Modulation of steroidogenic enzymes by orphan nuclear transcriptional regulation may control diverse production of cortisol and androgens in the human adrenal

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Abstract

The capacity of the adrenal to produce cortisol is controlled in part by 21-hydroxylase (CYP21) and the production of androgens by 17-hydroxylase/17-20-lyase (CYP17), in response to secretagogues including ACTH, angiotensin-II (A-II) and insulin. In this study we examined the capacity of human adrenocortical cells to produce cortisol and androgens in response to these secretagogues and their ability to regulate the expression of CYP21 and CYP17. In H-295 cells, forskolin and A-II were found to stimulate production of cortisol relative to androstenedione and a similar pattern of steroid production was noted in primary human adrenocortical cells. Both mRNA and protein expression of CYP21 was upregulated with forskolin and A-II alone and in combination, as detected by Northern and Western blotting. Whereas expression of CYP17 mRNA and protein was up-

regulated in the presence of forskolin and forskolin in combination with insulin. The ability of steroidogenic factor-1 (SF-1) and nur77 to regulate transcription of these enzymes was examined. Forskolin, A-II and insulin increased the protein expression of SF-1. Increased binding of SF-1 to its response element in the presence of forskolin, A-II and insulin was observed. Nur77 was expressed primarily in the zona glomerulosa and fasciculata. Increased protein expression of nur77 and the greatest binding of nur77 to its response element was seen when cells were stimulated with A-II in combination with forskolin. These data indicate that nur77 may preferentially regulate steroid enzyme genes relevant to cortisol production and thereby regulate differential cortisol and adrenal androgen production.

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Introduction

Differential production of adrenal steroids is relative rather than absolute. Cells of the adrenal cortex are capable of producing a range of steroids, but the relative production of cortisol, androgens and aldosterone differs (Young *et al.* 2003). Steroid production is controlled in response to secretagogues both at the level of the substrate through the conversion of cholesterol to pregnenolone and at the level of gene transcription of the steroidogenic enzymes (Peter & Dubuis 2000). Specific production of adrenal steroids results from the selective modulation of the steroid hydroxylases that catalyse the terminal steps of steroid biosynthesis (Parker & Schimmer 1994). This is illustrated physiologically at the adrenarche and clinically in polycystic ovarian syndrome, where characteristic hyperandrogenaemia is associated with an induction of CYP17 (Barnes & Rosenfield 1989, McKenna & Cunningham 1995, Gell *et al.* 1998).

Classic secretagogues including adrenocorticotrophin (ACTH), angiotensin II (A-II) and more recently novel

factors such as insulin, upregulate steroid production through the regulation of key steroidogenic enzymes (Bird *et al.* 1993b, Qin & Rosenfield 1998). 21-hydroxylase (CYP21) regulates cortisol and aldosterone production by conversion of 17-hydroxyprogesterone to 11-deoxycortisol and progesterone to 11-deoxycorticosterone in the aldosterone pathway, whereas 17-hydroxylase/17-20-lyase (CYP17) is primarily concerned with androgen production through the conversion of pregnenolone to 17 α -hydroxypregnenolone and on to dehydroepiandrosterone and progesterone to 17 α -hydroxyprogesterone.

Orphan members of the nuclear receptor superfamily play an important role in mediating transcriptional regulation of several steroid hydroxylase genes. Common binding motifs described within steroidogenic regulatory elements suggest that a shared promoter interacts with a mutual transcription factor to regulate the expression of the hydroxylase enzymes in a co-ordinated manner (Rice *et al.* 1991). Sequences matching the steroidogenic factor-1 (SF-1) binding site have been found upstream of all hydroxylase genes, suggesting that SF-1 is a

global regulator of steroidogenic cytochrome P450 gene expression (Lala *et al.* 1992, Morohashi *et al.* 1992).

Nur77 (also known as NGFIB/N10/NAK1/TR3) is a member of the immediate early response gene family and closely resembles SF-1 (Zhang & Mellon 1997). Recently nur77 response elements have been identified on the promoter region of CYP21 and the transcription factor has been shown to be involved in the regulation of CYP21-mediated cortisol production in Y1 mouse adrenal cortical cells (Wilson *et al.* 1993, Chang & Chung 1995).

