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The Orphan Nuclear Receptors NURR1 and NGFIB Regulate Adrenal Aldosterone Production

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Aldosterone biosynthesis in the zona glomerulosa of the adrenal cortex is regulated by transcription of *CYP11B2* (encoding aldosterone synthase). The effects of nerve growth factor-induced clone B (NGFIB) (NR4A1), Nur-related factor 1 (NURR1) (NR4A2), and steroidogenic factor-1 (SF-1) (NR5A1) on transcription of human *CYP11B2* (*hCYP11B2*) and *hCYP11B1* (11β-hydroxylase) were compared in human H295R adrenocortical cells. *hCYP11B2* expression was increased by NGFIB and NURR1. Although *hCYP11B1* was activated by SF-1, cotransfection with SF-1 inhibited activation of *hCYP11B2* by NGFIB and NURR1. NGFIB and NURR1 transcript and protein levels were strongly induced by angiotensin (Ang) II, the major regulator of *hCYP11B2* expression *in vivo*. Sequential deletion and mutagenesis of the *hCYP11B2* promoter identified two functional NGFIB response elements (NBREs), one located at −766/−759 (NBRE-1) and the previously studied Ad5 element at −129/−114. EMSAs suggested that both elements bound NGFIB and NURR1. In human adrenals, NURR1 immunoreactivity was preferentially localized in the zona glomerulosa and to a lesser degree in the zona fasciculata, whereas NGFIB was detected in both zones. The calmodulin kinase inhibitor KN93 partially blocked K^+-stimulated transcription of NGFIB and NURR1. KN93 partially inhibited the effect of Ang II on NURR1 mRNA levels but did not modify the effect on expression of NGFIB. Mutation of the NBRE-1, Ad5, and Ad1/cAMP response element (CRE) *cis*-elements reduced both basal and Ang II-induced levels of *hCYP11B2*, demonstrating that all three elements are important for maximal transcriptional activity. Our results suggest that NGFIB and NURR1 are key regulators of *hCYP11B2* expression and may partially mediate the regulation of *hCYP11B2* by Ang II. (*Molecular Endocrinology* 18: 279–290, 2004)

THE ADRENAL CORTEX is the primary site of mineralocorticoid and glucocorticoid biosynthesis. In humans, the synthesis of aldosterone, the major mineralocorticoid, relies on CYP11B2 (aldosterone synthase), a steroid-metabolizing cytochrome P450 (CYP). This enzyme converts deoxycorticosterone to aldosterone (1, 2). It is expressed only within the adrenal zona glomerulosa and is under the control of circulating levels of angiotensin (Ang) II and potassium (3–5). In contrast, CYP11B1 (11β-hydroxylase), which converts deoxycortisol to cortisol (1, 2), is regulated by ACTH and is expressed predominantly in the adrenal zona fasciculata (3, 6). Deoxycorticosterone is also synthesized in the fasciculata at levels that would lead to mineralocorticoid excess if it were converted to aldosterone. Thus, the ability of the adrenal cortex to control aldosterone production is the result of limiting the expression of human *CYP11B2* (*hCYP11B2*) to the zona glomerulosa. The zonal distribution of *hCYP11B2* is presumably due to the zone-specific expression of enhancer and/or repressor proteins that interact with specific elements in the promoter of this gene.

The *trans*-acting factors that regulate *hCYP11B2* expression remain poorly defined. The orphan nuclear receptor, steroidogenic factor-1 (SF-1), is a major regulator of other steroid hydroxylase genes including *hCYP11B1* (7, 8), but it fails to stimulate and indeed represses expression of *hCYP11B2* (9). Other transcription factors that are expressed in the adrenal cortex include the NGFIB family of orphan nuclear receptors (termed the NR4A subgroup for nuclear receptor subgroup 4) (10). This family includes NGFIB (nerve growth factor-induced clone B, also termed NR4A1), NURR1 (Nur-related factor 1, NR4A2) and neuron-derived orphan receptor 1 (NR4A3) (11). All three nuclear receptors are rapidly induced early response genes that enhance transcription by binding to a consensus sequence (AAAGGTCA) called a NGFIB response element (NBRE) (12, 13). Among steroidogenic genes, the human and mouse *CYP21* gene promoters contain

Abbreviations: Ad, Adrenal; Ang, angiotensin; ATF, activating transcription factor; CaM, calmodulin; CaMK, CaM-dependent protein kinase; CRE, cAMP response element; CREB, CRE binding protein; CYP, cytochrome P450; *hCYP11B2*, human *CYP11B2*; NGFIB, nerve growth factor-induced clone B; NBRE, NGFIB response element; NR, nuclear receptor; NURR1, Nur-related factor 1; SF-1, steroidogenic factor-1.

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canonical NBREs and a role for NGFIB in *CYP21* transcription has been proposed (14, 15).

In the current study, we examined the role of two NGFIB family members, NGFIB and NURR1, in the regulation of *hCYP11B2*. Our results show that NGFIB and NURR1 activate the *hCYP11B2* promoter. Both transcription factors are up-regulated by Ang II, the primary regulator of *hCYP11B2* expression *in vivo*. Both are expressed in the zona glomerulosa, the site of *hCYP11B2* expression. Finally, whereas NGFIB and NURR1 are potent activators of *hCYP11B2* expression, they do not stimulate transcription of *hCYP11B1*. Thus, NGFIB and/or NURR1 may contribute to the localized expression of *hCYP11B2* within the zona glomerulosa as well as agonist-regulated expression.

RESULTS

NGFIB and NURR1 Activate the Transcription of *hCYP11B2*

To determine whether NGFIB or NURR1 might contribute to the regulated expression of the *hCYP11B2* gene, we cotransfected H295R cells with a reporter construct containing 5′-flanking DNA from *hCYP11B2* and with increasing concentrations of expression vectors encoding NGFIB, NURR1, or SF-1 (Fig. 1A). The *hCYP11B2* promoter was activated by NGFIB and NURR1 in a concentration-dependent manner with activity increasing to 3.2- and 3.9-fold above basal levels, respectively, when cells were cotransfected with 1 μg/ml of expression plasmid. As expected from our prior study (9), SF-1 failed to activate the *hCYP11B2* promoter at any of the concentrations tested. In fact, 1 μg/ml of SF-1 reduced *hCYP11B2* reporter expression to 72% of basal levels.

In contrast to the results obtained with *hCYP11B2*, the *hCYP11B1* reporter construct was not activated by NGFIB or NURR1 but was strongly stimulated by SF-1 (Fig. 1B).

SF-1 Inhibits NGFIB- and NURR1-Stimulated Activation of *hCYP11B2*

Because SF-1 is a known repressor of *hCYP11B2*, we next demonstrated that SF-1 inhibited NGFIB and NURR1 activation of the *hCYP11B2* promoter in a concentration-dependent manner (Fig. 2A). When cotransfected in equal amounts (1 μg/ml), SF-1 reduced the activity achieved by NGFIB or NURR1 alone to that observed with the basal *hCYP11B2* reporter construct. As expected, cotransfection of H295R cells with SF-1 reduced both basal and Ang II-stimulated *hCYP11B2* reporter activity (Fig. 2B).

NGFIB and NURR1 Transcripts and Protein Are Induced by Ang II in H295R Adrenal Cells

Ang II and potassium are the primary regulators of *hCYP11B2* expression *in vivo* and in the H295R adrenal cell model (4, 5). To examine the effects of Ang II treatment on NGFIB and NURR1 expression, H295R cells were incubated in the presence or absence of Ang II (10 nM) for 6 and 24 h followed by analysis of NGFIB and NURR1 transcript levels on Northern blots (Fig. 3A). Low levels of NGFIB transcripts were present in untreated samples at 6 and 24 h, but NURR1 transcripts were not detectable in untreated samples.

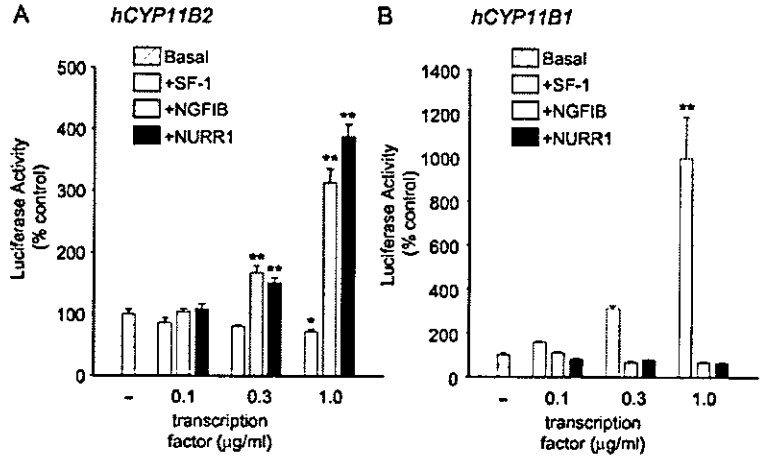

Fig. 1. Comparison of the Effects of NGFIB, Nurr1, or SF-1 on the Transcriptional Activity of *hCYP11B2* (A) or *hCYP11B1* (B) Reporter Gene Activity

H295R adrenocortical cells were transfected with luciferase reporter constructs containing *hCYP11B1* (pB1-1102) or *hCYP11B2* (pB2-1521) reporter constructs (1 μg/ml). Cells were cotransfected with either empty pRc/RSV expression vector or the indicated amounts of NGFIB, NURR1, or SF-1 expression plasmid along with a β-galactosidase expression vector (0.05 μg/ml). After recovery for 24 h, cells were lysed and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of pB1-1102 or pB2-1521. The results in each panel represent the mean ± SEM of data from three independent experiments, each one done in triplicate (**, $P < 0.0001$; *, $P = 0.0260$ compared with basal level).

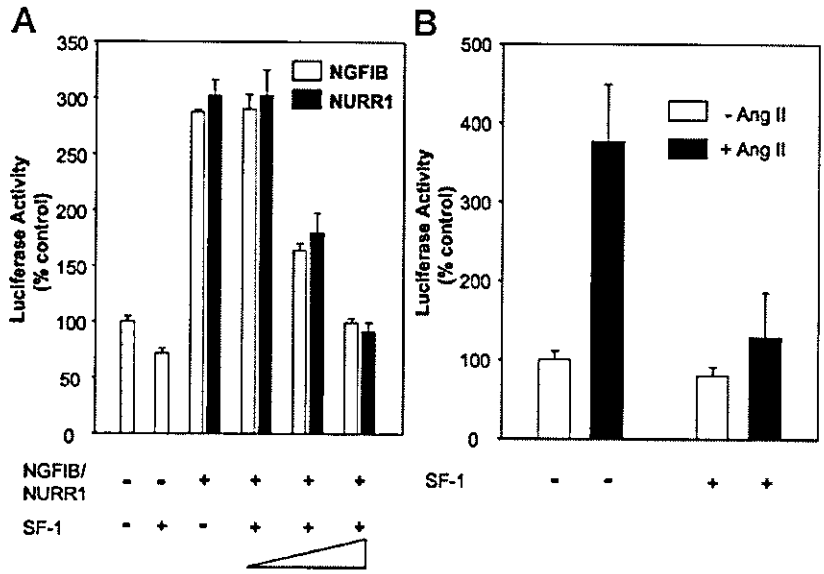

Fig. 2. SF-1 Blocks Ang II, NURR1, and NGFIB Stimulation of *hCYP11B2* Transcription

A, Inhibition of NGFIB- and NURR1-stimulated *hCYP11B2* reporter activity by SF-1. H295R adrenocortical cells were cotransfected with pB2-1521 5′-flanking DNA (1 μg/ml), NGFIB, or NURR1 (1 μg/ml) and increasing amounts of SF-1 (0.1, 0.3, 1.0 μg/ml) along with a β-galactosidase expression vector (0.05 μg/ml). After recovery for 24 h, cells were lysed and assayed for luciferase and β-galactosidase activity. B, Effect of SF-1 on Ang II-stimulated *hCYP11B2* reporter gene activity. H295R cells were cotransfected with pB2-1521 and SF-1 (1 μg each/ml) along with a β-galactosidase expression vector (0.05 μg/ml). After recovery, cells were treated (+) or untreated (−) with Ang II (10 nM) for 6 h, then assayed as above. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of the pB2-1521 construct. The results in both panels represent the mean ± SEM of data from three independent experiments each performed in triplicate.

