

Regulatory Mechanisms of Adrenal Gland Zona Glomerulosa-Specific 3 β -HSD

(副腎アルドステロン産生細胞特異的3 β -HSDアイソフォームの
発現制御機構)

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Prolegomenon

The enzyme 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD) is essential for the biosynthesis of all active steroid hormones, including those secreted from the adrenal gland. Whereas two distinct 3 β -HSD isoforms (type I 3 β -HSD, which is encoded by *HSD3B1*, and type II 3 β -HSD, which is encoded *HSD3B2*) exist in humans, it has long been thought that type II 3 β -HSD was the only isoform expressed in the human adrenal glands. However, this canonical view was recently revised due to the observation that the alternative isoform, HSD3B1, is expressed within zona glomerulosa (ZG) cells [1], where aldosterone is produced. Interestingly, the mouse also has two isoforms in the adrenal: one (*Hsd3b1*) is ubiquitous in the cortex, but the other (*Hsd3b6*) is ZG specific [2]. Thus, in both species, the adrenals possess a ZG-specific isoform, in addition to the ubiquitous one. However, it remains unknown why these different enzymes are expressed simultaneously in ZG cells. Since these isozymes catalyze the same enzymatic reaction, the question remains open why the newly identified ZG-specific isoform needs to be present in the ZG cells [3].

Because the ZG cells are the principal place where aldosterone is produced and *in vivo* aldosterone production is under multiple dynamic regulations by angiotensin II (AngII), potassium (K⁺), and circadian clock, this thesis is designed to investigate specific regulatory modes of this enzyme. The data shown for AngII (**chapter 1**), K⁺ (**chapter 2**), and circadian clock (**chapter 3**) illustrate the unique responsive properties of this gene.

References

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Chapter 1: Angiotensin II triggers expression of the adrenal gland zona glomerulosa-specific 3 β -HSD isoenzyme through de novo protein synthesis of the orphan nuclear receptors NGFIB and NURR1

Introduction

The enzyme 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD) is essential for the biosynthesis of all active steroid hormones, including those secreted from the adrenal gland [1-4]. Whereas two distinct 3 β -HSD isoforms (type I 3 β -HSD, which is encoded by *HSD3B1*, and type II 3 β -HSD, which is encoded by *HSD3B2*) exist in humans, it has long been thought that type II 3 β -HSD was the only isoform expressed in the human adrenal glands [1]. However, this canonical view was recently revised due to the observation that the alternative isoform, HSD3B1, is expressed within zona glomerulosa (ZG) cells [5,6], where aldosterone is produced. Interestingly, the mouse also has two isoforms in the adrenal: one (*Hsd3b1*) is ubiquitous in the cortex, but the other (*Hsd3b6*) is ZG-specific [6-9]. Thus, in both species, the adrenals possess a ZG-specific isoform, in addition to the ubiquitous one. However, it remains unknown why these different enzymes are

expressed simultaneously in ZG cells. Since these isozymes catalyze the same enzymatic reaction, the question remains open why the newly identified ZG-specific isoform needs to be expressed in ZG cells.

Angiotensin II (AngII) is the key peptide hormone in the renin-angiotensin-aldosterone system (RAAS). AngII is a potent secretagogue of the mineralocorticoid aldosterone, which is principally synthesized within the adrenal gland ZG cells through a series of enzymatic reactions that involve multiple enzymes, including 3 β -HSDs. The actions of AngII on ZG cells are often divided into two temporally different phases [10-12]: (i) an early (within minutes after stimulation) upregulation of aldosterone synthesis through posttranslational activation of steroidogenic acute regulatory (StAR) protein that facilitates the transfer of cholesterol (steroid precursor) to the mitochondria and (ii) a relatively late (hours after stimulation) enhancement of aldosterone synthesis by increasing the capacity to produce aldosterone through increased expression of relevant enzymes in ZG cells. It has been well documented that AngII triggers expression of CYP11B2 (aldosterone synthase), an enzyme also known to be expressed specifically within ZG cells. CYP11B2 and HSD3B1 are the only known steroidogenic enzymes whose expression is limited to ZG cells in the adrenal gland. However, virtually nothing is known about whether the expression

of HSD3B1 is under the control of AngII [13,14]. This paucity of knowledge is partly because of the high sequence similarity between *HSD3B1* and *HSD3B2* (93.6% identity, including the 5' and 3' untranslated regions [UTRs]) [5,6], which made it difficult to achieve isoform-selective quantification of their transcripts without the recent TaqMan MGB probe technology that was devised for single nucleotide discrimination between target genes [6,15].

A better understanding of *HSD3B1* and *HSD3B2* is also critical for the comprehension of adrenal disorders. The results of pathological investigations of human idiopathic hyperaldosteronism [5] and its animal model (circadian clock-deficient *Cry*-null mice) [6] revealed that abnormally increased expression of the ZG isoform (HSD3B1 for human, *Hsd3b6* for mouse) is closely associated with the enhanced aldosterone production of this disease. On the other hand, decreased expression of HSD3B1 and HSD3B2 was found in ZG cells located in a nontumor portion of an aldosterone-producing adenoma (APA)-containing adrenal gland [5,16,17], suggesting a feedback regulation of 3 β -HSDs as a compensatory response to excess aldosterone from the APA. Interestingly, aldosterone synthase CYP11B2 showed sustained expression in APA-associated ZG cells [5,18]. Thus, it appears that the expression of 3 β -HSDs and CYP11B2 is differently regulated and that the levels of 3 β -HSDs

might be an additional regulatory element for determining the capacity of steroid production in ZG cells. However, it is currently unknown how the expression levels of *HSD3B1* are regulated.

The present study was undertaken to elucidate whether AngII controls *HSD3B1* and/or *HSD3B2* and, if so, by what molecular mechanism(s). Using human adrenocortical H295R cells as a model system, I found that AngII is able to induce *HSD3B1* but not *HSD3B2*. Similarly, mouse adrenal glands showed selective induction of *Hsd3b6* (ZG isoform) but not *Hsd3b1* (ubiquitous isoform) after AngII treatment. This data also provide evidence that the acute induction of *HSD3B1* is fully dependent on AngII-stimulated *de novo* protein synthesis of the orphan nuclear receptors, NGFIB and NURR1. Interestingly, the NGFIB protein family has been implicated in the regulation of *CYP11B2* as well [19-22]. However, I found that the regulation of this gene is not entirely dependent on *de novo* protein synthesis. A mechanistic difference between *HSD3B1* and *CYP11B2* is discussed.

Materials and Methods

Animals

All animal studies were performed with protocols approved by the animal experimentation committee of Kyoto University. C57BL/6 male mice aged 8 weeks were purchased from local suppliers and housed in 12-h light/12-h dark cycle (lights on at 08:00, lights off at 20:00) for 7 days. Then, the animals were transferred to a low-sodium diet (0.001% elemental Na⁺ diet; CREA, Japan) at 08:00 (zeitgeber time zero), and the adrenal glands were removed by surgery after 24 or 48 h of sodium restriction. Since all samples were collected at 08:00, there is no confounding factor derived from circadian time on the expression of target genes. For AngII treatment, AngII (Peptide Institute, Japan) was injected into the mice at 08:00 intraperitoneally at a dose of 1 mg per kg body weight, and the adrenals were removed from the animals at 4 and 24 h after the injection. For the enucleation of the adrenal gland, the adrenals freed of adherent fat were mechanically separated into the capsular portions according to a conventional method [6,23]. The adrenal samples were harvested in either TRIzol reagent (Invitrogen) for subsequent RNA analysis or 4% paraformaldehyde-containing fixative solution for *in situ*

hybridization (see below).

Cell culture and treatments

Human adrenocortical H295R cells (ATCC CRL-2128) were cultured in Dulbecco modified Eagle medium (DMEM)–F-12 medium (Invitrogen) supplemented with 2.5% Nu serum (BD Biosciences) and 1% ITS premix (BD Biosciences). H295R cells are one of the best-characterized cellular models for the analysis of adrenal cell biology [24], since this is the human adrenal cell line that preserves the ability to secrete aldosterone in response to AngII [25]. For AngII stimulation, an aliquot of freshly reconstituted AngII (10 μ M) was added to the culture medium at a final concentration of 100 nM. To specify the type of AngII receptors involved, I also added either the AT1R blocker CV11974 (final concentration, 100 nM; a generous gift from Takeda Pharmaceutical) or the AT2R blocker PD123319 (final concentration, 1 μ M; Sigma) to the culture medium 1 h before AngII treatment. Pharmacological inhibition of *de novo* protein synthesis was also carried out by adding cycloheximide (CHX; final concentration, 10 μ g/ml) to the medium 15 min before AngII treatment. At 1, 2, 4, 6, 8, and 12 h after AngII treatment, the cells were harvested in TRIzol reagent (Invitrogen) for subsequent RNA analysis.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's protocol. Total RNA was converted to cDNA with random hexamer primers using SuperScript III first-strand synthesis Super-Mix (Invitrogen), and quantitative PCR (qPCR) was run in duplicate with the primers and probes shown below. For the analysis of human *HSD3B1* and *HSD3B2*, qPCR was done with the TaqMan Universal master mix (Applied Biosystems) using gene-specific TaqMan MGB probes. As I previously reported [6], these probes distinguish a few nucleotide differences at the region corresponding to the dehydrogenase catalytic Y-X-X-X-K motif [1] of the human 3 β -HSDs. On the other hand, SYBR green-based qPCR was done for the other genes with the aid of a Platinum SYBR green qPCR SuperMix-UDG kit (Invitrogen). As a qPCR device, I used a StepOnePlus real-time PCR monitoring system (Applied Biosystems), and the quantification of target cDNAs was achieved with a standard curve method as described previously [7]. The data were normalized to those for *Rplp0*. The sequences for the primers and probes are as follows: for mouse *Hsd3b1*, forward primer 5'-AGC ATC CAG ACA CTC TCA TC-3' and reverse primer 5'-GGA GCT GGT ATG ATA TAG GGT A-3'; for mouse *Hsd3b6*, forward