We hypothesise that differential production of cortisol and adrenal androgens is through the alternate transcriptional regulation of key steroidogenic enzymes. In this study we provide evidence that while ACTH, A-II and insulin all signal through SF-1 to regulate the expression of CYP21 and CYP17, ACTH and A-II can also signal through nur77 to regulate the gene transcription of CYP21 and thereby modulate cortisol production. Differential regulation of CYP21 and CYP17 may explain in part the divergence of cortisol and adrenal androgen production seen under physiological and pathophysiological conditions.

Materials and Methods

Preparation and stimulation of cell suspensions and H-295 cells

Twenty-three human adrenal glands, surgically removed at the time of resection for renal transplantation, were used in this study. Five adrenal glands were used for each experimental treatment. Adrenal glands were prepared as described previously (Clarke *et al.* 1996, Fearon *et al.* 1998). Excess fat and connective tissue were removed and the adrenal glands were diced. The tissue was incubated at 37 °C for 20 min in Eagle's Modified Essential Medium (EMEM) containing collagenase, type II (2 mg/ml). The tissue suspension was centrifuged at 400 *g* for 10 min and the supernatant discarded. The pellet was resuspended in 0.2% (w/v) BSA in EMEM and the cells passed through a nylon mesh (100 µm). The cell suspension was washed and centrifuged as before and resuspended in EMEM containing 0.5% BSA (w/v), 8 mM calcium and 1 mM ascorbic acid to a concentration of 10^6 cells/ml in the presence and absence of 10^{-7} M $\alpha 1-24$ ACTH, 10^{-7} M A-II or 10^{-7} M insulin. Cells were incubated for 2 h at 37 °C.

H-295 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in supplemented RPMI (5 µg/ml insulin, 10 µg/ml transferin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β -estradiol, 10 mM HEPES, 2 mM glutamine and 2% foetal calf serum (w/v)) grown in 75-cm² flasks at 37 °C under an atmosphere of 5% CO₂. Cells were serum- and steroid-depleted 24 h prior to treatment. Cells were incubated for either 2 or 24 h in the presence or absence of 10^{-7} M forskolin, 10^{-7} M

A-II, 10^{-7} M insulin, forskolin in combination with A-II or forskolin in combination with insulin. The supernatants and cells were removed and stored for steroid measurement and protein extraction respectively.

Steroid measurement

Cortisol and androstenedione were measured in human cell suspensions and supernatants from H-295 stimulated cells. Cortisol levels were measured using an 'in house' RIA, as previously described (Clarke *et al.* 1996). Cell lysates and supernatants were incubated with a specific anti-cortisol antibody (Guildhay, Guildford, Surrey, UK) and iodinated cortisol (Amersham) at 4 °C overnight. Bound and free fractions were separated with 1% dextran-gelatin coated charcoal in borate. Androstenedione was measured using commercially available RIA kits (Diagnostic Systems Laboratories, Webster, TX, USA). Androstenedione cross reactivity with cortisol < 0.04%.

Fixed H-295 cells

H-295 cells were seeded at 5×10^5 cells per well in six well culture plates (Costar, Acton, MA, USA) on adherent coverslips (Nunc, Naperville, IL, USA) in the presence and absence of 10^{-7} M forskolin for 2 h at 37 °C in 5% CO₂. At the end of the incubation period cells were fixed on coverslips in absolute alcohol for 10 min.

Immunohistochemistry and cytochemistry

An immunohistochemical technique was employed for the identification and localisation of CYP21, CYP17 and nur77 in human adrenal gland cryo-sections and H-295 cell cultures. Adrenal cryo-sections, 7 µm, were immunoblocked in serum for 90 min and incubated with a primary rabbit anti-porcine CYP17, 1:40; rabbit anti-ovine CYP21, 1:40 (Prof. J I Mason, Department of Clinical Biochemistry, Edinburgh, UK); goat anti-human nur77, 5 µg/ml (sc-7014, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at room temperature and subsequently with either anti-rabbit or anti-goat horseradish peroxidase secondary antibody, 1:200, for 1 h at room temperature. Sections were developed in 3,3'-diaminobenzidine tetrahydrochloride for 7 min and counter stained with Mayer's haematoxylin. Negative controls were performed either using blocking peptides to nur77 (sc-7014P, Santa Cruz Biotechnology Inc) or by omitting the primary antibody and no peroxidase staining was observed.