Both NGFIB and NURR1 expression levels were high after 6 h of incubation with Ang II, but they returned to control levels by 24 h of treatment. Western analysis using cell lysates from similarly treated H295R cells confirmed that immunoreactive NGFIB and NURR1 proteins increased in parallel with transcript levels (Fig. 3B). These data demonstrate that NGFIB and NURR1 are Ang II target genes.

Identification of NBREs by Deletion and Mutation Analyses

To identify potential NBREs within the *hCYP11B2* gene promoter, a series of deletion constructs were used containing progressively shorter fragments of *hCYP11B2* 5′-flanking DNA (pB2-1521, pB2-864, pB2-747, pB2-135 pB2-106, and pB2-65). Deoxyribonuclease I footprint analysis of the bovine CYP11B promoter had previously identified six *cis*-elements, termed Ad1 through Ad6 (Adrenal 1–6), which bound nuclear proteins (16–18); similar sequences have been identified in the *hCYP11B2* gene (19). These deletion constructs were transiently transfected into H295R cells along with empty pRc/RSV expression plasmid (basal) or pRc/RSV expression plasmid containing the coding sequence of NGFIB or NURR1 (Fig. 4). Both NGFIB and NURR1 stimulated the reporter activity of pB2-1521 and pB2-864 approximately 4-fold over basal levels. Further deletion to −747 bp decreased NGFIB and NURR1 stimulation to twice basal levels suggesting that DNA between −864 and −747 contained an NBRE. Further deletion to −135 bp, which eliminated an SF-1 binding site (Ad4), did not significantly affect NGFIB or NURR1 stimulation of *hCYP11B2* promoter activity. However, deletion to −106 bp abolished NGFIB and NURR1 stimulation of *hCYP11B2* reporter activity indicating that nucleotides between −135 and −106 contained an additional NBRE.

Examination of the sequence of the *hCYP11B2* 5′ flanking region indeed revealed two potential NBREs, one at −766/−759 (NBRE-1) and the other, the previously identified Ad5 site (19), at −129/−114 on the noncoding strand (Fig. 5A). To determine the relative importance of the NBRE-1 (−766/−759) and Ad5 (−129/−114) *cis*-elements, both were mutated in the context of the full-length (pB2-1521) *hCYP11B2* gene promoter. As shown in Fig. 5B, mutation of the NBRE-1 *cis*-element reduced both NGFIB- and NURR1-stimulated *hCYP11B2* activity by 52% and 55%, respectively, compared with basal level. Mutation of the Ad5 site further reduced both NGFIB- and NURR1-stimulated *hCYP11B2* reporter activities. Simultaneous mutations of both *cis*-elements reduced NGFIB- and NURR1-stimulated reporter activity to that observed with the pGL3Basic empty vector.

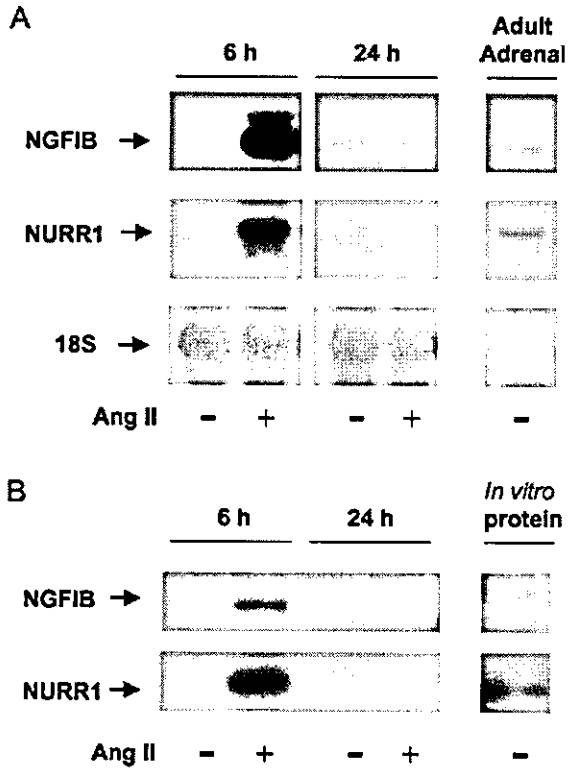

Fig. 3. Induction of NGFIB and NURR1 Transcript and Protein Levels by Ang II

A, Northern analysis of NGFIB and NURR1 mRNA in H295R cells and adult adrenal gland. Total RNA was prepared from H295R cells treated (+) with Ang II (10 nM) for the indicated times (6 and 24 h) or left untreated (−). Total RNA was also isolated from adult adrenal gland. After electrophoresis and transfer to nylon membrane, the RNA samples on the blot were probed sequentially with a cDNA for NURR1, NGFIB, and then a 18S ribosomal probe to control for loading and RNA transfer. B, Western analysis of NGFIB and NURR1 protein in H295R nuclear extracts. Nuclear extracts were prepared from H295R cells treated with Ang II as described above. Four micrograms of protein were electrophoresed and immunoblotted with antibodies specific for NGFIB or NURR1. *In vitro* prepared NGFIB or NURR1 protein was included on the blot as positive control.

Specific Binding of NGFIB and NURR1 to the NBRE-1 (−766/−759) and Ad5 (−129/−114) *cis*-Elements

To determine whether NGFIB and/or NURR1 interact directly with the NBRE-1 (−766/−759) and/or Ad5 (−129/−114) *cis*-elements, ^{32}P-labeled oligonucleotides containing these elements were prepared and used in EMSAs. Both elements bound *in vitro* synthesized NGFIB, NURR1, and SF-1 protein (Fig. 6). When H295R nuclear extract was incubated with the NBRE-1 probe (panel 1), protein/DNA complex C1 comigrated with the complex formed by *in vitro*-translated NGFIB or NURR1, whereas C2 migrated with the complex formed by SF-1. With the Ad5 probe, (panel 2), complex C3 did not comigrate with any of the *in vitro*-translated proteins whereas C4 migrated with the complex formed by SF-1. No binding of the Ad5 probe to proteins in the H295R nuclear extract corresponding to NGFIB or NURR1 was observed. Formation of C1–C4 was specifically inhibited by a 100-fold excess of unlabeled homologous oligonucleotide, indicating that all four complexes represented specific binding.

EMSA was also performed with mutated NBRE-1 and Ad5 oligonucleotides. The NBRE-1 and Ad5 mutant probes did not bind *in vitro*-prepared SF-1, NGFIB, or NURR1 (data not shown). Additionally, at least 10-fold more mutant NBRE-1 and Ad5 oligonucleotides were needed to displace H295R nuclear extract binding to their respective wild-type sequences.

Localization of NGFIB and NURR1 in Human Adrenal Gland

A previous study localized NURR1 to the mouse zona glomerulosa (20). To determine whether NGFIB or NURR1 might contribute to the zone-specific expression of *hCYP11B2*, we examined the expression of these transcription factors in human adrenal sections using immunohistochemistry (Fig. 7). NGFIB immunoreactivity was detected in nuclei of both glomerulosa and fasciculata cells. NURR1 immunoreactivity was highest in nuclei of glomerulosa cells with modest expression observed in the fasciculata. Neither NGFIB nor NURR1 immunoreactivity was detected in cells of the adrenal capsule or in inner medullary cells. No staining was observed in the absence of NGFIB or NURR1 antibody. These data support a potential role for NURR1 and/or NGFIB in glomerulosa-specific expression of *hCYP11B2*.

Effects of the Calmodulin (CaM) Kinase Inhibitor KN93 on Agonist-Stimulated Transcription of *hCYP11B2*, NGFIB, or NURR1

Ang II and K^+ use intracellular calcium signaling which often occurs through the action of the calcium binding protein, CaM. Of the various CaM-regulated kinases described to date, it is likely that CaM kinase I and/or IV are involved in Ang II and K^+ induction of aldosterone production (21). To determine whether calcium signaling plays a role in the transcriptional activation of NGFIB and/or NURR1, we examined the effects of the CaM kinase inhibitor, KN93, on agonist-stimulated transcription of *hCYP11B2*, NGFIB, and NURR1. KN93 completely blocked K^+ induction of *hCYP11B2* (Fig. 8A) and partially inhibited NGFIB and NURR1 expression (Fig. 8, B and C). KN93 also partially inhibited the effect of Ang II on *hCYP11B2* and NURR1 mRNA levels but had no effect on transcription of NGFIB. These data support the hypothesis that NURR1 may play a more important role than NGFIB in Ang II-regulated *hCYP11B2* expression.

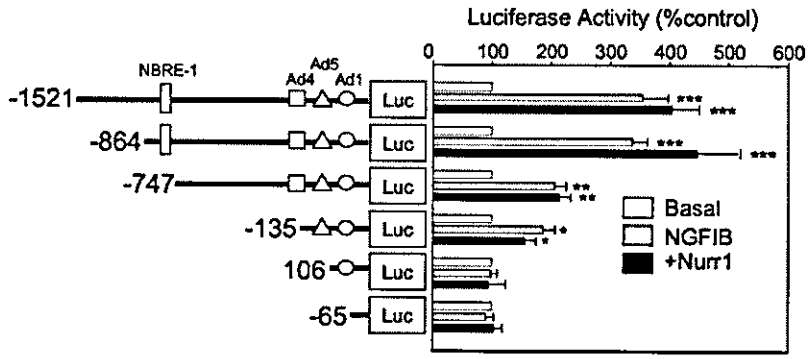

Fig. 4. Deletion Analysis of the *hCYP11B2* 5′-Flanking DNA to Identify Putative NBREs

H295R cells were transiently transfected with luciferase reporter constructs containing serial deletions of *hCYP11B2* 5′-flanking DNA. Transfection of reporter constructs was done with empty pRc/RSV expression vector (1 μg/ml) or expression vector containing the coding sequence for NGFIB or NURR1 (1 μg/ml) along with a β-galactosidase expression vector (0.05 μg/ml). After recovery for 24 h, cells were lysed and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of each *hCYP11B2* reporter construct. Results represent the mean ± SEM of data from a minimum of three independent experiments each performed in triplicate (***, $P < 0.0001$; **, $P = 0.0372$; *, $P = 0.0390$ compared with basal level).