primer 5'-TGA TGG GAA GAG GGT GGA G-3' and reverse primer 5'-AGG TGC TGA GAG GCT TGG A-3'; for mouse *Rplp0*, forward primer 5'-CTC ACT GAG ATT CGG GAT ATG-3' and reverse primer 5'-CTC CCA CCT TGT CTC CAG TC-3'; for human *StAR*, forward primer 5'-CCT GAG CAG AAG GGT GTT CA-3' and reverse primer 5'-CCA ACG GGT GAA GCA CCA T-3'; for human *CYP11A1*, forward primer 5'-AGC CAG CAT CAA GGA GAC ACT A-3' and reverse primer 5'-ACC AGT GTC TTG GCA GGA ATC A-3'; for human *CYP21A2*, forward primer 5'-CAC TGA GAC CAC AGC AAA CAC-3' and reverse primer 5'-CTG CAG TCG CTG CTG AAT C-3'; for human *CYP11B1*, forward primer 5'-GCC ATC AAC TAA TCA CGA CT-3' and reverse primer 5'-TGA TCT TAG CCT TCT AAG CCT T-3'; for human *CYP11B2*, forward primer 5'-ACT CGC TGG GTC GCA ATG-3' and reverse primer 5'-GTC TCC ACC AGG AAG TGC-3'; for human *NGFIB*, forward primer 5'-GCC TCC TGG AGG CTC TTC ATC-3' and reverse primer 5'-GAG AAG GCC AGG ATA CTG TCA ATC-3'; for human *NURR1*, forward primer 5'-GGC TCC CAG AGG GAA CTG-3' and reverse primer 5'-GAG TCC AGC CTG TCC AAT CTC-3'; for human *NOR1*, forward primer 5'-TCC GCT CCT CCT ACA CTC TC-3' and reverse primer 5'-GGT GTA TTC CGA GCT GTA TGT CTG-3'; for human *RPLP0*, forward primer 5'-ATG CAG CAG ATC CGC ATG T-3' and reverse primer 5'-TTG CGC ATC ATG GTG TTC TT-3'; for human *HSD3B1*, forward primer 5'-AGA AGA GCC TCT GGA AAA CAC

ATG-3', reverse primer 5'-TAA GGC ACA AGT GTA CAG GGT GC-3', and probe 5'-FAM-CCA TAC CCA CAC AGC-MGB-3' (where FAM is 6-carboxyfluorescein); and for human *HSD3B2*, forward primer 5'-AGA AGA GCC TCT GGA AAA CAC ATG-3', reverse primer 5'-CGC ACA AGT GTA CAA GGT ATC ACC A-3', and probe 5'-VIC-TCC ATA CCC GTA CAG CA-MGB-3'.

***In situ* hybridization**

Radioisotopic *in situ* hybridization was performed as described previously [6]. Briefly, paraformaldehyde-fixed adrenal glands were frozen and sectioned at a thickness of 30 μm . The free-floating sections were then transferred sequentially through 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer for 10 min, 1 $\mu\text{g}/\text{ml}$ proteinase K in 0.1 M Tris buffer (pH 8.0) with 50 mM EDTA for 15 min at 37°C, 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and 2 \times SSC buffer for 10 min. Then, the sections were transferred to hybridization buffer (55% formamide, 10% dextran sulfate, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.6 M NaCl, 0.2% N-laurylsarcosine, 0.5 g/ml tRNA, 1 \times Denhardt's solution, 0.25% SDS, 10 mM dithiothreitol [DTT]) containing radiolabeled riboprobes (4×10^6 dpm) and incubated at 60°C for 16 h. Following a high-stringency posthybridization wash, the sections were treated

with RNase A. Air-dried sections were exposed to X-ray films.

Western blotting

The H295R cells that had been treated with 100 nM AngII for periods ranging from 1 to 12 h were harvested in Laemmli buffer, and the lysates were subjected to Western blot analysis with antibodies to NGFIB (1:500 dilution; M-210 antibody; Santa Cruz Biotechnology), NURR1 (1:1,000 dilution; N1404 antibody; Perseus Proteomics), NOR1 (1:500 dilution; H7833 antibody; Perseus Proteomics), and β -actin (1:1,000 dilution; AC-15 antibody; Sigma) or α -tubulin (1:1,000 dilution; T6199 antibody; Sigma). For the detection of the dominant negative (DN) form of NGFIB (DN-NGFIB; which corresponds to the C-terminal 249-amino-acid portion of NGFIB), I employed an alternative antibody (1:500 dilution; E-20 antibody; Santa Cruz Biotechnology) that was raised against the C-terminal region of NGFIB. Immunoblotting was done according to our standard protocol [26].

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins extracted from H295R cells were subjected to a gel shift assay as described previously [27], with certain modifications. The sequence of the oligonucleotide probe

(5'-TAA CCC AAA GGT CAC TAT TTT-3') was designed to encompass the NGFIB-responsive element (NBRE) site of the human *HSD3B1* promoter (underlined). Radioactive probe was generated by end labeling the annealed oligonucleotide pairs with T4 polynucleotide kinase and [γ -³²P] ATP. The binding reactions were performed on ice for 30 min in a 20- μ l reaction mixture that contained 4 μ g of the nuclear extract proteins together with 4×10^5 dpm of the radiolabeled oligonucleotide probe in 10% glycerol, 250 mM NaCl, 25 mM Tris-HCl (pH 7.9), 0.5 mM DTT, 0.5 mM EDTA, 6.25 mM MgCl₂, 1 μ g/ μ l bovine serum albumin (BSA), and 0.2 μ g/ μ l poly(dG-dC). For antibody supershift assays, 1 μ g of specific antibodies that recognize NGFIB (anti-NGFIB; M-210 antibody), NURR1 (anti-NURR1; N-20 antibody), or NOR1 (anti-NOR1; A-20 antibody) or those recognizing both NGFIB and NURR1 (anti-NGFIB/NURR1; E-20 antibody) (all antibodies were from Santa Cruz Biotechnology) were preincubated with the nuclear extract on ice for 30 min before the addition of the radioactive probe. In control experiments, the antibody was replaced with nonimmune antibodies (IgG). For competition assay, a 100-fold molar excess of unlabeled probe was added to the reaction mixture simultaneously with the radiolabeled probe. The mutant oligonucleotide probe used for the competition assay was as follows: 5'-TAA CCC AGA ATT CAC TAT TTT-3' (underlining indicates

mutated NBRE). After the binding reactions, the resultant DNA/protein complexes were separated on a 4% native polyacrylamide gel in 1× Tris-glycine buffer containing 1% glycerol. The gel was dried and exposed to an X-ray film.

Chromatin immunoprecipitation (ChIP)

H295R cells grown to confluence on a 10-cm dish ($\sim 1 \times 10^7$ cells) were treated with either AngII (100 nM) or vehicle for 3 or 12 h. Then, the cells were homogenized in 1.2 ml (per dish) of phosphate-buffered saline (PBS) containing 2 mM disuccinimidyl glutarate (Pierce), and the homogenates were kept for 20 min at room temperature. Then, methanol-free formaldehyde (final concentration, 1%; Thermo) was added for 5 min at room temperature, and 125 μ l of 1.5 M glycine (final concentration, 150 mM) was added to stop the reaction on ice. The homogenates were centrifuged at 700× *g*, and the resultant nuclear pellets were washed twice with ice-cold PBS. The nuclei were resuspended in 2.5 ml of immunoprecipitation (IP) assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail) and sonicated 10 times for 30 s each time at 4°C using a Bioruptor UCW-201TM apparatus (Tosho Denki,

Yokohama, Japan). Approximately 1.5 μ g of fragmented chromatin was precleared by incubating with 40 μ l of protein A-agarose (Roche) for 2 h at 4°C on a rotating wheel. Precleared chromatin was then incubated with 1 μ g of anti-NGFIB/NURR1 antibody (E-20 antibody; catalog number sc-990; Santa Cruz Biotechnology) or control normal rabbit IgG (catalog number sc-2027; Santa Cruz Biotechnology) overnight at 4°C on a rotating wheel, 10 μ l of protein A/G Plus-agarose (Santa Cruz Biotechnology) was added to each sample, and the mixture was incubated for 2 h at 4°C. Beads were then washed once with IP assay buffer, once with high-salt wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF), once with LiCl wash buffer (20 mM Tris-HCl, pH 8.0, 250 mM LiCl, 2 mM EDTA, 0.5% Nonidet P-40, 1% sodium deoxycholate, 1 mM PMSF), and twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Coimmunoprecipitated DNA fragments were eluted with 100 μ l of elution buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% sodium dodecyl sulfate) and then reverse cross-linked at 65°C overnight, incubated with 10 μ g of RNase A for 30 min at 37°C and 50 μ g of proteinase K for 90 min at 55°C, and then purified using a QIAquick PCR purification kit (Qiagen). Immunoprecipitated DNA fragments were quantified by TaqMan qPCR for the NBRE site of *HSD3B1* with the following primers and

probe: forward primer 5'-CCT GTT AAG GCT AAA CCC AAG AC-3', reverse primer 5'-CAT TGC TCT CTC CTC CTA TGG G-3', and TaqMan probe 5'-VIC-TGC CAC ACT GCA GCA TTA GGA TGG G-NFQ-MGB-3'.