Fixed H-295 cells were immunoblocked in serum for 90 min and incubated with a primary goat anti-human nur77, 5 µg/ml (sc-7014, Santa Cruz Biotechnology Inc) for 1 h at room temperature and subsequently anti-goat horseradish peroxidase secondary antibody, 1:200, for 1 h at room temperature and developed as described above.

Immunofluorescence

H-295 cells were prepared as above and incubated in goat serum for 60 mins. Rabbit anti-human SF-1 (50 µg/ml in 10% human serum, PA1-800, Affinity Bioreagents, Golden, CO, USA) was placed on each slide for 90 mins. The sections were rinsed in PBS and incubated with the corresponding secondary fluorochrome conjugated antibody (1:100, TRITC labelled) for 60 min. Sections were rinsed in PBS and mounted (Dako, Glostrup, Denmark). Sections were examined under fluorescent light.

Northern analysis

Total RNA was isolated from stimulated H-295 cells using RNeasy extraction kit (Qiagen) at specific times after treatment. RNA was quantified by u.v. absorption. 10 µg of RNA was electrophoresed on a formaldehyde agarose gel and transferred to a nylon membrane (Bio-Rad) by pressure blotting overnight and was incubated for 3 h at 80 °C.

Prehybridisation was carried out at 42 °C for 3 h in a final buffer composition of 45% formamide, 4 × SSC, 0.1 M Na phosphate, 0.1% Na pyrophosphate, 0.1% SDS and 250 µg/ml Herring sperm. Hybridisations were performed in the same buffer at 42 °C overnight with anti-sense probes to CYP21 (Accession no. M12792, 16–324 b.p.) or CYP17 (Accession no. M14564, 48–349 b.p.) mRNA (Prof. W E Rainey, Department of Obstetrics and Gynecology, Dallas, TX, USA) using α -³²P-dCTP and a random primer-labelling system (Promega). The blots were then washed in 2 × SSC containing 0.5% SDS at room temperature for 10 mins and again at 42 °C, 55 °C for 10 mins before they were exposed to film at – 80 °C using intensifying screens. Results were assessed directly by Eagle Eye (Stratagene, La Jolla, CA, USA) image analysis and compared with levels of 28S RNA in the same lane.

Protein extraction and quantification

Protein was extracted from H-295 cells by incubation in lysis buffer (PBS, 1% Igepal and 0.5% deoxycholic acid) supplemented with PMSF 10 µg/ml and aprotinin 1.5 µg/ml for 30 mins. Protein was quantified using BCA assay kit (Pierce Chemicals Co., IL, USA). Nuclear protein extractions of H-295 were performed using NE-Per nuclear and cytoplasmic extraction kits (Pierce Chemicals, Rockford, IL, USA) according to manufacturer's instructions.

Western analysis

Western analysis was performed by SDS-PAGE on a discontinuous acrylamide gel. 30 µg of protein were electrophoresed through 4% stacking 12.5% resolving gel at 120 V for 2 h. Gels were transferred to nitrocellulose membrane at 250 mA for 1 h. Membranes were blocked

for 1 h at 37 °C in 5% non-fat dry milk and incubated overnight in either a primary rabbit anti-porcine CYP17, 1:500; rabbit anti-ovine CYP21, 1:1000; rabbit anti-human SF-1 (PA1-800, Affinity Bioreagents), 1:1000 and goat anti-human nur77 (sc-7014, Santa Cruz Biotechnology Inc), 1:500 and subsequently with either anti-rabbit, 1:1000 or anti-goat 1:1000 horseradish peroxidase secondary antibody (Santa Cruz Biotechnology Inc). Membranes were developed for 1 minute in luminol (Santa Cruz Biotechnology Inc), and autoradiographs were developed using standard photographic techniques.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein extracts were prepared as described above. For EMSA, 5 µg of nuclear extract was incubated for 20 min in the presence of 20 mM HEPES, pH 7.9, 5 mM MgCl₂, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 8% Ficoll, 600 mM KCl, 500 ng/µl poly dIdC (deoxyinosinic-deoxycytidylic) acid, 50 mM DTT and α -³²P-dCTP-labelled double-stranded oligonucleotide. For supershift experiments, antibodies to SF-1 (PA1-800, Affinity Bioreagents) or nur77 (sc-7014X, Santa Cruz Biotechnology Inc) were added following the initial 20 min incubation, then incubated for a further 20 min. The samples were electrophoresed through a 5.5% nondenaturing polyacrylamide gel in 0.5 × Tris Borate-EDTA buffer. For competition studies, the reaction was performed as described with 50 × molar excess of unlabelled probe. The following oligonucleotides were designed to incorporate the native human CYP21 (for nur77 binding) and CYP17 (for SF-1 binding) promoter sequences: SF-1 (CYP17, NM_000102) 5'-TTGCAAGCCTTGACTCCTGAG CCCAGATACCA-3', nur77 (CYP21, NM_000500) 5'-CTGGATGCAGGAAAAAGGTCAGGGTTGCACTT-3'. The consensus sequence for the SF-1 and nur77 binding elements is underlined (Wilson *et al.* 1991, Hanley *et al.* 2001).