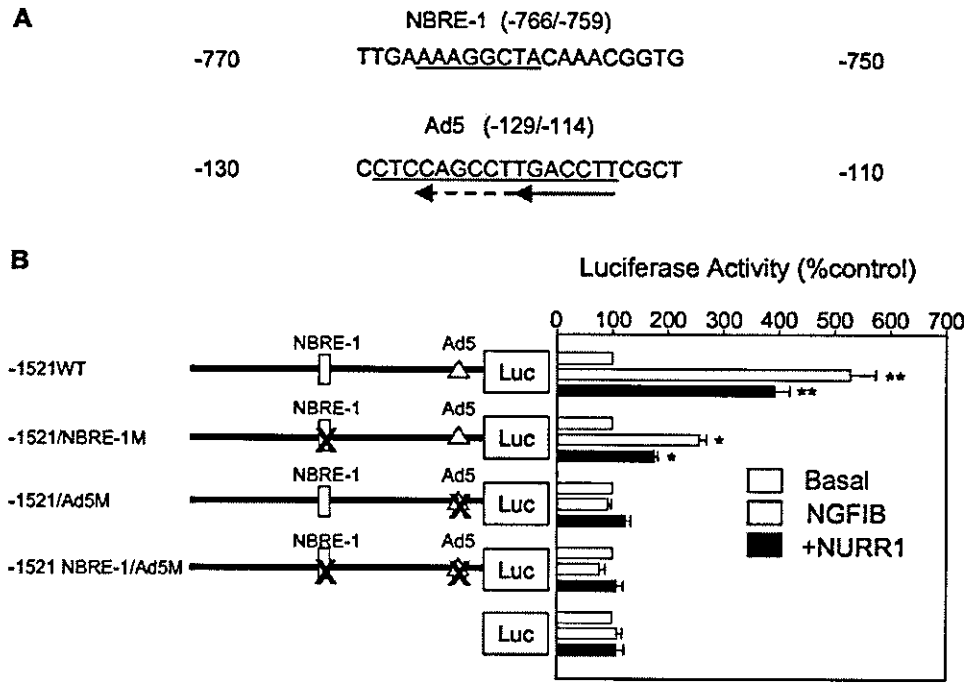

Fig. 5. Characterization of NBREs Present within the *hCYP11B2* Gene Promoter

A, Nucleotide sequence of *cis*-elements, NBRE-1(−766/−759) and Ad5(−129/−114), two putative NBREs. Bases are numbered relative to the *hCYP11B2* transcriptional start site. *Arrows* denote the variant (*dashed*) and near-consensus (*solid*) NBREs present at −129/−114 (Ad5) on the noncoding strand of *hCYP11B2*. The NBRE-1, present on the coding strand of *hCYP11B2*, is underlined. B, Mutational analysis of the NBRE-1 and Ad5 *cis*-elements. H295R cells were transiently transfected with a luciferase reporter vector driven by pB2-1521 containing the wild-type NBRE-1 and Ad5 sequences (−1521 WT), the mutated NBRE-1 sequence (−1521/NBRE-1M), mutated Ad5 sequence (−1521/Ad5M), or NBRE-1/Ad5 double mutant (−1521 NBRE-1/Ad5M). Cells were transfected with the indicated *hCYP11B2* reporter construct and either pRc/RSV empty expression vector (1 μg/ml) or with expression vector containing the coding sequence for NGFIB or NURR1 (1 μg/ml) along with a renilla expression vector (0.05 μg/ml). After recovery for 24 h, cells were lysed and assayed for luciferase and renilla activity. Data were normalized to renilla and expressed as a percentage of the basal reporter activity of each *hCYP11B2* reporter construct. Results represent the mean ± SEM of data from three or more independent experiments each performed in triplicate (**, $P < 0.0001$; *, $P = 0.0091$ compared with basal level).

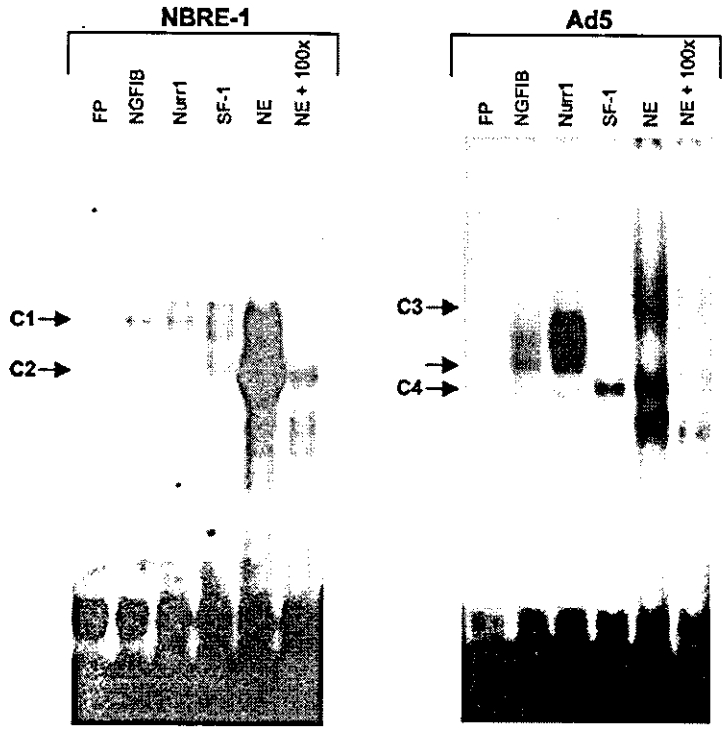

Fig. 6. EMSA of NBRE-1 and Ad5 *cis*-Elements

EMSA was performed using ^{32}P-labeled oligonucleotide probes containing either the NBRE-1 or Ad5 NBRE consensus sequence of *hCYP11B2* (Table 1). Radiolabeled probe alone (FP; free probe) is shown in lane 1 of each panel. Lanes 2–4 of each panel correspond to labeled probe incubated with *in vitro*-translated NGFIB, Nurr1 or SF-1 as indicated. Probe incubated with H295R nuclear extract (NE; 1 μg) is shown in lane 5 of each panel. Nonradiolabeled self-competitor DNA was added to the nuclear extract reaction mixture in a 100-fold molar excess (NE + 100x) to identify nonspecific protein/DNA interactions (lane 6 of each panel). Protein/DNA complexes (C1–C4) were separated from free probe by gel electrophoresis. In panel 1, the protein/DNA complex designated C1 migrated with the complex formed by *in vitro*-translated NGFIB or Nurr1, whereas C2 migrated with the complex formed by SF-1. In panel 2, the C3 complex did not migrate with any of the *in vitro* proteins, whereas C4 migrated with the complex formed by SF-1. The *unlabeled arrow* in panel 2 denotes a protein that was present in the rabbit reticulocyte lysate extract used for *in vitro* protein preparation.

Effects of Ang II Treatment upon Mutated NBRE-1, Ad5, and/or Ad1 *hCYP11B2 cis*-Elements

A cAMP response element (CRE, sequence TGACGTGA), also called Ad1, is crucial for transcriptional regulation of the *CYP11B* genes of several species (22). To determine the relative role of the NBRE-1, Ad5, and Ad1/CRE *cis*-elements in agonist stimulation of *hCYP11B2*, we mutated all three elements alone or in combination in the context of the pB2-1521 luciferase construct. Wild-type and mutated constructs were transfected into H295R cells. After recovery, cells were treated or untreated with Ang II for 6 h, then lysed and assayed for luciferase activity. Mutation of the NBRE-1, Ad5, and Ad1/CRE *cis*-elements reduced both basal and Ang II-induced levels of *hCYP11B2*, demonstrating that all three elements are required for maximal transcriptional activity (Fig. 9). The fold induction by Ang II was similar in the wild-type *hCYP11B2* (4.4-fold) and the mutated NBRE-1 (5.3-fold) and Ad5 (4-fold) constructs, whereas the Ad1/CRE mutant was induced by Ang II only 2.2-fold over basal. Ang II stimulated the *hCYP11B2* triple mutant construct only 1.8-fold over basal, which was similar to that observed with the pGL3Basic empty vector (2-fold).

DISCUSSION

Temporally, the regulation of aldosterone biosynthesis can be divided into two phases. Acutely (minutes to hours after a stimulus), aldosterone production is controlled by the movement of cholesterol into the mitochondria, which is mediated by increased expression of steroidogenic acute regulatory protein (23). Chronically (hours to days), aldosterone production is regulated at the level of expression of aldosterone synthase (CYP11B2). The studies reported here, together with previous work, provide a detailed picture (Fig. 10) of the mechanisms that control *hCYP11B2* expression and thus, aldosterone biosynthesis.

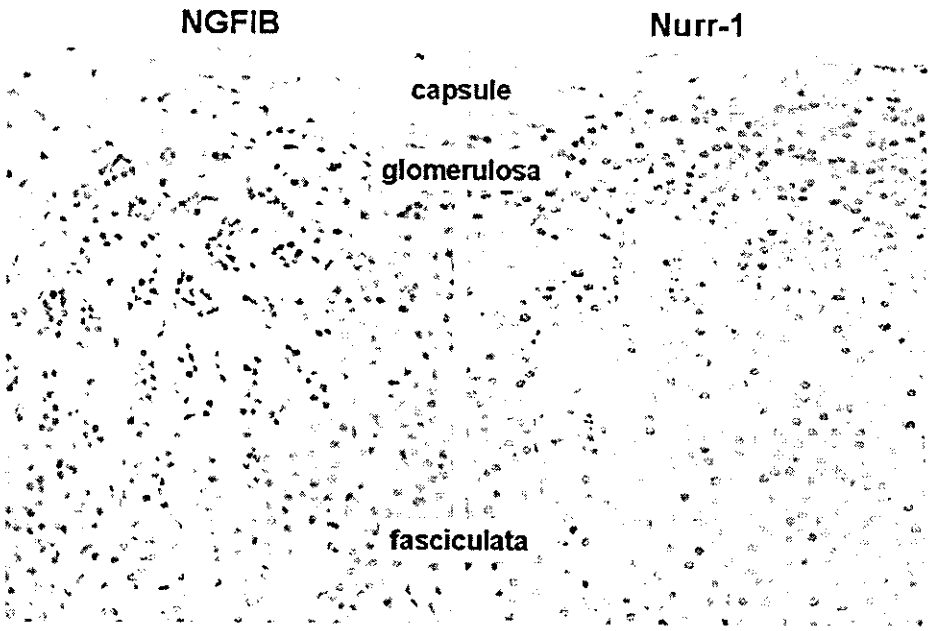

Fig. 7. Immunohistochemical Localization of NGFIB or NURR1 in Human Adult Adrenal Gland

NGFIB and NURR1 were studied, using immunohistochemistry, with antibodies specific for either NGFIB (*left panel*) or NURR1 (*right panel*). The areas of the capsule, zona glomerulosa, and zona fasciculata, are indicated. NURR1 exhibited expression that was localized primarily to the glomerulosa, whereas NGFIB was found in both the glomerulosa and fasciculata.