Reporter assays

Expression vectors for human NGFIB (hNGFIB/pEZ; catalog number EX-A0227-M02) and NURR1 (hNURR1/pEZ; catalog number EX-I0539-M02) were purchased from GeneCopoeia. The following reporter plasmids were used in this study: (i) pGL4.10 *HSD3B1* promoter-Luc (1332), in which a 1,332-bp genomic DNA fragment upstream of the translation start site (position +274) of the human *HSD3B1* was cloned into the pGL4.10 (luc2) vector (Promega); (ii) pGL4.10 *HSD3B1* promoter-Luc (599), in which a 599-bp sequence upstream of the transcription start site of *HSD3B1* was cloned into pGL4.10 (luc2); (iii) pGL4.10 *HSD3B1* promoter-Luc (200) and pGL4.10 *HSD3B1* promoter-Luc (100), which are deletion constructs of pGL4.10 *HSD3B1* promoter-Luc (599) containing either 200 or 100 bp of the 5'-flanking sequence; (iv) pGL4.10 *HSD3B1* mut NBRE promoter-Luc (599), in which the NBRE sequence of pGL4.10 *HSD3B1* promoter-Luc (599) was mutated to 5'-AGAATTCA-3' with a standard sequential PCR method [28]; (v) pGL4.23 NBRE-Luc, in which a DNA fragment containing nine tandem copies of the

sequence corresponding to the *HSD3B1* NBRE with its flanking sequences (positions -141 to -72) was inserted into the pGL4.23 (luc2/minP) vector (Promega); and (vi) pGL4.23 mut NBRE-Luc, which is the same as pGL4.23 NBRE-Luc, except that every NGFIB-binding site was mutated to 5'-AGAATTCA-3'. Cell culture and transfection assays were carried out as previously described [6], except that the Lipofectamine LTX/Plus reagent (Invitrogen) was used as the transfection reagent according to the manufacturer's protocol.

siRNA transfection

H295R cells were transfected using Nucleofector technology (Amaxa Biosystems). Three million log-phase cells were resuspended in 100 μ l Nucleofector solution R containing 2 μ M (final concentration) small interfering RNA (siRNA) mixtures for NGFIB (siRNAs s6978 and s6979), NURR1 (siRNAs s9785 and s9786), and NOR1 (siRNAs s15541 and s15543) or negative-control siRNA (catalog number 12935112) (all siRNAs were from Life Technologies) and electroporated using the proprietary program P-20. Cells were allowed to recover for 15 min in RPMI 1640 medium at 37°C and then plated in 48-well plates with 0.5 ml H295R cell complete medium at a density of 5×10^5 cells/well. One day after the

electroporation, dead cell debris was removed by refreshing the medium, and the cells were cultured for 48 h. Then, the culture medium was removed and the cells were incubated in serum-free medium (DMEM–F-12 medium) for 24 h, followed by a treatment with or without AngII for 4 h. At the end of the treatment, the cells were washed with PBS and immediately lysed in TRIzol reagent for RNA extraction or boiled in Laemmli buffer for Western blotting.

Isolation of DN-NGFIB-transfected H295R cells by the MACSelect system

DN-NGFIB (the C-terminal 249-amino acid portion of NGFIB) was produced by deleting the N-terminal transactivation domain of human NGFIB. This protein is defective in transactivation function but shares the same DNA sequence binding specificity with the NGFIB family. Thus, the DN-NGFIB protein acts as an inhibitor for all three subfamily members: NGFIB, NURR1, and NOR1 [29,30]. For coexpression of DN-NGFIB with a cell surface marker protein, H-2K^k (Miltenyi Biotec), I used the pMACS K^k.II expression vector (Miltenyi Biotec), which allows the simultaneous expression of H-2K^k and the gene of interest from a single vector. Since in H295R cells the simian virus 40 (SV40) promoter shows lower levels of activity than the cytomegalovirus (CMV) promoter, the SV40 promoter

region of this vector was replaced by the CMV promoter. Then, the modified vector (pMACS K^k.II CMV) was used for cloning and expression of DN-NGFIB. Transient transfection of H295R cells was done by lipofection using the Lipofectamine LTX/Plus reagent (Invitrogen), for which I routinely observed an ~25% transfection efficiency (an estimation from green fluorescent protein-transfected H295R cells). The cells seeded in 10-cm dishes (5×10^6 cells/dish) were transfected with 24 μ g of the DN-NGFIB/pMACS K^k.II CMV plasmid. After transfection, the cells were allowed to recover for 72 h prior to AngII treatment. Following 3 h treatment with AngII (100 nM) or vehicle, the cells were washed and removed with Versene solution (Invitrogen) and then dissociated by gentle pipetting and passed through a 40- μ m-pore-size cell strainer (BD Falcon). The resultant cell suspensions were resuspended in ice-cold PBS (150 μ l) containing 0.5% BSA and 1 mM EDTA and mixed with MACSelect K^k microbeads (1:375 dilution; Miltenyi Biotec). After incubation on ice for 15 min, the cells were isolated on a magnetic separation column (Miltenyi Biotec) under a magnetic field according to the manufacturer's protocol. The cells underwent two consecutive rounds of purification. Then, the second round of eluted cells and the first round of flowthrough cells were washed twice with ice-cold PBS, and these cell samples were either lysed in TRIzol reagent for

qRT-PCR or boiled in Laemmli sample buffer for Western blotting.

Statistical analysis

I used Student's t test for the comparison of two test groups (see Fig. 1B and D). For the experiments in which three or more test groups were compared, I used one-way analysis of variance (ANOVA) with Bonferroni's post hoc test (see Fig. 1A). The differences arising from two independent parameters (see Fig. 1E, 4G, 6A, and 7C) were assessed through two-way ANOVA, followed by Bonferroni's multiple-comparison test.

Results

AngII triggers expression of *Hsd3b6* but not *Hsd3b1* in the mouse adrenal gland.

The mouse *Hsd3b6* gene is the specific 3 β -HSD isoform whose adrenal expression is confined to ZG cells [6,7]. To study whether the activation of the renin-angiotensin system influences this gene's expression profile in the adrenal, mice were placed on a low-sodium diet (0.001% elemental Na⁺) for 1 or 2 days, and the levels of adrenal expression of *Hsd3b6* and *Hsd3b1* mRNA were examined by qRT-PCR with gene-specific primers (Fig. 1A). I found that sodium restriction significantly increased *Hsd3b6* mRNA levels in the whole adrenal gland within 24 h after treatment (Fig. 1A). In contrast, *Hsd3b1* mRNA levels did not show any appreciable increase even after 48 h of sodium restriction (Fig. 1A). Analogous results were also observed for the enucleated adrenal gland sample (i.e., the capsular portions of the adrenal gland), which consists largely of ZG cells (Fig. 1B): the capsular mRNA levels of the ZG-specific isoform, *Hsd3b6*, were significantly increased by more than 3-fold after 2 days of sodium restriction, whereas those of the canonical isoform, *Hsd3b1*, remained unchanged. *In situ* hybridization of the whole adrenal sections with a radiolabeled