Chemicals

All chemicals were obtained from Sigma unless otherwise specified.

Statistics

Statistics were performed using the Wilcoxon Signed Rank test for non-parametric data, with significance accepted at the 5% level.

Results

Functional analysis of human adrenocortical cells and H-295 cell line

The ability of primary human adrenal cortical cells and the ability of adrenocortical tumour cell line, H-295, to

Figure 1 (A) Ratio of cortisol to androstenedione production and androstenedione to cortisol production in human adrenocortical cells in the presence (+) and absence (–) of 10^{-7} M ACTH, 10^{-7} M A-II and 10^{-7} M insulin. Mean $\pm$ S.E.M., $n=5$ adrenals for each treatment group. (B) Ratio of cortisol to androstenedione production and androstenedione to cortisol production in H-295 cell line in the presence (+) and absence (–) of 10^{-7} M forskolin, 10^{-7} M A-II and 10^{-7} M insulin. Mean $\pm$ S.E.M., $n=10$ individual experiments; *significantly different from control cells without addition of secretagogues, $P<0.05$.

differentially produce cortisol and androstenedione in response to the secretagogues ACTH, A-II and insulin were examined. Human adrenocortical cells were found to produce both cortisol and androstenedione. The production of these steroids differed following stimulation with ACTH compared with insulin. ACTH produced more cortisol relative to androstenedione compared with control unstimulated cells, whereas insulin produced more androstenedione relative to cortisol when compared with control, though these differences did not reach statistical significance (Fig. 1A). There was no alteration in steroid production in primary adrenocortical cells treated with A-II compared with control. H-295 cells were used as a

model to examine the response of adrenocortical cells to these secretagogues. Forskolin, the adenylate cyclase activating agent, was used as a functional analogue of ACTH as the H-295 cell line does not express a functional ACTH receptor. A-II was used to activate the protein kinase C (PKC) pathway. Forskolin and A-II were found to produce more cortisol relative to androstenedione compared with control cells (Fig. 1B) ($P<0.05$). Forskolin in combination with A-II also significantly increased the production of cortisol relative to androstenedione ($P<0.05$). There was, however, no alteration in steroid production in cells treated with both insulin and forskolin compared with cells treated with forskolin alone.

Figure 2 Photomicrographs showing immunohistochemical stain for (A) CYP21, (B) CYP17 in human adrenocortical cryosections counterstained with haematoxylin (left) and matched negative controls (right). Positive cells are indicated by a brown stain. G, glomerulosa; F, fasciculata; R, reticularis. Original magnification $\times 200$.

Conversely, insulin alone did not alter steroid production in H-295 cells.

Immunolocalisation of hydroxysteroid enzymes

Differential production of adrenal steroids is through the specific regulation of key steroidogenic enzymes. The traditional functional and histological characterisation of the human adrenal cortex was correlated with the expression of key steroidogenic enzymes concerned with the production of cortisol and androgens. CYP21 was localised in the zona fasciculata, zona reticularis and to a lesser extent the zona glomerulosa (Fig. 2A). CYP17 was localised to the zona fasciculata and zona reticularis, and was found to be absent from the zona glomerulosa (Fig. 2B). Both of these steroid enzymes were expressed within the cytosol of adrenocortical cells.

mRNA expression of hydroxysteroid enzymes

The ability of forskolin, A-II and insulin to regulate CYP21 mRNA following 16 h incubation was examined.