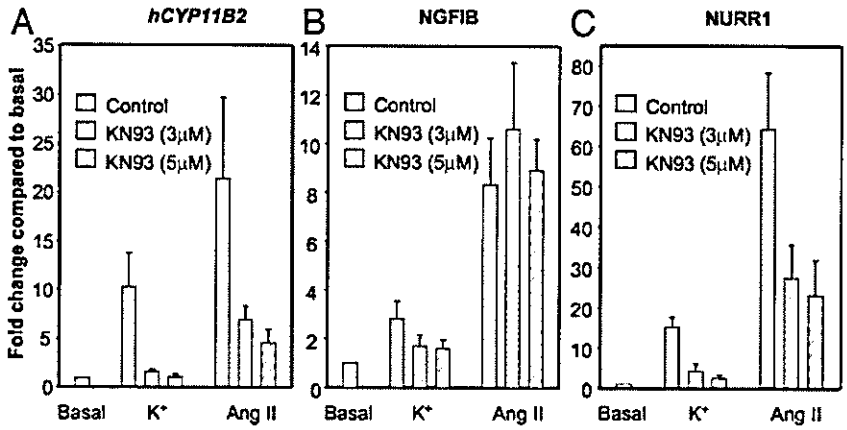

Fig. 8. Effect of Treatment with the CaM Kinase Inhibitor KN93 upon Transcription of *hCYP11B2* (A), NGFIB (B), or NURR1 (C)

H295C cells were treated for 6 h with K^+ (20 mM), Ang II (10 nM), and/or KN93 (3 μM or 5 μM). After isolation of total RNA, real-time RT-PCR was used to quantify the transcripts for *hCYP11B2*, NGFIB, and NURR1 as described in *Materials and Methods*. Fold change was adjusted to the amount of 18S ribosomal RNA in each sample. Results represent data pooled from four independent experiments. Note the different scales in the three panels.

Signaling Pathways that Regulate *CYP11B2* Expression

The primary regulators of *CYP11B2* expression and aldosterone production are Ang II and potassium (K^+) that act, in part, by increasing intracellular calcium ($[Ca^{2+}]i$) (24). Ang II acts via the type 1 Ang II (AT1) receptor, which is coupled to Gq. Triggering the receptor activates phospholipase C-β, resulting in increased levels of diacylglycerol and inositol triphosphate (IP3); the latter mobilizes intracellular calcium stores (25). Potassium, on the other hand, increases $[Ca^{2+}]_i$ through activation of voltage-sensitive L- and T-type Ca^{2+} channels, resulting in the influx of calcium from extracellular sources.

Whereas protein kinase C-dependent pathways activated by diacylglycerol apparently do not play a major role in the regulation of *hCYP11B2* (26), increases in intracellular calcium activate CaM, which in turn activates several CaM-dependent protein kinases

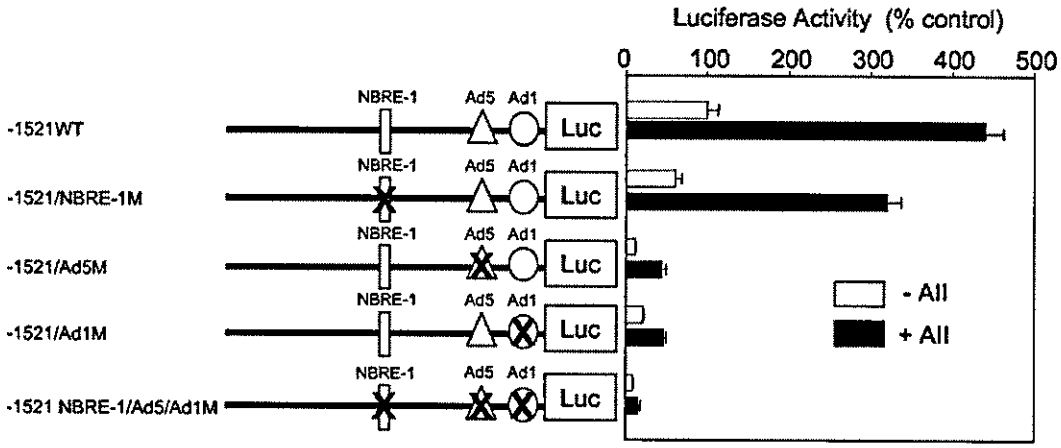

Fig. 9. Mutational Analysis to Determine the Role of the NBRE-1, Ad5, and/or Ad1 *cis*-Elements Present in *hCYP11B2*

H295R cells were transiently transfected with a luciferase reporter vector driven by pB2-1521 containing the wild-type NBRE-1, Ad5, and Ad1 sequences (−1521 WT), the mutated NBRE-1 sequence (−1521/NBRE-1M), mutated Ad5 sequence (−1521/Ad5M), mutated Ad1 sequence (−1521/Ad1M) or NBRE-1/Ad5/Ad1 triple mutant (−1521 NBRE-1/Ad5/Ad1M). Cells were transfected with the indicated *hCYP11B2* reporter construct along with a β-galactosidase expression vector (0.05 μg/ml). After recovery, cells were treated or untreated with Ang II (10 nM) for 6 h, then lysed and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of the −1521 WT reporter construct. Results represent the mean ± SEM of data from three or more independent experiments each performed in triplicate.

(CaMKs). Calcium appears to act through CaMKI and possibly CaMKIV to regulate *hCYP11B2* transcription (21). CaM and CaMKs also appear to regulate the acute steps of aldosterone production (27).

Important *cis*-Elements in *hCYP11B2*

The *cis*-elements and *trans*-acting factors that regulate the differential expression of *hCYP11B2* in the adrenal zona glomerulosa have been an area of ongoing study in this laboratory. We have identified three important *cis*-elements in the *hCYP11B2* promoter: a CRE at −71/−64, a *cis*-element termed Ad5 at −129/−114 (19), and, herein, a third *cis*-element termed NBRE-1 (−766/−759). The CRE is common to both *hCYP11B1* and *hCYP11B2* and is regulated by both protein kinase A- and CaMK-dependent mechanisms.

Neither the NBRE-1 nor Ad5 *cis*-elements found in the 5′-flanking region of *hCYP11B2* are present in *hCYP11B1*, making these elements likely candidates in the specific regulation of *hCYP11B2*. Herein, we found that mutation of either the NBRE-1 or Ad5 element dramatically decreased the basal levels of reporter construct expression and blunted maximal agonist stimulation. The NBRE-1 site is a nuclear receptor half-site that we demonstrate has the ability to bind NGFIB family members. The Ad5 *cis*-element represents a direct repeat of two nuclear receptor half-sites in tandum (DR-0), which we show can bind members of the NGFIB family as well as SF-1 and COUP-TF (chicken ovalbumin upstream promoter-transcription factor) (19).

The Ad1/CRE is highly conserved between species and is important for bovine, rodent (mouse, rat, hamster) and human *CYP11B2* gene activity (16, 17, 28–30, 19). Whereas sequence analysis of 2 kb of the mouse and hamster promoters failed to locate the −766/−759 NBRE-1 *cis*-element seen in *hCYP11B2*, variant and consensus NBREs were found more distal, at about −1500 bp, in the mouse and hamster promoters, respectively. However, the most intriguing of the *cis*-elements, the Ad5 site, does not appear to be conserved among *CYP11B2* of different species (22). Thus, these data support the hypothesis that there are species variations in the key *cis*-elements regulating *CYP11B2* gene transcription.

Trans-Acting Factors Regulating *CYP11B2*

Previous work has shown that the *hCYP11B2* CRE binds activating transcription factors, ATF-1 and ATF-2, and CRE binding protein (CREB) (31). Phosphorylation of ATF-1 and/or CREB by CaMKI and CaMKIV increases the ability of these factors to enhance gene transcription (32, 33). In a recent report, it was shown that mutation of the CRE was able to block CaMKI induction of *hCYP11B2* reporter activity (21). Thus, it is likely that activated CaMKI phosphorylates ATF-1 and/or CREB leading to enhanced transcription of *hCYP11B2*.

We showed in the present study that treatment of H295R adrenal cells with Ang II and K^+ rapidly and dramatically increased the levels of NGFIB and NURR1 mRNA and protein. Inhibition of the CaMKs with KN93 reduced agonist-stimulated NGFIB (only K+ stimulation), NURR1 and *hCYP11B2* transcription. Activity of CaMKs are known to increase NGFIB expression in other cell model systems (34). We therefore propose that the effects of K^+ and Ang II on

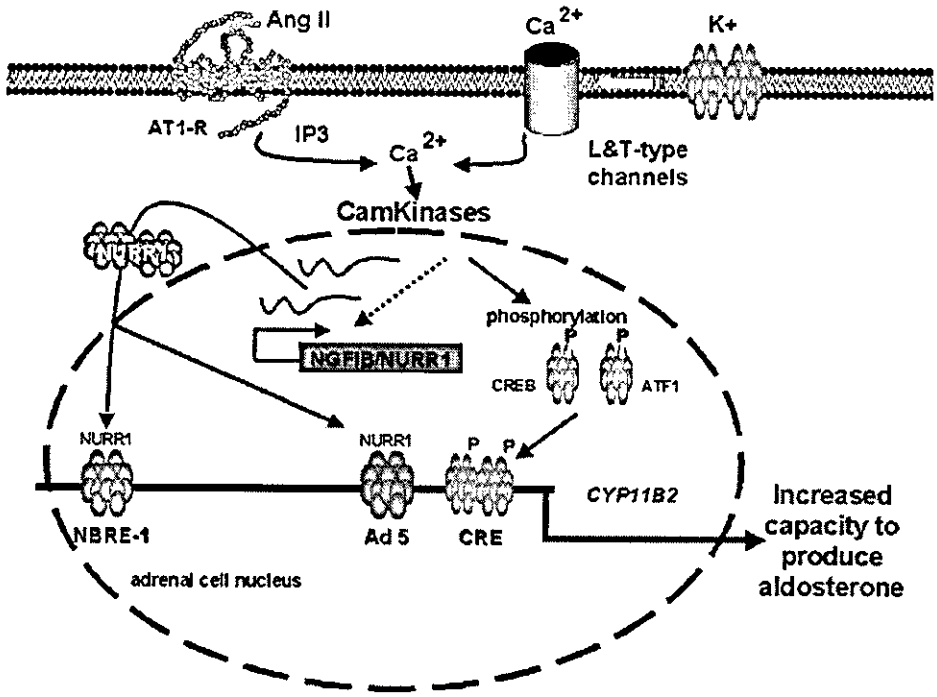

Fig. 10. Schematic Model Showing Ang II and K^+ Regulation of *hCYP11B2* Expression

The CRE (Ad1), Ad5 and NBRE-1 *cis*-elements are indicated. Ang II, acting through the type 1 Ang II receptor (AT1), and K^+ increase intracellular calcium which, in turn, activates CaMKI and CaMKIV. CaMKI and/or CaMKIV phosphorylates ATF-1 and CREB, which increases binding to the *hCYP11B2* CRE. Expression of NGFIB and NURR1 mRNA and protein are induced by Ang II, in part through the action of CaMKI and/or CaMKIV. NGFIB and/or NURR1 bind to the Ad5 and NBRE-1 *cis*-elements. Bound ATF-1/CREB and NGFIB/NURR1 activate *hCYP11B2* gene expression. This increase in *hCYP11B2* transcription directly determines the capacity of the adrenal glomerulosa to produce aldosterone.

hCYP11B2 transcription occur through two pathways: increased expression of NURR1/NGFIB and phosphorylation of ATF-1/CREB (Fig. 10).

NGFIB is abundantly expressed in brain, thymus, muscle, and some peripheral tissues (35, 36), whereas NURR1 is expressed predominantly in the central nervous system, where it is responsible for the differentiation and maintenance of dopaminergic neurons (37, 38). NGFIB and NURR1 have been detected in human adrenal, and NURR1 has been localized to the murine adrenal zona glomerulosa by *in situ* hybridization (20). Herein, we found that NURR1 was expressed in the human adrenal glomerulosa and to a lesser degree in the fasciculata, whereas NGFIB was expressed in both fasciculata and glomerulosa zones.