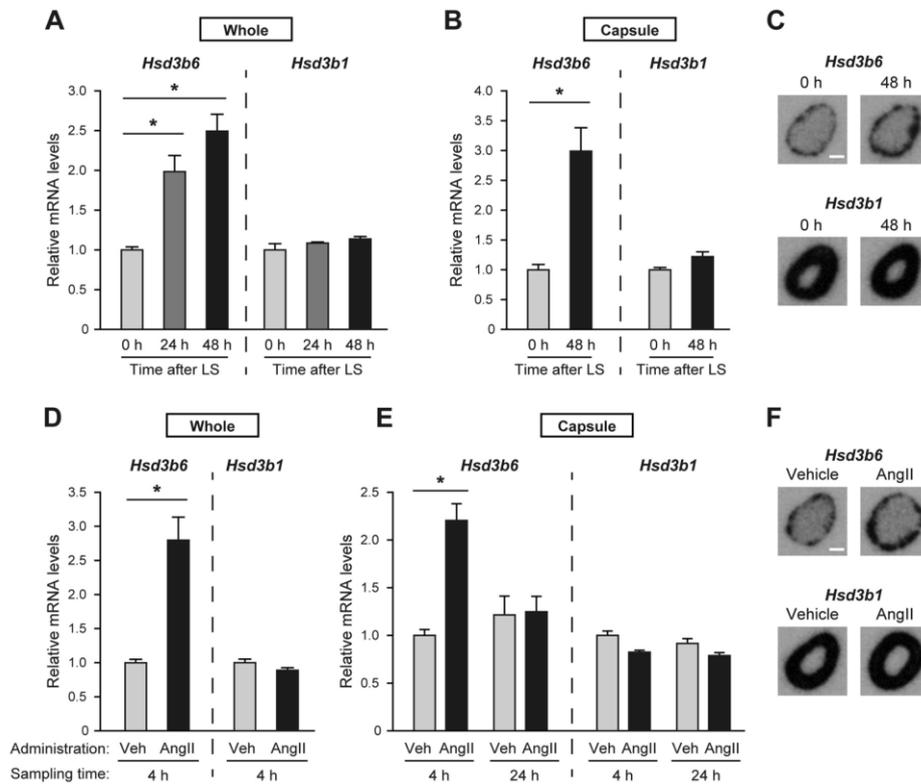


Fig. 1. The mouse adrenal *Hsd3b6* increases in response to AngII treatment and a low-salt diet. (A to C) Adrenal *Hsd3b6* and *Hsd3b1* mRNA levels in mice after a change to a low-salt (LS) diet. The adrenals were removed from animals at 0, 24, and 48 h after sodium restriction. Total RNA was prepared from either whole adrenal (A) or the ZG cell layer-containing capsular portion of the adrenal gland (B). The mRNA levels of *Hsd3b6* and *Hsd3b1* were determined by gene-selective qRT-PCR. All the values (means \pm SEM, $n = 3$) were normalized to the levels of *Rplp0*, and the values at time zero were set equal to 1. *, $P < 0.01$. Autoradiographs (C) show mouse adrenal sections hybridized with a radioisotopic hybridization probe for *Hsd3b6* and *Hsd3b1* before and after 48 h of sodium restriction. Bar, 0.5 mm. (D to F) Adrenal *Hsd3b6* and *Hsd3b1* mRNA levels in mice after a single intraperitoneal administration of AngII (1 mg/kg) or the vehicle (Veh) control (saline). Adrenals were removed from the animals at 4 and 24 h after administration, and total RNAs prepared from either whole adrenal (D) or the capsule portion (E) were analyzed by qRT-PCR, as described in the legends to panels A and B. *, $P < 0.01$. Autoradiographs (F) show the sections of the adrenal collected at 4 h after the administration of AngII or vehicle. Bar, 0.5 mm.

probe for *Hsd3b6* (Fig. 1C) confirmed selective induction of this gene in ZG cells, which constitute the outer layer of the cortex.

In order to determine whether the expression of *Hsd3b6* is directly regulated by AngII, mice were then treated with AngII (Fig. 1D to F). I found that a single intraperitoneal administration of AngII (1 mg/kg) caused a rapid and transient induction of *Hsd3b6* (Fig. 1D): the expression of *Hsd3b6* mRNA in the whole adrenal gland increased approximately 3-fold over the basal levels (vehicle treatment) after 4 h of administration, but it returned to near basal levels within 24 h after AngII treatment (Fig. 1E). No significant increase was observed for *Hsd3b1* (Fig. 1D and E). Selective induction of *Hsd3b6* was also observed in the qPCR analysis of the enucleated adrenal gland (Fig. 1E), as well as with the isoform-selective *in situ* hybridization of adrenal gland sections (Fig. 1F).

AngII triggers expression of *HSD3B1* but not that of *HSD3B2* in human adrenocortical H295R cells.

Our laboratory recently showed that *HSD3B1* is exclusively expressed in ZG cells in the human adrenal gland [5,6]. This identification raises the question of whether the expression of this gene is under the regulation of AngII. Using adrenocortical H295R cells as a model system, I next sought to investigate AngII

responsiveness of the human 3 β -HSD isoform genes (see ref. [31]). I treated H295R cells with AngII (100 nM) over a range of times (1, 2, 4, 8, and 12 h), and the gene expression profiles of human *HSD3B1* and *HSD3B2* were examined by qRT-PCR with the aid of isoform-specific TaqMan MGB probes and primers that our laboratory developed previously (6) (see also Materials and Methods) (see ref. [31]). Because of the high degree of cDNA sequence similarity between the two isoforms (93.6% identity, including the 5' and 3' UTRs) [5,6], studies aimed at isoform-selective comparison between *HSD3B1* and *HSD3B2* have not been available in the past for H295R cells [13,14]. Interestingly, I observed that AngII caused a marked transient induction of *HSD3B1* (see ref. [31]): after 4 h AngII treatment, the levels of *HSD3B1* mRNA were dramatically increased up to about 10-fold over basal levels. On the other hand, *HSD3B2* was not responsive to AngII (see ref. [31]): no appreciable increase was observed for this gene over 12 h of AngII treatment. Thus, these results indicate that the ZG-specific isoform *HSD3B1* differs from *HSD3B2* in the ability to respond to AngII stimulation.

There are two different AngII receptor subtypes, AT1 and AT2. Previous studies have shown that AT1 is the principal AngII receptor for the regulation of aldosterone biosynthesis in the human

adrenal gland as well as in H295R cells [24,32]. Consistent with this, AngII-induced induction of *HSD3B1* mRNA in H295R cells was completely blocked by pharmacological treatment with the AT1 receptor antagonist, CV11974, whereas no inhibition was observed in response to treatment with the AT2 receptor antagonist, PD123319 (see ref. [31]). These results indicate that the AT1 receptor is responsible for the AngII-induced *HSD3B1* mRNA expression.

As reported, AngII stimulation-dependent acute mRNA induction was observed for *StAR*, *CYP21A*, *CYP11B1*, and *CYP11B2* but not for *CYP11A1* (see ref. [31]) [14,33-36]. Not all steroidogenic enzymes were increased upon AngII stimulation [11]. Moreover, the expression profiles were time dependent. For example, *HSD3B1* showed the maximum mRNA expression at 4 h after AngII stimulation, while expression of the other genes, such as *CYP11B2*, increased continuously for 8 h after AngII stimulation (see ref. [31]). These results indicate that AngII controls different steroidogenic genes with different kinetics.

AngII-driven transcriptional activation of *HSD3B1* relies on *de novo* protein synthesis.

In the process of the gene expression time course studies (see ref. [31]), I noticed that *HSD3B1* did not show any

increase during the first hour of AngII stimulation. The levels of *HSD3B1* mRNA began to increase 2 h after AngII treatment. This late onset of *HSD3B1* induction might be ascribed to a need for *de novo* protein synthesis of the relevant trans-acting regulator(s) of this gene. I therefore studied whether cycloheximide (CHX) treatment affects the AngII-responsive mRNA expression profiles of *HSD3B1* in H295R cells (see ref. [31]). Notably, pharmacological blockade of new protein synthesis by CHX treatment completely blocked the AngII response of *HSD3B1*, while the induction profiles of *StAR*, *CYP11B1*, and *CYP11B2* were essentially unimpaired by this treatment (see ref. [31]). These data clearly indicate that *HSD3B1* differs from *StAR*, *CYP11B1*, and *CYP11B2* in requiring *de novo* protein synthesis for the response to AngII stimulation.

I observed that *HSD3B2* was again AngII stimulation insensitive, regardless of the CHX treatment. In addition, the data presented in this study reproduced a previously reported CHX-sensitive AngII response of *CYP21A* [35], while this gene's induction was relatively modest compared to that of *HSD3B1* (see ref. [31]).

Upon AngII stimulation, NGFIB and NURR1 protein levels increase synchronously with *HSD3B1* mRNA levels.

The 5'-flanking genomic sequence of *HSD3B1* contains a

consensus NGFIB-binding motif in the vicinity of the transcription initiation site (Fig. 2A). Specifically, the element at position –117 contains 9 nucleotides that closely resemble the consensus NGFIB-responsive element [NBRE; (A/T)AAAGGTCA] [37]. This potential NBRE site attracted my attention, since all NGFIB nuclear receptor gene family members, which include *NGFIB*, *NURR1*, and *NOR1*, are known to be AngII stimulation responsive in H295R cells [21,38,39]. Importantly, the induction of these genes occurred rapidly, within 1 h, after stimulation even in the presence of CHX (Fig. 2B to D). Such features fulfill the criteria for the potential regulators of *HSD3B1*. In contrast, while previous studies [21,38,39] showed that AngII can also induce the expression of *FOS*, *JUNB*, and *EGR*, neither a potential AP-1 site (to which *FOS* and *JUNB* bind) nor the consensus *EGR* binding motif was found in the sequence of the 5'-flanking region of *HSD3B1* [1].

Western blot analysis of NGFIB (Fig. 2E) further demonstrated that the levels of the NGFIB protein in H295R cells were increased rapidly, within 2 h, after AngII stimulation. Following the peak of expression at about 4 h, the levels of NGFIB expression began to decrease by 8 h and returned to near basal levels after 12 h of AngII treatment. These profiles temporally correlate with the kinetics of the rise and fall of the levels of the *HSD3B1* transcript (Fig. 2E).

These data support the hypothesis that the regulated expression of *HSD3B1* mRNA involves the rapid *de novo* protein synthesis of the short-lived transcription factor, NGFIB.

This was supported by the fact that the CHX treatment that blocked *HSD3B1* induction indeed blocked AngII-stimulated induction of the NGFIB protein (Fig. 2H). I also examined the other members of the NGFIB protein family: NURR1 was increased upon AngII stimulation in a pattern similar to that of NGFIB (Fig. 2F and H). In comparison, the NOR1 protein showed a relatively slow accumulation (Fig. 2G and H): NOR1 peaked at 8 h and remained expressed even 12 h after AngII treatment. Thus, the expression profile of the NOR1 protein does not account for the transient expression of *HSD3B1* mRNA.