Figure 3 Representative Northern blot of (A) CYP21 and (B) CYP17 mRNA expression following stimulation in the H-295 cells following stimulation with 10^{-7} M forskolin, 10^{-7} M A-II and 10^{-7} M insulin. The presence and integrity of the 28S major RNA component for each lane was examined and found to be similar. Optical density readings were obtained. Control values were normalised to one and experimental groups were expressed as a ratio. Values are expressed as mean $\pm$ S.E.M. ($n=3$).

The action of forskolin and A-II alone and in combination resulted in an increase in CYP21 mRNA expression. No increase in expression was observed in the presence of insulin, whereas insulin in combination with forskolin was seen to inhibit the stimulatory effect of forskolin (Fig. 3A). The expression of CYP17 mRNA following 20 h incubation was examined. CYP17 mRNA was increased in

Figure 4 Representative protein immunoblot of (A) CYP21, (B) CYP17 total protein expression in H-295 cells following 24 h stimulation with 10^{-7} M forskolin, 10^{-7} M A-II and 10^{-7} M insulin. β -actin represents equal loading of samples. Optical density readings were obtained. Control values were normalised to one and experimental groups were expressed as a ratio. Values are expressed as mean $\pm$ S.E.M. ($n=3$).

response to forskolin and insulin. There was no alteration observed in the presence of A-II (Fig. 3B).

Protein expression of hydroxysteroid enzymes

The ability of the secretagogues to regulate protein expression of CYP21 and CYP17 was examined. H-295 cells were incubated in the presence or absence of forskolin, A-II and insulin. Increases in protein expression of CYP21 were seen in the presence of forskolin, A-II and forskolin in combination with A-II (Fig. 4A). Protein expression of CYP17 was increased in the

presence of forskolin and insulin alone, and in combination (Fig. 4B).

Regulation of SF-1 and nur77 in the adrenal cortex

To examine whether forskolin, A-II and insulin mediate their effects through SF-1 and nur77, protein expression of these transcription factors in adrenocortical cells was assessed. SF-1 was found to be expressed predominantly within the nucleus of H-295 cells. Following treatment with forskolin an increase in nuclear expression of SF-1 was observed (Fig. 5A). Forskolin, A-II and insulin all induced an increase in the protein expression of SF-1 (Fig. 5B).

Nur77 was localised within the human adrenal cortex and the protein expression following stimulation examined. Nur77 was expressed predominantly within the nuclei of cells of the zona fasciculata and zona glomerulosa (Fig. 6A). Immunocytochemical studies of nur77 expression in H-295 cells demonstrated an increase in the nuclear expression of nur77 following 2 h incubation with forskolin (Fig. 6B). Protein expression of nur77 in H-295 cells was found to be low as determined by Western blotting, with an increase in expression observed when cells were incubated with all secretagogues tested, in particular forskolin in combination with A-II (Fig. 6C).

Activation of SF-1 and nur77

A consensus-binding site for the nuclear receptor SF-1 has been located within the proximal promoter region of the steroid hydroxylase enzymes (Parker & Schimmer 1997). Because of the role of ACTH, A-II and insulin in inducing the expression of these genes and the ability of these secretagogues to increase the protein expression of SF-1, a role for ACTH, A-II and insulin in signalling through SF-1 was sought. Using published oligonucleotide sequences (Wilson *et al.* 1991, Hanley *et al.* 2001), the ability of nuclear extracts from non-stimulated H-295 cells to bind to the DNA-binding motif compared with cells stimulated for 2 h with forskolin, A-II and insulin was examined. Binding was specific as demonstrated by successful competition with excess homologous oligonucleotide. A super shift induced by pre-incubation of the nuclear extracts with anti-SF-1 established that SF-1 was present in the protein-DNA complex. The protein-DNA complex was differentially inducible with forskolin, and to a lesser extent insulin and A-II (Fig. 7A).