Both NGFIB and NURR1 markedly increased transcription of *hCYP11B2*, but neither factor had any effect on transcription of *hCYP11B1*. This presumably reflects transcriptional regulation through the NBRE-1 and Ad5 sites, which are unique to *hCYP11B2*. The ability of NGFIB and NURR1 to regulate transcription of *hCYP11B2* extends the role of these nuclear receptors in the hypothalamic-pituitary-adrenal axis. Both NGFIB and NURR1 are known mediators of CRH function in the hypothalamus (39). In the adrenal, a role for NGFIB in the regulation of 21-hydroxylase (*CYP21*) transcription has been proposed (14, 15). The site of expression of each factor within the adrenal may influence the relative role of NGFIB or NURR1 on target gene selection. The overlap in expression and similarity in effects on gene transcription may explain why previous studies using targeted disruption of single NGFIB family members have not demonstrated an adrenal phenotype (37, 40). Nevertheless, these transcription factors are not functionally equivalent. Subtle changes in the consensus NBRE can differentially affect the *trans*-activation ability of each family member (41). Moreover their activities, and consequently their functional roles *in vivo*, can be modulated by post-translational modifications and differing heterodimerization abilities (35, 42–45). Our data suggest that NURR1 may play the more specific role in *hCYP11B2* regulation.

In summary, Ang II and K^+ regulation of adrenal aldosterone production appears to converge on calcium signaling pathways (Fig. 10). Both agonists increase $[Ca^{2+}]_i$, the activation of CaM, CaM kinases, and the expression of NGFIB family members. NURR1 and/or NGFIB along with CRE-binding transcription factors subsequently increase the transcription of *hCYP11B2* thus controlling the long-term capacity of the adrenal gland to produce aldosterone.

MATERIALS AND METHODS

Preparation of Reporter Constructs and Expression Vectors

The *hCYP11B1* (pB1-1102) and *hCYP11B2* (pB2-1521) promoter constructs were previously described (9). The *hCYP11B2* 5′-deletion constructs were prepared using available restriction endonuclease sites or by PCR as described (19). For the NBRE-1 mutant construct, the sequence 5′-AAAGGCTA-3′ (−766/−759) was changed to 5′-gAAttCTA-3′; for the Ad5 mutant construct, the sequence 5′-GAC-CTT-3′ (−119/−114) was changed to 5′-GAtaTc-3′; for the Ad1/CRE mutant construct, the sequence 5′-TGACGTGA-3′ was changed to 5′-gGtaccGA-3′ (Table 1). All bases were numbered relative to the *hCYP11B2* transcriptional start site.

Plasmids encoding rat NGFIB and mouse NURR1 were kindly provided by Colin Clyne (Prince Henry's Institute of Medical Research, Victoria, Australia). A human SF-1 plasmid was provided by Meera Ramayya (University of Washington, Seattle, WA). All three coding sequences were subcloned into the pRc/RSV expression plasmid (Invitrogen, Carlsbad, CA) or into pcDNA3.1 Zeo+ (Invitrogen).

Cell Culture and Transfection Assay

Cell culture and transfection assays were carried out as previously described except that Transfast (Promega, Madison, WI) was used as the transfection reagent according to the manufacturer's protocol (9).

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cells using the Ultraspec RNA isolation system (Biotecx Laboratories Inc., Houston, TX) and aliquots (10 μg) were resolved by electrophoresis on a 1% agarose/formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech Inc., Piscataway, NJ) by blotting overnight at 10 V and cross-linked under UV light. Prehybridization was carried out overnight at 42 C in formamide prehybridization/hybridization solution supplemented with 50 mM NaH_2PO_4, 0.5 mg/ml salmon sperm DNA and 5% dextran sulfate. The membrane was hybridized with a NGFIB or NURR1 cDNA probe (Rediprime II; Amersham) in formamide prehybridization/hybridization solution supplemented with 20 mM NaH_2PO_4, 0.25 mg/ml salmon sperm DNA and 10% dextran sulfate at 42 C overnight. The membrane was washed in 0.1× SSC (sodium chloride/sodium citrate)/0.1% SDS (sodium dodecyl sulfate) twice each at room temperature and at 42 C and then exposed to x-ray film. The membrane was subsequently rehybridized with ^{32}P-radiolabeled 18S probe to confirm equal loading of RNA samples.

Western Blot Analysis

Nuclear extracts were prepared from H295R cells untreated or treated with Ang II as described (8). PAGE was carried out using precast 4–12% bis-Tris NuPage gels (Novex, San Diego, CA). After electrophoresis, proteins were electrophoretically transferred onto polyvinylidene difluoride membranes for 1 h at 25 V. After transfer, the membranes were incubated with NGFIB (1:500) or NURR1 (1:1000) antibodies overnight at 4 C. The NGFIB antibody (catalog no. 1600045) was obtained from Geneka Biotechnology (Montreal, Quebec, Canada) and the NURR1 antibody (sc-991) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). In addition to the manufacturer's characterization, we tested both antibodies for specificity using *in vitro*-translated NGFIB or NURR1. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence Western blotting detection reagents from Amersham Biosciences (Piscataway, NJ).

EMSA

H295R nuclear extracts were prepared and EMSA was carried out, as described, with certain modifications (8). For NBRE-1, 0.1 mg/ml poly(dG-dC) (deoxyguanosine-deoxycytidine) was added to the binding buffer as nonspecific competitor. For the Ad5 probe, binding was carried out in 25 mM Tris, 100 mM KCl, 0.125% Nonidet P-40, 15% glycerol, 2.5 mM dithiothreitol, and 0.05 mg/ml poly(dI-dC) deoxyinosine-deoxycytidine). After binding, the resulting DNA/protein complexes were separated from free probe by electrophoresis through a 4% native polyacrylamide gel in either 1× Tris-glycine (NBRE-1) or 0.50× Tris-borate-EDTA (Ad5) running buffer. The gel was dried and visualized after autoradiography at −70 C for 24 h. Rat NGFIB, mouse NURR1, and human SF-1 were prepared using an *in vitro* transcription/translation system (Promega). The *hCYP11B2*-specific NBRE-1 (−766/−759) and Ad5 (−129/−114) wild-type oligonucleotide sequences used for EMSA are listed in Table 1.

Immunolocalization of NGFIB and NURR1 in Human Adrenal Cortex

Nonpathologic human adrenals were retrieved from autopsy files of Tohoku University Hospital (Sendai, Japan). Tissues were fixed in 10% formalin and embedded in paraffin. Histological examinations revealed no significant pathologic abnormalities including nodules or neoplasms. Review of the charts revealed that these patients had not received any forms of adrenocortical steroids before their demise.

Rabbit polyclonal antibodies for NURR1 (sc-991, Santa Cruz Biotechnology) and NGFIB (NAK1/Nur77;1600045, Geneka Biotechnology) were used for immunohistochemical analysis employing the streptavidin-biotin amplification method and a Histofine Kit (Nichirei, Tokyo, Japan). Briefly, deparaffinized sections were pretreated by heating the slides in an autoclave at 120 C for 5 min in citric acid buffer [3 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)]. The slides were then treated with 1% normal goat serum for 20 min at room temperature and incubated with anti-NURR1 (dilution; 1/250) or anti-NGFIB (dilution: 1/200) for 18 h at 4 C. The slides were subsequently reacted with Envision (DAKO,

Table 1. Oligonucleotide Sequences Used as Primers for Site-Specific Mutagenesis or as Probes for EMSAs

hCYP11B2	NBRE-1/MUT	5′ GTC ACT TTG AgA Att CTA CAA ACG GTG TC 3′ (sense)
hCYP11B2	Ad5/MUT	5′ CTC CAG CCT TGA taT cCG CTC TGA GAG TC 3′ (sense)
hCYP11B2	Ad1/CRE/MUT	5′ GTT CTC CCA gGt acc GAT ATG TTT CCA G 3′ (sense)
hCYP11B2	wtNBRE-1	5′ TTT GAA AAG GCT ACA AAC GGT GTC ATT C 3′ (sense)
hCYP11B2	wtAd5	5′ CCA GCC TTG ACC TTC GC 3′ (sense)

The *hCYP11B2* reporter constructs with mutated NBRE-1, Ad5, or Ad1/CRE bases (*lowercase letters*) were produced using the indicated primers. Mobility shift assays were performed using wtNBRE-1 or wtAd5 oligonucleotides corresponding to the indicated sequence in *hCYP11B2*. Oligonucleotides used for EMSA were double stranded.

Table 2. Sequences of Primer and Probe Oligonucleotides Used for Real-Time RT-PCR

Gene	Primer (Sense)	Primer (Antisense)	Probe
hCYP11B2	5′-GGCAGAGGCAGAGATGCTG-3′	5′-CTTGAGTTAGTGTCTCCACCAGGA-3′	5′-CTGCACCACGTGCTGAAGCACT-3′
NR4A1 (NGFIB)	5′-ATACACCCGTGACCTCAACCA-3′	5′-TTCTGCACTGTGCGCTTGAA-3′	
NR4A2 (NURR1)	5′-TTCGCCCCCGGTGAGT-3′	5′-ATAGTCAGGGTTCGCCTGGAA-3′	

Copenhagen, Denmark), then visualized with 3.3′-diaminobenzidine solution [1 mM diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] For negative controls, the sections were incubated with normal rabbit IgG instead of the primary antibodies and no specific immunoreactivity was detected in these sections.

RNA Extraction and Real-Time RT-PCR

Total RNA was extracted from tissue, using the method of Chirgwin (46), followed by deoxyribonuclease I treatment. Two micrograms of total RNA were reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers and probes for real-time RT-PCR were designed using the Primer Express computer program (Applied Biosystems) (Table 2). For NGFIB or NURR1 quantitation, a double-stranded DNA dye, SYBR Green I (Molecular Probes Inc., Eugene, OR) was used along with 15 μl 2× SYBR Green Universal PCR Master Mix (Applied Biosystems) and 0.1 μM of each primer. *hCYP11B2* and 18S quantitation were performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems) and 10 μl of primer/probe mix. For *CYP11B2*, the final concentrations of primer and probe used were 0.1 μM each. For 18S, the final concentrations of primer and probe were 0.05 μM and 0.1 μM, respectively. All real-time RT-PCRs were carried out, in two steps, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the dissociation protocol. Step 1: 50 C for 2 min followed by 95 C for 10 min, one cycle. Step 2: 95 C for 15 sec, followed by 60 C for 60 sec, 40 cycles. Standard curve cDNA plasmids for NGFIB, NURR1, and *CYP11B2* were used to quantitate transcript levels. As an internal standard, each individual sample was normalized to its 18S ribosomal RNA content. mRNA levels were expressed as attomoles per microgram 18S rRNA.