AngII-induced NGFIB and NURR1 bind to the NBRE site on the *HSD3B1* promoter.

There are many studies describing the AngII response of *NGFIB*, *NURR1*, and *NOR1* in H295R cells [19,40,41], but it remains unexplored whether the corresponding proteins bind to the cognate DNA element in an AngII stimulation-dependent manner. I therefore performed an electrophoretic mobility shift assay (EMSA) to determine whether the AngII-induced NGFIB family proteins bind to

the potential NBRE site of the *HSD3B1* promoter (Fig. 3A). Nuclear extracts were prepared from H295R cells after 3 h of incubation with AngII or vehicle (3 h treatment was chosen to see the proteins bound to NBRE during the phase of the increase in *HSD3B1*). I observed that the H295R cell nuclear extracts were able to form two separate complexes, C1 and C2, with the radiolabeled *HSD3B1* NBRE oligonucleotide probe (Fig. 3A). Competition assays with nonlabeled probes demonstrated that the complexes C1 and C2 both compete with the wild-type but not the mutant NBRE oligonucleotide probes (see lanes 9 to 12), indicating that both complexes are formed through the sequence of the NBRE.

Notably, the amount of C1 complex profoundly increased upon AngII stimulation (Fig. 3A, lanes 1 to 4; see the bands indicated by a filled arrowhead). Also, the amount of C2 complex increased upon stimulation (indicated by an open arrowhead), but the increase was modest and appreciably less than the extent of the C1 induction. Thus, it was crucial to determine whether or not the C1 and C2 complexes contained a member of the NGFIB protein family. To this end, I performed gel mobility supershift assays (Fig. 3B) with antibodies against NGFIB (anti-NGFIB; lanes 7 and 8), NURR1 (anti-NURR1; lanes 9 and 10), and NOR1 (anti-NOR1; lanes 11 and 12). I found that whereas neither the C1 complex nor the C2

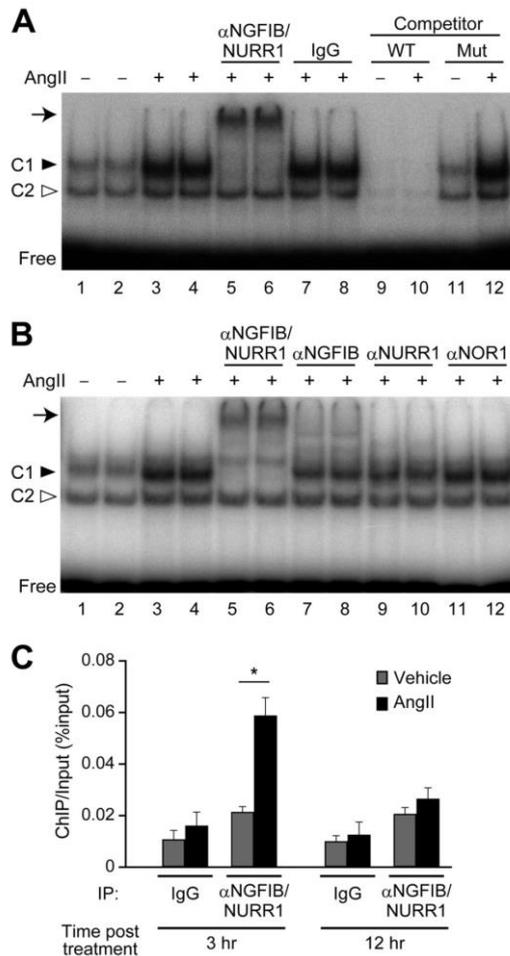


Fig. 3. AngII-induced NGFIB and NURR1 bind to the NBRE site of the *HSD3B1* promoter. (A and B) EMSA analysis for NGFIB binding at the NBRE. A radiolabeled oligonucleotide probe for the *HSD3B1* NBRE (positions -130 to -110) was incubated with the nuclear extracts from AngII-treated or vehicle-treated H295R cells. Supershift assays were performed by preincubating the nuclear extracts with the indicated antibodies. Normal IgG was used as a control. A cold competition assay was performed by adding a 100-fold molar excess of unlabeled wild-type (WT) or mutant (Mut) NBRE probe. (C) ChIP of *HSD3B1* promoter. After 3 or 12 h treatment with vehicle or AngII, cross-linked nuclear extracts from H295R cells were subject to ChIP assays with anti-NGFIB/NURR1 antibody and analyzed by qPCR using specific primers and a TaqMan probe targeting the DNA fragments containing the *HSD3B1* NBRE. Normal rabbit IgG was used as a control for immunoprecipitation. ChIP values are expressed as a percentage of the input amount of chromatin. The results are the means \pm SEM for three independent samples. *, $P < 0.01$, Bonferroni test.

complex was diminished by anti-NOR1, the antibodies specific to NGFIB (anti-NGFIB) and NURR1 (anti-NURR1) both induced a selective reduction of the C1 complex (Fig. 3B, lanes 7 to 10). Moreover, I found that EMSAs with the antibody recognizing both NGFIB and NURR1 (anti-NGFIB/NURR1) led to a complete loss of the C1 complex with the concomitant generation of a slowly migrating supershifted band (Fig. 3A and B, lanes 5 and 6). Control IgG did not induce any noticeable shifting or reduction of the complexes in the EMSAs (Fig. 3A, lanes 7 and 8). The antibody shift assays thus strongly suggest that the AngII stimulation-induced protein complexes formed on the NBRE site of *HSD3B1* are yielded primarily from NGFIB and NURR1.

The recruitment of the NGFIB/NURR1 protein to the promoter region of *HSD3B1* was further analyzed by chromatin immunoprecipitation (ChIP) assays (Fig. 3C). H295R cells were incubated with or without AngII. Then, cross-linked, sheared chromatin fragments were immunoprecipitated with anti-NGFIB/NURR1. DNA fragments from the immunoprecipitates were examined by qPCR with a sequence-specific TaqMan probe. I found that 3 h AngII treatment significantly increased the levels of NGFIB/NURR1 binding to the promoter region of *HSD3B1* (Fig. 3C). I observed that this AngII-induced binding was decreased to nearly

basal levels after 12 h AngII treatment (Fig. 3C). This transient binding property is in parallel with the kinetics of *HSD3B1* gene expression. These data indicate that AngII-induced NGFIB/NURR1 protein binds to the *HSD3B1* promoter at the time of activation of this gene.

NGFIB and NURR1 have the ability to enhance transcription of *HSD3B1*.

Next, I performed reporter gene expression assays in H295R cells to know whether NGFIB and NURR1 have the ability to increase the promoter activity of *HSD3B1* (Fig. 4A). I found that the reporter activities under the control of the *HSD3B1* promoter (a 1,332-bp DNA fragment upstream of the translation start site of *HSD3B1*) were significantly increased by ectopically expressed *NGFIB*. Deletion and mutational analyses of the 5'-flanking region of *HSD3B1* further demonstrated that the DNA sequence to which NGFIB and NURR1 proteins bound in the EMSA as well as in the ChIP assay (Fig. 3) was indeed responsible for the NGFIB-mediated transactivation of *HSD3B1* (Fig. 4A). Importantly, similar to NGFIB, NURR1 increased the activity of the *HSD3B1* promoter in a manner that depended on the NBRE sequence (Fig. 4A). In addition, I also found that both NGFIB and NURR1 can activate transcription from

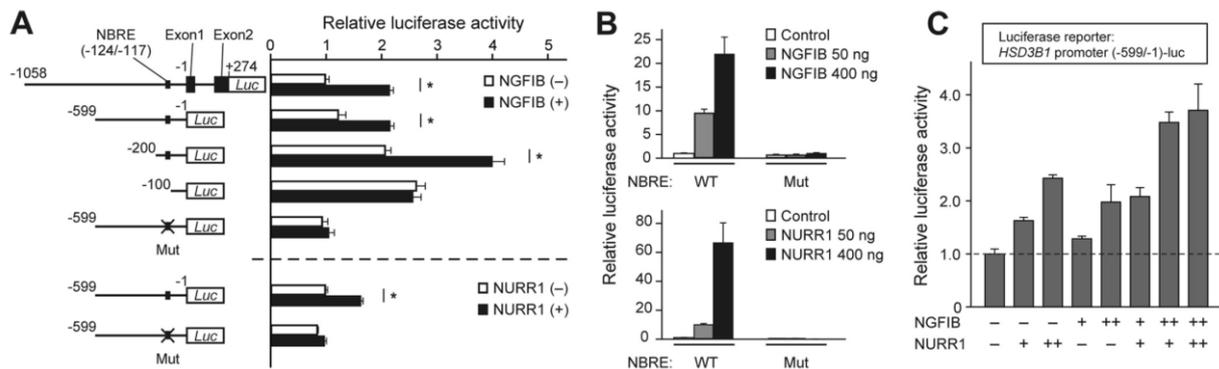


Fig. 4. NGFIB and NURR1 possess the ability to enhance *HSD3B1* promoter activity. (A) Luciferase reporter assays of NGFIB and NURR1 for the *HSD3B1* promoter. Reporter constructs containing serial deletions of the *HSD3B1* promoter (positions -1059 to +274, -599 to -1, -200 to -1, and -100 to -1) as well as the mutant derivative for the NBRE (Mut; positions -124 to -117) were transiently introduced (20 ng plasmid/well for each) into H295R cells with either an empty pEZ expression vector (500 ng) or an expression vector containing the coding sequence of human NGFIB or NURR1 (500 ng). After recovery for 24 h, cells were lysed, and luciferase activity was measured. Deletion constructs are numbered relative to the transcription initiation site. Shown are representative data from replicate experiments with similar results. Values represent the means \pm SEM ($n = 4$). *, $P < 0.01$, Bonferroni test. (B) Luciferase reporter assays of NGFIB and NURR1 for the isolated NBRE. A luciferase reporter construct (20 ng) that contains either nine copies of the NBRE sequence of *HSD3B1* (wild type) or those of the mutated sequence was transiently introduced into H295R cells with increasing doses of the expression vector for NGFIB or NURR1 (50, 400 ng). Values are the means \pm SEM ($n = 4$). (C) Luciferase reporter assays examining the effect of coexpression of NGFIB and NURR1 on the promoter activities of *HSD3B1*. The reporter plasmid containing the *HSD3B1* promoter (positions -599 to -1) (20 ng) was transiently introduced into H295R cells with the indicated combinations of expression plasmids for NGFIB and NURR1. +, 200 ng, ++, 400 ng. Values are the means \pm SEM ($n = 4$).