The classic consensus-binding site for nur77 (NBRE) has previously been described on the promoter region of CYP21 (Wilson *et al.* 1993, Chang & Chung 1995). Since forskolin and A-II can induce the protein expression of CYP21 the ability of these secretagogues to signal through nur77 was examined. Specific binding was demonstrated as above. Pre-incubation of the nuclear extracts with an antibody directed against nur77 induced a shift in the

Figure 5 (A) Immunofluorescence stain for SF-1 in H-295 adrenocortical cells. Original magnification $\times 200$ (B) Representative protein immunoblot of SF-1 protein expression in H-295, following 6 h stimulation with forskolin 10^{-7} M, A-II 10^{-7} M and insulin 10^{-7} M. Results are representative of those obtained in three separate experiments. β -actin represents equal loading of samples.

protein–DNA complex, verifying that nur77 was present in the complex. Increased binding was observed in the protein–DNA complex with all secretagogues, in particular forskolin in combination with A-II (Fig. 7B).

Discussion

Differential production of adrenal steroids has been thought to be achieved at least in part through cell specific production of individual steroids (Parker & Schimmer 1994, Zhang & Mellon 1997). However, we have recently reported that all adrenocortical cells are capable of producing a range of steroids, though the relative production of cortisol, androgens and aldosterone differs (Young *et al.* 2003). The relative production of adrenal steroids is achieved through the differential expression of key steroidogenic enzymes, under the influence of secretagogues (Hu *et al.* 2001). ACTH is the classic secretagogue for both cortisol and adrenal androgens, whereas A-II, though primarily concerned with the production of aldosterone, has also been shown to upregulate

cortisol production (Bird *et al.* 1993a, Rainey *et al.* 1993). Both clinical and *in vitro* studies have implicated insulin in the regulation of adrenal androgen production (Kristiansen *et al.* 1997, Qin & Rosenfield 1998). In this study, we found that H-295 cells produced significantly more cortisol relative to androstenedione in the presence of forskolin and forskolin in combination with A-II. These patterns of steroid production were reflected in human adrenocortical cell suspensions though differences did not reach statistical significance.

The relative production of cortisol and adrenal androgens is a function of the differential expression of CYP21 and 3β -hydroxysteroid dehydrogenase (3β HSD) to CYP17. Where ACTH upregulates all steroid enzymes, A-II signalling via the PKC pathway positively regulates both CYP21 and 3β HSD, and negatively regulates CYP17 (Bird *et al.* 1998). In this study, secretion of cortisol and the adrenal androgen androstenedione was examined, and as 3β HSD is involved in the production of androstenedione and cortisol, the relative expression of CYP21 to CYP17 was examined. The spatial localisation of these enzymes was determined in the adult human

Figure 6 (A) Photomicrograph of immunohistochemical stain for nur77 in human adrenal cryosections counterstained with haematoxylin (left) and matched negative controls (right). Brown indicates positive stain, F, fasciculata; G, glomerulosa; C, capsule. (Original magnification $\times 400$). (B) Immunocytochemical stain for nur77 in unstimulated H-295 cells (left) and following 2 h stimulation with forskolin 10^{-7} M (right). (C) Representative protein immunoblot of nur77 total protein expression in H-295 cells. Results are representative of those obtained in three separate experiments. β -actin represents equal loading of samples.

adrenal cortex. CYP21 and CYP17 were both localised to the cytoplasm of zona fasciculata and zona reticularis cells. Low expression of CYP21 was also detected within the zona glomerulosa whereas CYP17 was found to be

expressed exclusively in the cytoplasm of cells in the zona fasciculata and reticularis. These classic patterns of steroid enzyme expression are consistent with those described by others (Bird *et al.* 1996, Morley *et al.* 1996).

In this study ACTH, A-II and insulin were found to differentially regulate cortisol and adrenal androgen production. Increases in CYP21 at the mRNA level have been described in the presence of both ACTH and A-II (Bird *et al.* 1998), though little has been shown regarding alterations in protein expression. Here we found increased CYP21 mRNA and protein with forskolin and A-II individually and forskolin in combination with A-II. The role of insulin in the regulation of CYP21 expression remains controversial (Klein *et al.* 1992, Endoh *et al.* 1998). We observed no alteration in the expression of CYP21 at either the mRNA or protein level in the presence of insulin, moreover insulin appeared to abrogate increases in CYP21 protein seen in the presence of forskolin. Conversely, forskolin and insulin have been implicated in the upregulation of CYP17 (Bird *et al.* 1993b, Qin & Rosenfield 1998), whereas the role of A-II remains to be fully elucidated (Lebrethon *et al.* 1994, Parker & Schimmer 1994, Viard *et al.* 1994). In this study CYP17 mRNA and protein expression were found to be upregulated in the presence of forskolin and insulin. These data implicate ACTH, A-II and insulin in the differential regulation of cortisol and adrenal androgens through their relative modulation of CYP21 and CYP17 expression.