Acknowledgments

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Dioxin Inhibition of Estrogen-Induced Mouse Uterine Epithelial Mitogenesis Involves Changes in Cyclin and Transforming Growth Factor-β Expression

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A single dose of dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD; 5 μg/kg, ip) inhibits 17β-estradiol (E2)-induced uterine epithelial mitogenesis, apparently through disruption of stromal-epithelial interactions. To understand if TCDD alters early uterine (Ut) responses to E2, young adult C57BL/6J mice were ovariectomized and given (ip) either oil or 5 μg/kg TCDD. After 24 h, TCDD-treated mice received E2, and oil-treated mice were given E2 or oil. Body and Ut weights were collected 6 and 18 h later. Ut were flash-frozen at 6 h. E2 increased Ut weight ($p < 0.0001$) and Ut/body weight ratio ($p < 0.0001$), compared to mice given oil alone. Ut cyclin expression was assessed by an RNase protection assay. E2 increased mRNA expression for cyclin A2 and B1 ($p < 0.05$), in addition to D1, D2, and D3 ($p < 0.001$), while cyclin C was unchanged from oil controls and cyclins A1 and B2 were undetectable. In contrast, TCDD completely abolished E2-induced cyclin A2, which has been associated with S phase initiation, and reduced B1 and D2 ($p < 0.05$). Interestingly, TCDD did not alter E2-induced Ut weight increases at 6 h, but inhibited E2-induced Ut weight gain at 18 h. A 10-μg/kg TCDD dose was necessary for attenuation of the early E2-induced Ut weight increases ($p < 0.01$). Since TGF-β regulates cyclins, Ut TGF-β was also assessed in TCDD + E2-treated and control mice. TGF-β mRNA levels were increased after TCDD compared to E2 alone ($p < 0.01$), suggesting a possible mechanism for TCDD inhibition of Ut cyclin A2. Thus, TCDD alters specific E2-regulated Ut G_1 phase activities and may inhibit E2-induced Ut epithelial mitogenesis by disrupting specific cell signaling mechanisms necessary for S phase initiation *in vivo*.

Key Words: cytokine; uterus; estrogen; proliferation; antiestrogen; aryl hydrocarbon receptor; ovariectomy.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (dioxin; TCDD) is the most potent congener within a large class of chlorinated hydrocarbon environmental contaminants known as dibenzo-*p*-dioxins. TCDD elicits a variety of biochemical and toxicological outcomes, including immunotoxicity, carcinogenicity and alterations in endocrine responses (Barsotti *et al.*, 1979; Geyer *et al.*, 2000; Huff *et al.*, 1991). Most TCDD effects have been attributed to its ability to bind the cytosolic aryl hydrocarbon receptor (AhR), a member of the basic helix-loop-helix/PAS superfamily of DNA binding proteins (Swanson and Bradfield, 1993). The AhR is a promiscuous receptor in that its activity is modulated by not just one but numerous compounds, and dioxins compose just one class in a large family of manmade polyhalogenated chemicals that interact with it; polychlorinated dibenzofurans and coplanar polychlorinated biphenyls are examples of other types of compounds that also have strong affinity for AhR (Geyer *et al.*, 2000).

As an endocrine disruptor, TCDD has antiestrogenic activity both *in vitro* and *in vivo* (Gallo *et al.*, 1986; Safe, 2001). TCDD decreases mouse uterine wet weight (Gallo *et al.*, 1986), suggesting inhibitory effects on estrogen-controlled cellular processes contributing to water imbibition. Whereas estrogen induces proliferation and secretory protein productions in uterine epithelia, these responses are inhibited by TCDD in ovariectomized wild-type but not AhR gene knockout mice (Buchanan *et al.*, 2000). Both TCDD and unbound AhR downregulate cell cycle progression in absence of hormone administration *in vitro*, albeit through different mechanisms (Kolluri *et al.*, 1999; Puga *et al.*, 2000). Mechanistic influences of TCDD and AhR on stimulation and inhibition of cell cycle progression in nonreproductive tissues *in vitro* and *in vivo* have been described (Abbott *et al.*, 1987; Lucier *et al.*, 1991; Stohs *et al.*, 1990). Yet, TCDD effects are cell type- and species-specific (DeVito and Birnbaum, 1994), and the cellular and molecular mechanisms underlying antiestrogenic TCDD effects on processes such as uterine epithelial proliferation have not been reported.

Within the first 24 h, a single injection of 17β-estradiol (E2), the most potent endogenous estrogen, stimulates multiple responses in the rodent uterus. For example, uterine epithelial proliferation and differentiation depend on stromal cell re-

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sponses to E2 (Buchanan *et al.*, 1999; Cooke *et al.*, 1986, 1997). Shortly after E2 treatment, stromal cells undergo differentiative changes associated with dramatic increases in uterine fluid uptake. Subsequently, endothelial, along with luminal and glandular epithelial, cells enter G_1 in preparation for DNA synthesis before proceeding to mitosis. The greatest proportion of maximal responses to E2 in mouse uterus correspond to G_1 since they occur within 6 h after exposure (Buchanan, unpublished report) and include hyperemia, water imbibition, and variations in expression of delayed early genes (e.g., cytokines and cyclins; Geum *et al.*, 1997; Martin *et al.*, 1976; Takahashi *et al.*, 1994). These early preparatory events are critical for onset and normal progression of S phase, which is confined to epithelia in the uterus and begins about 8.5 h after E2 treatment (Martin *et al.*, 1973). As an antiestrogen, TCDD may alter events necessary for these early uterine responses.

Uterine epithelial S phase peaks between 13 and 25 h after E2 treatment in ovariectomized mice (Martin *et al.*, 1973), and E2 acts through uterine stromal estrogen receptor-α (ERα) to induce epithelial mitogenesis *in vivo* (Cooke *et al.*, 1997). We recently determined that a single TCDD exposure of 5.0 μg/kg (ip) inhibits E2-induced uterine epithelial mitogenic activity in ovariectomized mice and that this inhibition requires stromal AhR, while epithelial AhR is not involved (Buchanan *et al.*, 2000). Thus, TCDD inhibits epithelial proliferation prior to S phase *in vivo,* and liganded AhR alters stromal-epithelial interactions by disrupting stromal responses to E2. While TCDD inhibition of uterine epithelial proliferation may be initiated through alterations in E2-induced stromal responses, factors under E2 control that regulate epithelial proliferation and differentiation have not yet been identified either *in vitro* or *in vivo.*

The use of AhR ligands to understand E2/TCDD interactions and control E2 signaling has been suggested (Safe and Krishnan, 1995). To better understand the mechanisms responsible for the TCDD effect and the E2 signaling pathway *in vivo,* TCDD influence on uterine responses during the early E2-induced preparatory period was examined in the absence of endogenous hormonal influences. We determined whether TCDD alters early E2 regulation of uterine events such as wet weight, in addition to G_1 phase activity, as indicated by cyclin and cytokine gene expressions. Our data show that TCDD alters early E2-regulated uterine processes critical for cell cycle progression *in vivo* and emphasize the use of TCDD as a tool for understanding steroid regulatory pathways.

MATERIALS AND METHODS

Animals. Adult C57BL/6J mice (40- to 50-day-old; Chu-bu Kaggaku Shizai, Nagoya, Japan) were given CE-2 rodent chow (Clea Japan, Tokyo) and tap water *ad libitum.* Animals within similar treatment groups were housed 2 to 4 per cage with controlled lighting (12L:12D), temperature (22–24°C), and humidity (50 ± 5%) in clear plastic cages with hardwood bedding and were maintained in accordance with the *Guiding Principles in the Use of Animals in Toxicology.* The Animal Research Committees at Okazaki National Research Institutes and National Institute for Environmental Studies, Japan, approved all experiments.

TCDD and E_2 treatments. TCDD (>99.5% pure; Cambridge Isotope Laboratory, Andover, MA) was dissolved in n-Nonane (Nacalai Tesque, Kyoto, Japan) and diluted in sesame oil to the appropriate concentration. The final formulation for each TCDD dose included 10% n-Nonane. To determine the influence of TCDD on E2-stimulated increases in uterine (Ut) weight and regulation of early gene expression, mice were ovariectomized and one week later given 1 of 4 TCDD doses (0.2, 1.0, 5.0, or 10.0 μg TCDD + 10% nonane/kg body weight) or 10% nonane + oil vehicle by ip injection. The next day, 10% nonane + oil-treated animals received pure oil vehicle or E2 (30 ng/mouse in 0.05 ml corn oil, which is equivalent to 1.5 μg/kg); all TCDD-treated animals received E2 (30 ng). Body, liver, and Ut weights were collected 6 or 18 h later. After weighing, Ut were flash frozen in liquid N_2.

Probe synthesis, RNA isolation. For RNase protection assay, antisense biotin-labeled probe syntheses was carried out using PharMingen multiprobe template sets for mouse cyclin and cytokine mRNAs (mCYC and mCK-3b; BD-PharMingen International, San Diego, CA) with Biotin RNA Labeling Mix (10×; Roche Diagnostics GmbH, Mannheim, Germany) by *in vitro* transcription at 37°C according to standard protocols. Labeled probe was extracted by phenol-chloroform and then precipitated with 4 M LiCl and 100% EtOH to remove free nucleotides. Total RNA was isolated from whole frozen Ut using TRIzol reagent (Gibco BRL, Rockville, MD) according to the manufacturer's protocol. To remove residual contaminants, RNA was further purified using the Qiagen RNeasy total RNA kit (Qiagen K.K., Tokyo, Japan).

RNase protection assay. Ut mRNA expression levels for cyclins (A2, B1, C, D1, D2, D3, A1, and B2) and cytokines (TGF-β1, TGF-β2, TGF-β3, MIF, TNF-α, IL-6, and IFN-γ) were assessed in 5.0 μg TCDD-exposed and control Ut after 6 h E2 or oil by RNase protection assay. Yeast control and target RNAs (12 μg) were incubated with 20 ng labeled probe at 90°C for 10 min, then 56°C for 15 h and 37°C for a final 30 min. Unhybridized probe and unprotected RNA were digested in a 1:100 dilution of RNase A + T1 mix in hybridization buffer (Ambion, Inc., Austin, TX) for 30 min at 37°C. RNases were inactivated with RNase Inactivation/Precipitation III Solution (Ambion, Inc.). Precipitation of yeast control RNA that had been incubated with or without RNase and protected probe samples was achieved at −20°C. Samples were then resolved on a denaturing 5% polyacrylamide gel and blotted by semidry electro-transfer to a nylon (+) membrane (Hybond-N+, Amersham Pharmacia Biotech, Buckinghamshire, UK). Biotin signals from blot membranes were captured on X-ray film by chemiluminescence (BrightStar Bio-Detect, Amersham) and converted to electronic form, using an Epson ES2000 scanner interfaced with a Macintosh G4 computer utilizing Adobe Photoshop software. Autoradiograms for cyclins and cytokines were quantitated using Image Gauge software (Fuji Photo Film Co., Ltd., Tokyo, Japan). Relative mRNA transcript levels were normalized based on densitometric analysis of hybridization signals for mRNA of the ribosomal protein L32 to compensate for loading differences between gel lanes. For all treatment groups, RNase Protection assays were replicated at least 3 times in duplicate for cyclins and 2 times in triplicate for cytokines.

Statistical analysis. Data on Ut and liver weights were evaluated by ANOVA (StatView, version 5.0; SAS Institute, Cary, NC) followed by Dunnett's test for pairwise comparisons. Ut mRNA expressions were evaluated by Student's *t*-test using StatView. All data are reported as means ± SEM. In all cases, means were considered significantly different when $p < 0.05$. For the various endpoints, data are from 6 to 12 mice per treatment group.