an artificial promoter that contains multiple copies of the isolated NBRE sequence of *HSD3B1* (Fig. 4B). These activities of NGFIB and NURR1 were entirely abolished when the NBRE sequences were mutated, indicating that this NBRE site is essential and sufficient to induce the transactivation function of NGFIB and NURR1.

I next examined the potential cooperation between NGFIB and NURR1 (Fig. 4C), since these two proteins synchronously accumulate after AngII stimulation and bind to the same NBRE site of the *HSD3B1* promoter (Fig. 3). Interestingly, reporter luciferase assays with the *HSD3B1* promoter (Fig. 4C) demonstrated that while both NGFIB and NURR1 could independently enhance the promoter activities of *HSD3B1* in a dose-dependent fashion by approximately 2-fold, concomitant expression of NGFIB and NURR1 resulted in a further enhancement of the promoter activities to approximately less than 4-fold. It seems likely that NGFIB and NURR1 can additively activate *HSD3B1* transcription in a dose-dependent manner.

Inactivation of NGFIB and NURR1 attenuates AngII-stimulated *HSD3B1* induction.

In the promoter assays, luciferase reporter plasmids were recombinant and episomal. Thus, it remains uncertain whether NGFIB and NURR1 are indeed involved in the regulation of

the endogenous native promoter of *HSD3B1*. In an attempt to clarify this point and further explore a potential contribution of NGFIB and NURR1 to the AngII-responsive induction of *HSD3B1*, I finally investigated the expression of the endogenous *HSD3B1* mRNA under the conditions where NGFIB and NURR1 were inactivated. For this analysis, I used two different experimental tools, small interfering RNAs (siRNAs) targeting *NGFIB* and *NURR1* (Fig. 5) and a dominant negative NGFIB (DN-NGFIB) that is known to competitively inhibit all members of the NGFIB family [29,30] (Fig. 6).

Because the transfection efficiency of H295R cells is extremely low [42,43], electroporation with the Nucleofector system (Amaxa Biosystems) was performed for efficient introduction of siRNAs [44,45]. I observed that electroporation of siRNA mixtures, each directed against *NGFIB* or *NURR1*, led to a specific knockdown of the target gene without any apparent off-target cross-reactions between the members of the NGFIB family (Fig. 5A and B for mRNA and protein, respectively). In agreement with the previously described role for the NGFIB family in *CYP11B2* expression [19-22], knockdown of NGFIB and NURR1 decreased AngII-stimulated induction of *CYP11B2* (Fig. 5C, right). Importantly, I also observed that knockdown of NGFIB and NURR1, either each alone or both, led to a significant attenuation of *HSD3B1* mRNA induction at any of the

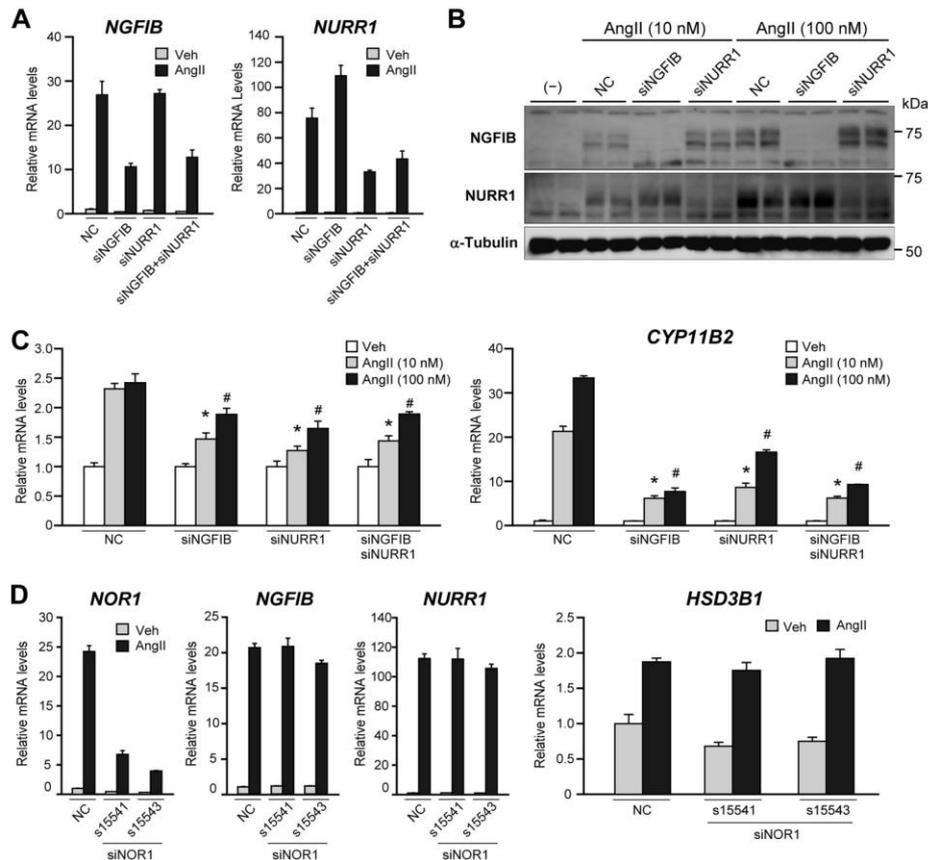


Fig. 5. Knockdown of NGFIB and NURR1 attenuates AngII-stimulated *HSD3B1* induction. (A) Gene-selective knockdown by electroporation of siRNAs against *NGFIB* and/or *NURR1*. H295R cells were transfected by electroporation using the indicated siRNA mixtures, each directed against *NGFIB* (siNGFIB) or *NURR1* (siNURR1), and treated with 100 nM AngII or vehicle for 4 h. Total RNA was isolated, and expression of *NGFIB* and *NURR1* was determined by qRT-PCR. All values (means \pm SEM, $n = 3$) were normalized to the levels of *RPLP0*. As a control, the electroporation was also performed with a negative-control siRNA (NC). (B) Reduction of protein expression of *NGFIB* and *NURR1* by siRNA-mediated knockdown. H295R cells transfected with the indicated siRNAs were stimulated by 4 h AngII treatment (10 nM or 100 nM) and subjected to Western blotting with antibodies against *NGFIB* and *NURR1*. α -Tubulin was used as a loading control. (C) Attenuated AngII response of *HSD3B1* and *CYP11B2* by knockdown of *NGFIB* and *NURR1*. H295R cells were transfected with the indicated siRNAs and then stimulated with either 100 nM or 10 nM AngII or vehicle for 4 h. The levels of *HSD3B1* and *CYP11B2* mRNA were determined by qRT-PCR ($n = 3$ for each), and the means of vehicle treatment were set equal to 1 after normalization to the level of *RPLP0*. Error bars indicate SEMs. *, $P < 0.05$ (versus 10 nM AngII induction of negative-control siRNA-transfected cells); #, $P < 0.05$ (versus 100 nM AngII induction of negative-control siRNA-transfected cells). (D) Unimpaired AngII response of *HSD3B1* by electroporation of siRNAs targeting *NOR1* (siNOR1). H295R cells transfected with the indicated siRNAs (s15541, s15543, or the negative-control siRNA) were treated with 100 nM AngII or vehicle for 4 h. Total RNA was isolated, and expression of *NGFIB*, *NURR1*, *NOR1*, and *HSD3B1* was determined by qRT-PCR. All values (means \pm SEM, $n = 3$) were normalized to the level of *RPLP0*.

dosages of AngII used for stimulation (10 nM and 100 nM) (Fig. 5C, left). These data demonstrate that NGFIB and NURR1 can each play a role in increasing the expression of *HSD3B1* in response to AngII. I observed that NOR1 knockdown did not attenuate AngII-induced *HSD3B1* expression in H295R cells (Fig. 5D).

