Biochem J 390:749–759
59. **Samten B, Townsend JC, Weis SE, Bhoumik A, Klucar P, Shams H, Barnes PF** 2008 CREB, ATF, and AP-1 transcription factors regulate IFN-γ secretion by human T cells in response to mycobacterial antigen. J Immunol 181:2056–2064
60. **Wilhelm D, van Dam H, Herr I, Baumann B, Herrlich P, Angel P** 1995 Both ATF-2 and c-Jun are phosphorylated by stress-activated protein kinases in response to UV irradiation. Immunobiology 193: 143–148
61. **Hoeffler JP, Lustbader JW, Chen CY** 1991 Identification of multiple nuclear factors that interact with cyclic adenosine 3′,5′-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions. Mol Endocrinol 5:256–266