RESULTS

Body, Liver, and Uterine Weights

Eighteen h of E2 exposure induced a marked hyperemia and increased Ut weight by 42% ($p < 0.0001$) and Ut-to-body weight ratio by 41% ($p < 0.0001$), compared to oil-treated

TABLE 1
TCDD Effect on Uterine Weight Increases after 6 or 18 Hours Estradiol

Treatment	LW (g), 6 h	LW/BW, 6 h	BW (g)		UtW (mg)		UtW/BW	
			6 h	18 h	6 h	18 h	6 h	18 h
Oil	1.09 ± 0.07	0.054 ± 0.002	20.23 ± 0.77	17.78 ± 0.88	15.16 ± 1.85	17.5 ± 1.29	0.75 ± 0.10	0.99 ± 0.09
E2	1.09 ± 0.11	0.055 ± 0.003	17.17 ± 0.52	17.99 ± 0.27	28.86 ± 1.01*	30.40 ± 5.42*	1.68 ± 0.09*	1.69 ± 0.29*
TCDD								
0.2 μg	1.03 ± 0.05	0.055 ± 0.002	18.84 ± 0.38		28.09 ± 4.24		1.49 ± 0.20	
1.0 μg	1.17 ± 0.04	0.063 ± 0.002***	17.79 ± 0.43		31.08 ± 4.36		1.75 ± 0.28	
5.0 μg	1.10 ± 0.04	0.059 ± 0.002***	18.81 ± 0.51	18.5 ± 0.56	26.27 ± 5.79	19.39 ± 5.41**	1.4 ± 0.28	1.04 ± 0.29**
10.0 μg	1.10 ± 0.17	0.061 ± 0.009***	17.98 ± 0.48		25.23 ± 2.28**		1.47 ± 0.52	

Note. Ovariectomized mice received TCDD or oil. After 24 h, TCDD-treated mice were given estrogen (E2, 30 ng), and oil-treated mice received either E2 or oil. Body weight (BW), uterine weight (UtW), and liver weight (LW) were collected 6 or 18 h after E2 or oil; 6–12 mice per treatment group.

*Significant increase compared to oil ($p < 0.0001$).

**Significant decrease compared to E2 ($p < 0.01$).

***Significant change compared to E2 ($p < 0.05$).

mice. In contrast, when 5.0 μg/kg TCDD was given prior to the 18 h E2 exposure, Ut weight increased by only 10% and Ut-to-body weight ratio increased by only 5% compared to oil-treated mice (Table 1). Thus, both absolute and relative Ut weights achieved 18 h after E2 were dramatically decreased by TCDD ($p < 0.01$). The shorter 6-h E2 exposure elicited responses similar to those seen 18 h after E2. Specifically, 6 h of E2 exposure induced Ut hyperemia and increased Ut weight by 45% ($p < 0.0001$) and Ut-to-body weight by 42% ($p < 0.0001$), compared to oil. Importantly, 0.2, 1.0, or 5.0 μg/kg TCDD did not alter E2-induced increases in hyperemia, Ut weight, or Ut-to-body weight ratio 6 h after E2. However, although 10.0 μg/kg TCDD had no effect on relative uterine weight, 10.0 μg/kg TCDD abrogated the early (6 h) E2-induced hyperemic response and diminished the early E2-induced Ut weight increase ($p < 0.01$; Table 1). Thus, at least 10.0 μg/kg TCDD was required to achieve inhibition of the early E2-stimulated gain in Ut weight and hyperemia while only 5.0 μg/kg TCDD was sufficient to inhibit Ut weight gain and hyperemia 18 h after E2 treatment.

Absolute and relative liver weights were unchanged by E2 given alone or by 0.2 μg/kg TCDD compared to oil controls. Liver-to-body weight ratios were slightly increased (9%; $p < 0.05$) in the 1.0, 5.0, and 10.0 μg/kg TCDD groups 6 h after E2, but absolute liver weights in these groups were not altered by TCDD when compared to mice given E2 alone (Table 1). Relevant to previous findings that antiestrogenic TCDD effects may not be related to changes in liver enzyme activity (DeVito *et al.*, 1992), the lack of effect on absolute liver weight by TCDD minimizes the probability of a general toxicological response in this study.

Uterine Cyclin Gene Expression

Uterine epithelial cells are in G_1 phase through 6 h of E2 treatment. Cyclin mRNA expression levels were determined and compared between uteri from mice given 5.0 μg/kg TCDD + 6 h E2, oil vehicle, or 6 h E2 alone. In the absence of TCDD, 6 h of E2 exposure significantly induced Ut mRNA expression for cyclins A2 and B1 ($p < 0.01$ and $p < 0.05$, respectively), and also cyclins D1, D2, and D3 ($p < 0.001$) relative to oil controls. Cyclin C expression was unchanged compared to oil control levels, and cyclins A1 and B2 were undetectable. In contrast, 5.0 μg/kg TCDD given 24 h before E2 completely abolished E2-induced cyclin A2 expression ($p < 0.05$), and cyclin B1 and D2 expression levels were significantly reduced compared to those of E2 alone ($p < 0.05$ and $p < 0.01$, respectively; Fig. 1). Expression levels for cyclins C, D1, and D3 did not change after TCDD compared to E2 levels, suggesting that the inhibitory effect of TCDD on the E2-induced increases in cyclins A2, B1, and D2 was specific.

Uterine Cytokine Gene Expression

Total RNA collected from Ut of mice given 5.0 μg/kg TCDD + 6 h E2, oil vehicle, or 6 h E2 alone was also used to determine cytokine mRNA expression levels. Ut mRNA expression levels for TGF-β1 were not altered after 6 h of E2 compared to oil control animals, and TGF-β3 mRNA levels were suppressed ($p < 0.01$) below control levels after 6 h of E2. After TCDD, TGF-β1 expression was increased ($p < 0.0001$) compared to mice that received E2 alone, and TGF-β3 suppression by E2 was reversed and appeared similar to the level of oil controls ($p < 0.01$; Figure 2). Uterine mRNA expression levels for the cytokines macrophage migration inhibiting factor (MIF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ) were also evaluated. MIF increases in response to E2 (Suzuki *et al.*, 1996). The E2-induced increase in MIF mRNA over oil-treated mice ($p < 0.01$) was further increased by 5.0 μg/kg TCDD ($p < 0.0001$; Figure 2). Neither E2 nor 5.0 μg/kg TCDD + E2 altered uterine mRNA levels for TNF-α, IL-6, or IFN-γ compared to

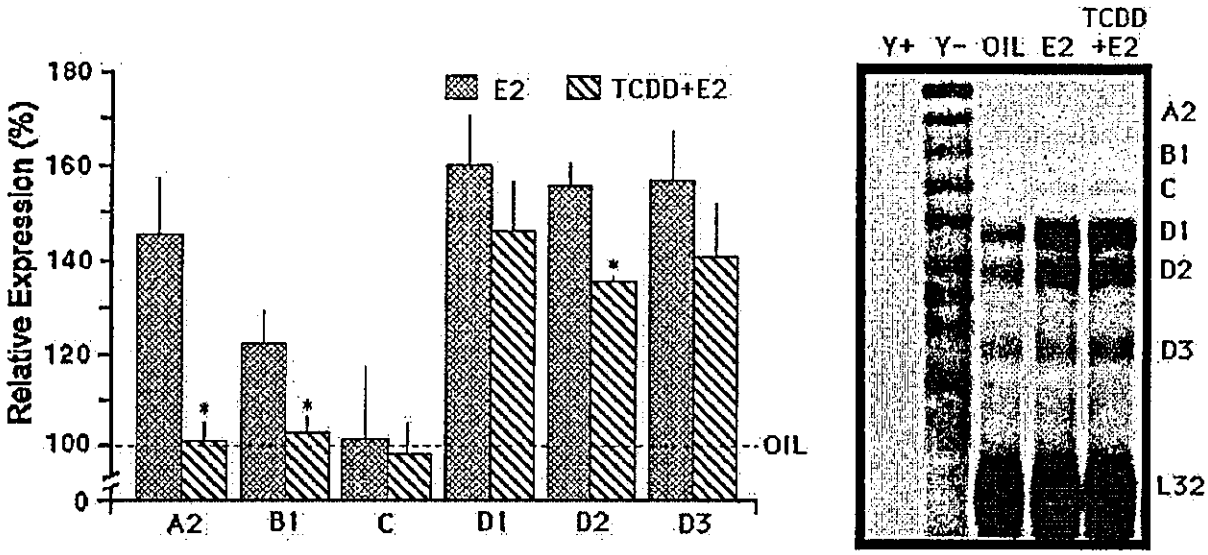

FIG. 1. RNase protection assay for cyclin expression in mouse uteri. Representative blot with normalized densitometric data shown graphically. Mice were given oil or TCDD (5 μg/kg, ip) 24 h before estrogen treatment (E2). Uteri were collected 6 h post-E2 or oil. Uterine mRNA levels from oil-treated mice were considered baseline as indicated by a line through the data. The E2 dose increased cyclin A2 and B1 ($p < 0.01$ and $p < 0.05$, respectively) and cyclins D1, D2, and D3 ($p < 0.001$), while cyclin C was unchanged compared to oil. TCDD dosing completely abolished E2-induced cyclin A2 expression ($p < 0.05$) and reduced B1 and D2 levels ($p < 0.05$ and $p < 0.01$, respectively), compared to E2 alone (asterisks). RNA was pooled from 8 to 12 mice for each treatment. Densities were normalized to that of ribosomal protein L32, which served as a loading control. Inset: Y−, yeast RNA minus RNase; Y+, yeast RNA plus RNase.

uteri from oil control mice (data not shown), confirming specificity of the E2 and TCDD effects.

DISCUSSION

To better understand the mechanisms underlying the antiestrogenic TCDD effect and the E2 signaling pathway *in vivo*, the influence of TCDD on early E2-induced uterine fluid uptake and G_1 phase activity was examined. As seen in the present study, uterine fluid uptake approaches maximum by 6 h after E2 treatment, and the resulting increase in wet weight is maintained at a similar level by 18 h (Martin *et al.*, 1976). DeVito *et al.* (1992) found no change in mouse serum E2 levels after TCDD and proposed that observed decreases in uterine ER levels after TCDD may be responsible for the antiestrogenic TCDD effect on uterine weight seen in nonovariectomized (intact) mice (Gallo *et al.*, 1986). However, uterine ER levels were high and were not altered by TCDD in ovariectomzed mice (DeVito *et al.*, 1992). Taken together, these findings suggested ovariectomy renders mice insensitive to the TCDD effect on uterine weight. Nonetheless, TCDD completely reduced uterine weight gain and hyperemia 18 h after E2 in ovariectomized mice to near control levels in the present study. This indicates ER levels may not contribute to TCDD effects on uterine weight in intact mice.

TCDD (5 μg/kg) did not alter E2-induced increases in uterine weight at 6 h but did alter G_1 phase gene transcription for factors associated with epithelial cell cycle progression. When considered with findings by Buchanan *et al.* (2000) that TCDD inhibits E2-induced uterine epithelial proliferation, the inhibition of E2-induced uterine weight gain by 5 μg/kg TCDD at 18 h but not at 6 h suggests normal epithelial proliferative functioning may be critical for maintenance of uterine fluid uptake and weight at 18 h. Further, 10.0 μg/kg TCDD reduced uterine weight after 6 h E2, even though 5 μg/kg TCDD was insufficient, demonstrating a lower sensitivity to TCDD at this earlier time point and suggesting the antiestrogenic TCDD effects on early weight increases and G_1 phase activity may be elicited through different mechanisms.