In order to further confirm the roles of NGFIB and NURR1 in *HSD3B1* expression, I used DN-NGFIB (Fig. 6). To see the effect of DN-NGFIB on the endogenous gene expression profiles in H295R cells, I used a magnetic cell separation system (MACSelect H-2K^k) that allows selective enrichment of transfected cells and thereby circumvents the inherent problems associated with the low-level transfection efficiency observed for H295R cells. As shown in Fig. 6A, 3 days after cotransfection of DN-NGFIB with a cell surface selection marker (H-2K^k), the cells were treated with either AngII or vehicle for 3 h. Then, immediately after the treatment, the cells were subjected to the MACSelect separation system and isolated into two cell fractions: H-2K^k-positive, transfected cells (eluate) and H-2K^k-negative, nontransfected cells (flowthrough) (Fig. 6A). The preparation of each cell sample was accomplished within 1 h to minimize potential alterations of target gene induction. Importantly, immunoblot analysis of DN-NGFIB confirmed the successful fractionation of the cells (Fig. 6B). Moreover, the

expression levels of DN-NGFIB were profoundly higher than those of the endogenously induced NGFIB (Fig. 6C), suggestive of the predominance of DN-NGFIB over the endogenous pathway. Under these conditions, I assessed the influence of AngII stimulation on endogenous *HSD3B1* mRNA expression. Notably, qRT-PCR analysis (Fig. 6D) revealed that the induction of this gene was completely blocked by the DN-NGFIB expression (compare the results for the eluate versus the flowthrough in Fig. 6D). Importantly, the transfected cells (eluate) still possessed the ability to respond to AngII stimulation, as in these cells DN-NGFIB did not inhibit the induction of *StAR* and endogenous *NGFIB* mRNA (Fig. 6D). On the other hand, DN-NGFIB caused a suppression of *CYP11B2* induction (Fig. 6D), a result congruent with that of a previous study [20] (also see Discussion). Finally, I also confirmed that *HSD3B2* was not responsive to AngII, irrespective of the presence of DN-NGFIB.

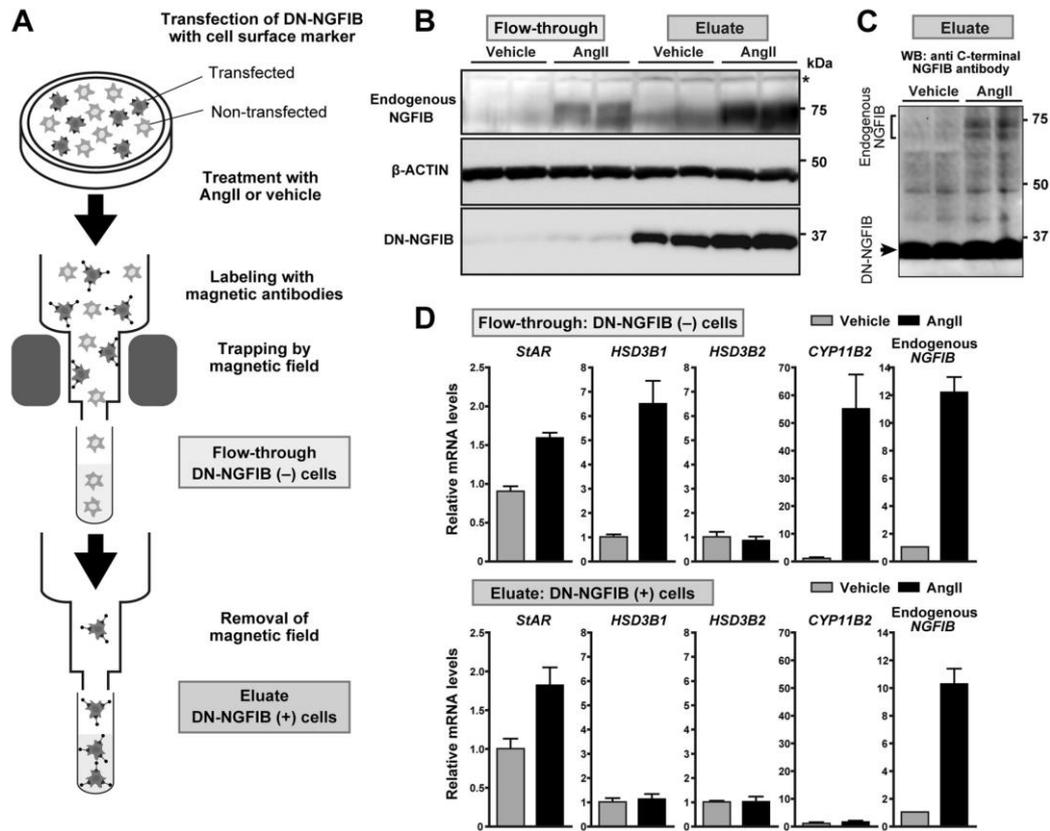


Fig. 6. DN-NGFIB interferes with AngII-induced *HSD3B1* expression in H295R cells. (A) Cell separation scheme for DN-NGFIB-transfected cells (eluate) and control nontransfected cells (flowthrough). The cells were treated with AngII or vehicle and separated with a cell surface marker antibody-based immunomagnetic microbead system. (B) Western blots of the separated cell samples with antibodies against endogenous NGFIB, β -actin, and DN-NGFIB. *, a nonspecific band. (C) Immunoblot of DN-NGFIB-transfected cells (eluate) with anti-C-terminus NGFIB antibody that allows the simultaneous detection of endogenous NGFIB and N-terminally truncated DN-NGFIB. Note that in the transfected cells DN-NGFIB predominates over the endogenously induced NGFIB. WB, Western blotting. (D) Levels of *HSD3B1*, *HSD3B2*, *StAR*, *CYP11B2*, and *NGFIB* mRNA in the separated cell samples. All the values (means \pm SEM, $n = 3$) were determined by qRT-PCR (the primers used for *NGFIB* do not cross-react with *DN-NGFIB*), and the value for vehicle treatment in eluate cells for each gene was set equal to 1 after normalization to the levels of *RPLP0*.

Discussion

The 3 β -HSD enzyme family is comprised of multiple, structurally similar isozymes that are encoded by different genes. The major finding of this study is that the ZG-specific isozyme *HSD3B1* (*Hsd3b6* for mouse) has a different characteristic from *HSD3B2* (*Hsd3b1*) in terms of the ability to respond to AngII stimulation (see Fig. 7). I showed that AngII stimulation triggers expression of *HSD3B1* but not *HSD3B2* in human adrenocortical H295R cells. Pharmacological studies demonstrated that the induction of *HSD3B1* relies entirely on the proteins that are newly synthesized upon AngII stimulation. The orphan nuclear receptor NGFIB family, proteins NGFIB and NURR1, showed a rapid and transient increase in expression in a pattern similar to that of *HSD3B1*. Moreover, the results from EMSAs, CHIP assays, and reporter-based promoter assays revealed that the *HSD3B1* promoter contains a functional NGFIB/NURR1-responsive element to which these proteins bind in response to AngII. Both knockdown of these proteins and overexpression of a dominant negative NGFIB resulted in a reduction of AngII-induced expression of *HSD3B1*. Taken together, data presented here demonstrate that *HSD3B1* is an AngII- responsive 3 β -HSD isoform gene and that the mechanism by

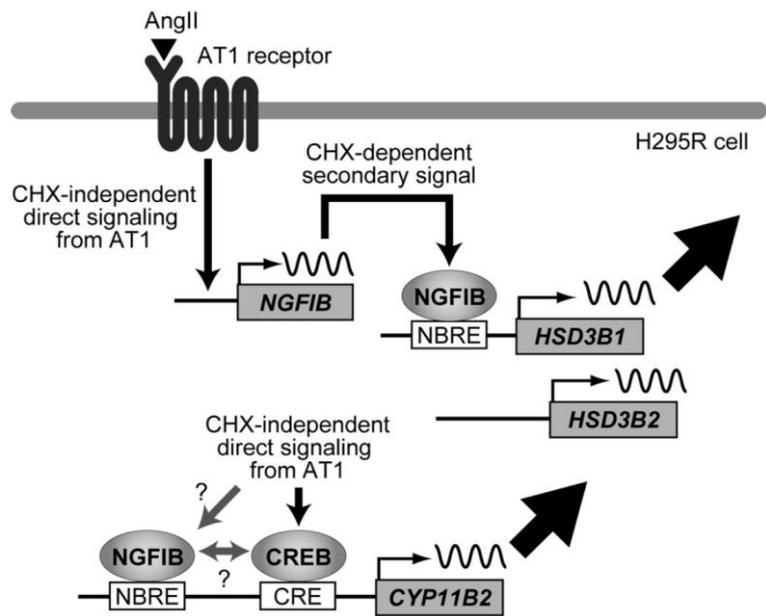


Fig. 7. Schematic model showing AngII regulation of *HSD3B1* in H295R cells. The AngII-AT1 receptor-NGFIB/NURR1 pathway leads to the upregulation of *HSD3B1*. *CYP11B2* differs from *HSD3B1* in relying on a CHX-independent pathway(s) from the AT1 receptor.

which AngII promotes *HSD3B1* expression involves the activation of the AT1 receptor downstream pathway, leading to rapid *de novo* protein synthesis of the nuclear orphan receptors NGFIB and NURR1 (Fig. 7).