SF-1 is an important regulator of the expression of cytochrome P450 steroid hydroxylase enzymes in humans. This shared regulatory protein can interact with a number of variations of an AGGTCA motif on the promoter region of the steroid enzymes (Rice *et al.* 1991, Morohashi *et al.* 1992). Using *in situ* hybridisation the expression of SF-1 has been demonstrated throughout the adrenal cortex (Ikeda *et al.* 1993), correlating with its proposed role in regulating steroid hydroxylases. Hormonal regulation of SF-1 remains controversial; it has been suggested that SF-1 transcripts are not altered in response to treatment with secretagogues (Parker & Schimmer 1997), whereas others have shown increases in SF-1 mRNA in the presence of both forskolin and A-II (Enyeart *et al.* 1996). In this study protein expression of SF-1 was found to be expressed at a basal level and was elevated in the presence of all secretagogues tested.

C-terminal regions of mammalian SF-1 are highly conserved within their putative ligand-binding domains, which is consistent with the theory that SF-1 is activated by a ligand. A specific SF-1 ligand remains elusive, although hydroxycholesterol has been suggested (Janowski *et al.* 1996). Alternatively regulation of SF-1 activity may be indirect through specific upstream events or may be activated through post-translational modifications. A conserved phosphorylation motif for cAMP-dependent protein kinase provides a candidate at which a ligand-independent modulation of activity may be affected.

Figure 7 Electrophoretic mobility shift analysis of nuclear extracts from H-295 cells. Nuclear protein extracts from H-295 cells in the presence and absence of forskolin 10^{-7} M, A-II 10^{-7} M and insulin 10^{-7} M were compared for increased binding to (A) α^{32} P-dCTP-labelled SF-1 response element and (B) α^{32} P-dCTP-labelled nur77 response element (NBRE). DNA protein interactions were assayed in the presence of $\times 50$ molar excess of homologous oligonucleotide (every second lane). Nuclear protein extracts were pre-incubated in the presence of anti-SF-1 or anti-nur77. Results are representative of those obtained in three separate experiments.

In these studies the adenylate cyclase activator forskolin, and to a lesser extent insulin and A-II, induced SF-1 binding to its response element in H-295 adrenocortical cells.

Other transcription factors, which are expressed in the adrenal cortex, include the nurr family of orphan nuclear receptors. The role of the family in adrenal cortical steroidogenesis is gathering interest. Recent studies have demonstrated a role for nurr1 and nur77 in the transcriptional regulation of CYP11B2 (encoding aldosterone synthase) implicating nurr in the regulation of aldosterone production (Bassett *et al.* 2004). Binding regions for the orphan nuclear receptor nur77 have been identified on the promoter region of CYP21 (Wilson *et al.* 1993, Chang & Chung 1995). Regulation of this nuclear receptor has been observed in the presence of ACTH and A-II in both murine and bovine adrenocortical cells (Enyeart *et al.*

1996, Li & Lau 1997). In this study nur77 was found to be expressed predominantly within the nuclei of cells of the human zona glomerulosa and fasciculata. In contrast to the response of SF-1 observed in the presence of secretagogues, a particular increase in nur77 protein expression was detected in response to forskolin in combination with A-II. Similarly, the greatest binding of nur77 to its response element was observed in the presence of forskolin in combination with A-II, thus implicating these secretagogues in the regulation of nur77. Therefore, differences between SF-1 and nur77 in spatial localisation within the adrenal cortex, expression and DNA interaction in response to secretagogues suggest that these transcription factors may differentially modulate the transcription of genes in the human adrenal cortex.

These data provide evidence that secretagogues can signal through nur77 to regulate the production of

key steroidogenic enzymes. We propose that SF-1 is responsible for regulating all hydroxylase enzymes under basal conditions and in the presence of secretagogues. Stimulation through the adenylate cyclase and the PKC pathways however also leads to the induction of nur77, resulting in a further activation of CYP21. Therefore in the human adrenal, ACTH and A-II may preferentially stimulate cortical production by signalling through nur77.

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