Cyclin activity has been well characterized *in vitro*, yet cyclin regulation and function in the uterus and *in vivo* are not well understood. Consistent with our data, mRNA for cyclins D1 and D3 are maximally increased 6 h after E2 in mouse uterus (Geum *et al.*, 1997). D-type cyclins regulate passage through G_1, and B cyclins are initially active during late S phase (Hunter and Pines, 1994). In MCF-7 cells, TCDD inhibits E2-induced cyclin D1 (Wang *et al.*, 1998) but had no effect on 6-h E2-induced uterine D1 mRNA levels *in vivo*. Transcription of cyclin A2, which is required for initiation and progression of S phase, is confined to proliferating cells, and deregulation of cyclin A2 expression can disturb normal G_1-S transition (Huet *et al.*, 1996; Resnitzky *et al.*, 1995). In mice, uterine cyclin A2 increases by 6 h after E2 and is predominately epithelial (Tong and Pollard, 1999). Of note, cyclin E regulates G_1-S transition *in vitro* (Hunter and Pines, 1994). In mouse uterus, E2-induced cyclin E protein levels are 2- to 5-fold lower than those of cyclin A2 throughout G_1 and S

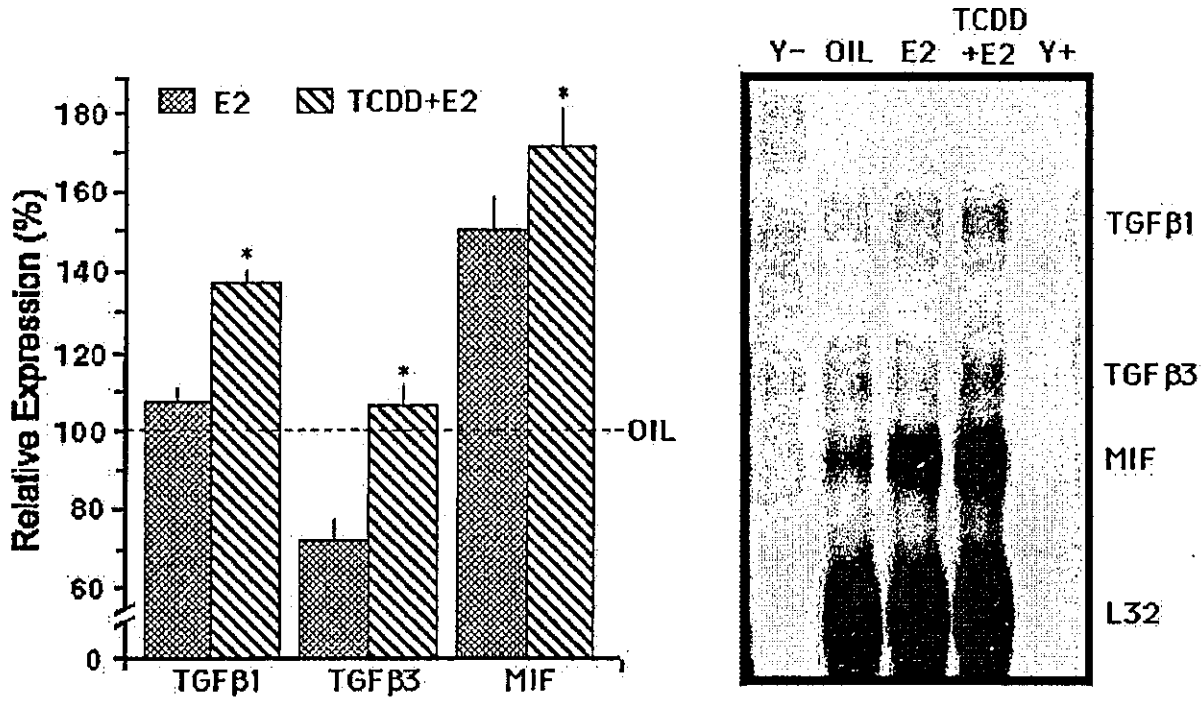

FIG. 2. TCDD effect on estrogen (E2)-induced uterine TGF-β mRNA expression by RNase protection assay. Representative blot with normalized densitometric data shown graphically. Animals received oil or TCDD (5 μg/kg, ip) 24 h before E2. Uteri were collected 6 h post-E2 or oil; mRNA levels from oil controls were considered baseline. TGF-β1 was unchanged and TGF-β3 was suppressed by E2 ($p < 0.01$) compared to baseline. However, TCDD dosing completely abolished E2 suppression of TGF-β3 ($p < 0.01$) and increased TGF-β1 by 28% ($p < 0.0001$), compared to E2 (asterisks). E2 induction of migration inhibiting factor (MIF) was augmented by TCDD ($p < 0.0001$). Total RNA was pooled from 8 to 12 mice for each treatment. Densities were normalized to mRNA for the ribosomal protein L32. Inset: Y–, yeast RNA minus RNase; Y+, yeast RNA plus RNase.

(Tong and Pollard, 1999), pointing to cyclin A2 as a substantial E2-induced cyclin for controlling the initiation of DNA synthesis. When mice were given TCDD in the present study, cyclin A2 mRNA induction by E2 was specifically abolished, while cyclin D2 and B1 expressions were decreased compared to animals given E2 alone. This antimitogenic effect during G_1 by TCDD has never before been described in the uterus, nor in opposition to hormone treatment *in vivo*. Although it is not clear how E2 regulates cyclin A2 or how TCDD disrupts E2 signaling necessary for cyclin expression, the TCDD effect on cyclin A2 is maximal and may be useful for understanding E2 signaling. To further investigate this, the TCDD antiestrogenic effect on uterine cytokine gene expression was examined.

TGF-β promotes stromal growth and extracellular matrix deposition (Sporn and Roberts, 1990). In epithelial cell cultures, TGF-β is a potent antimitogen and inhibits several G_1 phase cyclins (Geng and Weinberg, 1993). TGF-β blocks proliferation early by preventing hyperphosphorylation of retinoblastoma (Rb) protein, but it loses this effect by late G_1 (Geng and Weinberg, 1993). Importantly, TGF-β specifically suppresses cyclin A2 gene expression *in vitro* (Feng *et al.*, 1995; Yoshizumi *et al.*, 1997). The present results show *in vivo* that low uterine TGF-β levels are associated with increased cyclin expression. These events are inversely correlated as indicated by TCDD treatment. TCDD results in the opposite response to E2 so that increased TGF-β1 and β3 levels are accompanied by decreases in mRNAs for specific E2-induced cyclins that are important for normal G_1 phase progression and S phase initiation. This correlation is indicative of alterations in normal uterine E2 signaling and further underscores the use of TCDD to understand E2 signaling *in vivo*.

Uterine TGF-β1, β2, and β3 mRNAs are maximally increased by 3 h after E2, but β1 and β3 are suppressed below oil control levels and β2 is undetectable at 6 h after injection of an E2 analogue in weanling mice (Takahashi *et al.*, 1994). Consistent with this, TGF-β1 and β3 were similar to or suppressed below oil control levels, respectively, while β2 was undetectable after 6 h of E2 exposure in the present study. Importantly, TCDD increased E2-regulated TGF-β1 and β3 levels, suggesting a possible mechanism for TCDD suppression of E2-induced uterine cyclins and consequently epithelial mitogenesis. Unexpectedly, E2 induction of MIF was augmented by TCDD. MIF expression is increased by TGF-β in murine colon carcinoma cells (Takahashi *et al.*, 1998). Thus, the additional increase in uterine MIF after TCDD likely tracks the TGF-β increase and evidently results from increased TGF-β protein signaling. These findings, are the first indication that TCDD alters E2 regulation of uterine TGF-β.

TGF-β1 and β3 upregulation following TCDD has been reported in MCF-7 cell and thymus organ cultures (Lai *et al.*, 1997; Vogel and Abel, 1995), but the mechanism leading to the increase is not known. Mouse TGF-β1 contains dioxin response elements (DREs) in its gene promoter (Lai *et al.*, 1996), but no DREs have been found in the β3 promoter. Similar to the results of Kover *et al.* (1995), E2 did not alter uterine expression of cytokines TNF-α, IL-6, or IFN-γ, and their levels were unaffected by TCDD under the present conditions, even when their promoters contained a DRE (IL-6 and IFN-γ; Lai *et al.*, 1996). This lack of effect by TCDD is consistent with the theory that increased TGF-β levels after TCDD may not be due to TGF-β gene induction, but could result from stabilization of TGF-β mRNA levels reported to be maximum at 3 h post-E2 (Takahashi *et al.*, 1994) and/or inhibition of E2-induced suppression at 6 h. Alternatively, since the TGF-β1 promoter has AP-1 elements, it may be upregulated by that mechanism (Fos-Jun; Kim *et al.*, 1990) since TCDD upregulates both c-fos and c-jun and increases AP-1 DNA binding (Hoffer *et al.*, 1996). TCDD may also behave as an AhR antagonist for TGF-β regulation. Liver abnormalities in AhR-null mice are attributed to TGF-β production by hepatocytes, resulting in apoptosis (Zaher *et al.*, 1998). Thus, possible AhR suppressive effects may be antagonized by TCDD *in vivo* to allow TGF-β upregulation. Studies are now under way to better understand the TCDD effect and role of AhR on uterine TGF-β gene expression and protein signaling.

Several explanations for the TCDD inhibitory effect on cyclins are possible. *In vitro*, the Rb protein is required for cyclin A2 and cyclin D activity during G_1 (Feng *et al.*, 1995; Hunter and Pines, 1994). Puga and colleagues (2000) found that AhR interaction appears to maintain Rb in the hypophosphorylated state and contributes to cell cycle arrest in cultures. AhR may exhibit such effects in the presence of hormone *in vivo*, but this remains to be investigated. Another possibility is that liganded AhR can interfere with liganded ER binding at the gene promoter (Krishnan *et al.*, 1995). However, a DRE has not been described in either mouse or human cyclin A2 genes.

The changes we observed in cyclin and MIF levels, which correlated with changes in TGF-β following E2 and TCDD treatments in the present study, are consistent with TGF-β protein signaling (Slingerland *et al.*, 1994). Moreover, the TCDD effect on TGF-β and cyclin mRNAs are actual fundamental changes that likely upset transcript level balance and cellular signaling, and as such, could alter cellular functioning as demonstrated by TCDD inhibition of E2-induced uterine epithelial proliferation and secretory protein activity (Buchanan *et al.*, 2000).

The findings herein are the first to show that TCDD may inhibit uterine epithelial mitogenesis by altering E2-regulated cellular signaling necessary for cyclin production and S phase initiation. We have demonstrated that a TCDD dose capable of inhibiting uterine epithelial proliferation 18–24 h after E2 also increases TGF-β mRNAs and abolishes cyclin A2 mRNA at 6 h after E2. Thus, TCDD alters E2-regulated transcription for cell signaling factors associated with normal cyclin gene expression. Further, TCDD-induced decreases in mRNAs of cyclins critical for G_1 phase progression and S phase entry suggest increased TGF-β protein activity and provide a possible explanation for TCDD inhibition of E2-induced uterine epithelial proliferation *in vivo*. Additional research on the antiestrogenic TCDD effect *in vivo* should continue to provide valuable clues for understanding steroid signaling pathways.

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