Adrenal ZG cells are the principal place where aldosterone is biosynthesized from cholesterol via a series of enzymatic reactions involving 3 β -HSD. CYP11B2 (aldosterone synthase) is the last and unique enzyme in the aldosterone biosynthetic pathway exclusively expressed in ZG cells. This spatially limited expression of the enzyme CYP11B2 precludes aldosterone production in the region outside ZG cells [46-48]. Moreover, the centripetal blood flow in the adrenal cortex also prevents the precursors of aldosterone in the fasciculata cells from being supplied to ZG cells (which are located in the outer layer of the cortex). Therefore, all the steroid precursors devoted to aldosterone production are synthesized locally in ZG cells. This locality assigns a potential role of importance to the ZG-specific 3 β -HSD isozyme in the regulation of aldosterone synthesis. I previously showed that in both human and mouse, the adrenals express two distinct 3 β -HSD isoform genes with different zone specificities [5-7]: one is a ubiquitous isoform expressed in both ZG and zona fasciculata (ZF) (*HSD3B2* for human and *Hsd3b1* for mouse), but the other is

expressed exclusive in the ZG cells (*HSD3B1* for human and *Hsd3b6* for mouse). The data presented in this study therefore extend the difference between the two enzymes from zonal specificity to AngII stimulation reactivity. I showed that *HSD3B1* (*Hsd3b6*) has the ability to respond to AngII. A low-sodium diet also upregulated the expression of the ZG isoform *Hsd3b6* in the mouse adrenal gland without affecting the expression of the canonical adrenal isoform *Hsd3b1*. These data imply that the subtype-specific AngII responses of 3 β -HSDs represent a feature that is evolutionally conserved in humans and mice.

At present, it remains unknown whether the enzymes catalyzing the intermediate steps of aldosterone synthesis have a role in changing the capacity of AngII-induced aldosterone production in human adrenal cells. A study based on RNA interference knockdown might be a way to evaluate whether HSD3B1 plays a role. Unfortunately, however, our efforts to achieve 3 β -HSD isoform-selective silencing were unsuccessful: all siRNAs that I tested (three independent siRNAs for each isoform) had a severe off-target cross-reaction between the isoforms, a consequence perhaps due to the extremely high sequence similarity between *HSD3B1* and *HSD3B2* [5,6]. Recently developed subtype-selective monoclonal antibodies to HSD3B1 and HSD3B2

provide clinical evidence that altered expression of the ZG-specific isoform HSD3B1 correlates with the pathology of idiopathic hyperaldosteronism [5]. On the basis of the evolutionary conservation, it would be of interest to explore the role of this enzyme by using genetically modified mice specifically lacking *Hsd3b6*.

The NGFIB nuclear orphan receptor superfamily includes three members, NGFIB (also termed NR4A1), NURR1 (NR4A2), and NOR1 (NR4A3) [49]. These transcription factors belong to the nuclear receptor family but have no identified ligand. They regulate transcription through changes in their expression level and phosphorylation [49]. Importantly, all three family members are expressed in adrenal ZG cells, while they are also expressed in the ZF with different intensities [19,50-52]. Moreover, previous transcriptome studies demonstrate that all the three NGFIB family members are acutely induced upon AngII stimulation in H295R cells [21,38,39] as well as in primary cultures of rat and bovine ZG cells [38,53]. These common responses of *NGFIB* family members across different species suggest that the AngII induction of these transcription factors may be necessary for the coordinate regulation of steroid synthesis. Consistent with this hypothesis, Nogueira *et al.* previously demonstrated that overexpression of a dominant

negative NGFIB mutant causes a significant reduction of aldosterone synthesis in AngII-stimulated H295R cells [20]. However, probably because NGFIB family members have redundant roles, prior studies using targeted disruption of individual family members have not found adrenal phenotypes [54,55]. In the present study, I showed that both NGFIB and NURR1 have the potential to activate *HSD3B1* promoter activity. AngII-induced protein induction of NGFIB and NURR1 was indeed accompanied by a concomitant increase of *HSD3B1* mRNA expression. In contrast, NOR1 showed a relatively delayed accumulation compared with that of NGFIB and NURR1. Moreover, *in vitro* EMSA data presented in this study further demonstrate that NGFIB and NURR1 are the major components of the protein-DNA complex formed on the NBRE site of the *HSD3B1* promoter in H295R cells (note that essentially no residual C1 complex was found after incubation with anti-NGFIB/NURR1 antibody, which does not cross-react with NOR1). Thus, the NOR1 protein in H295R cells appears to target NBRE sites of other genes under AngII control. It has been shown that subtle changes in the consensus NBRE can affect the transactivation ability of each family member differently [56]. The target gene selectivity of NGFIB family members might also be modulated through posttranslational modifications and different heterodimerization

abilities [49].

I showed that AngII can induce *HSD3B1* but not *HSD3B2*. Mechanistically, the AngII-NGFIB/NURR1 pathway is integral to the activation of *HSD3B1*. However, it remains unknown why the same signal pathway does not induce *HSD3B2*. The *HSD3B2* promoter contains NGFIB-responsive elements [1,13], and these elements have already been implicated in the regulation of this gene's induction upon adrenocorticotrophic hormone (ACTH) stimulation [13]. Thus, the different responsiveness to AngII that was observed between the two 3 β -HSD isoforms cannot be explained by NGFIB alone, suggesting that a yet unknown additional mechanism may be involved in the isoform-selective induction of 3 β -HSD. In this respect, it is interesting to note that in the plasmid-based reporter assays, NGFIB could induce only a modest fold increase in the promoter activity of *HSD3B1*. The same was also true for NURR1. Thus, it is likely that despite the necessity of *de novo*-synthesized NGFIB and NURR1, the protein accumulation of these transcription factors alone may not be sufficient for inducing the maximal expression of *HSD3B1*. This probably suggests the requirement for other molecules to be synthesized or posttranslationally modulated upon AngII signaling. Further studies will be needed to understand the isoform-selective

AngII regulation of different 3 β -HSDs.

I also found that the mechanisms controlling *HSD3B1* and *CYP11B2* are not identical. Notably, the induction of *HSD3B1* required rapid *de novo* protein synthesis, but that of *CYP11B2* did not. Moreover, the induction kinetics of *HSD3B1* was relatively transient, but *CYP11B2* had a continuous increase even after *HSD3B1* was decreased to nearly basal levels. These behaviors of *CYP11B2* would be somewhat interesting, given a number of studies in the literature showing that the NGFIB family members are involved in the mechanisms regulating *CYP11B2* [19-22]. The *CYP11B2* promoter contains two canonical NBRE sites, for which I confirmed anti-NGFIB/NURR1 binding in ChIP assays (T. Ota, M. Doi, and H. Okamura, unpublished results). Moreover, I reproduced a reported reducing effect of DN-NGFIB on AngII-induced *CYP11B2* expression in H295R cells [20], indicating that the members of the NGFIB protein family are indeed involved in the upregulation of *CYP11B2*. However, it is currently difficult to explain how they contribute to the AngII-induced transcription of *CYP11B2* that occurs without relying on *de novo* protein synthesis. Considering that activator transcription factor (ATF)/CREB family members, namely, ATF1, ATF2, CREB, and CREM, have already been shown to activate *CYP11B2* transcription via a posttranslational

modification-based transactivation mechanism [44,57,58], one possible mechanism is that the NGFIB family members may interact with AngII-activated ATF/CREB family members to modulate their transactivation function (Fig. 7 depicts this model for discussion): the *CYP11B2* promoter contains not only NBRE sites but also a Ca^{2+} /cyclic AMP-responsive element (CRE) to which the ATF/CREB family members bind [44,58], and a potential interplay between NGFIB and ATF/CREB has been proposed for the regulation of the transcription of *CYP11B2* [44] and other genes [59]. An alternative explanation is also possible. For example, CHX-independent posttranslational direct modification of the NGFIB family proteins might be involved (Fig. 7). Thus, I would propose that a posttranslational protein-protein interaction(s) and/or modification(s) of the preexisting, basal-level NGFIB family proteins (but not of the newly synthesized ones) might be a part of the mechanisms regulating *CYP11B2*. Additional experiments will be required to test these hypotheses.

In conclusion, this study revealed that *HSD3B1* (*Hsd3b6*) is an adrenal gland zona glomerulosa-specific 3β -HSD isoform gene that is regulated by AngII. *HSD3B1* (*Hsd3b6*) differs from *HSD3B2* (*Hsd3b1*) in the ability to respond to AngII. I found that the induction of *HSD3B1* occurs through a mechanism differing from

that of *CYP11B2*. Notably, the AngII-induced induction of *HSD3B1* relies entirely on the *de novo* protein synthesis of NGFIB and NURR1. Aldosterone synthesis is a complex process potentially subject to many levels of regulation. Future studies aimed at deciphering the mechanism underlying the differential regulation between *HSD3B1* and *CYP11B2* will help to provide a better understanding of the AngII-dependent dynamic and coordinated regulation of the capacity of aldosterone production by the human adrenal gland.

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Chapter 2: Differential regulation of the type I 3 β -hydroxysteroid dehydrogenase gene expression by angiotensin II and potassium in human adrenocortical H295R cells

Next, I find that *HSD3B1* does not increase in response to K^+ , despite showing a drastic increase in response to AngII. In contrast, the aldosterone synthase (*CYP11B2*) gene expression is responsive to both AngII and K^+ . Promoter analyses reveal that although both AngII and K^+ activate transcription from the Ca^{2+} /cAMP-responsive element (CRE) located in the *CYP11B2* promoter, the NBRE in the *HSD3B1* promoter that is an essential *cis*-element for the AngII-responsiveness of this gene does not provide any K^+ reactivity. Thus, the reactivity of the NBRE is confined to AngII, but that of the CRE is common for AngII and K^+ . Consistent with this, AngII treatment increases expression of the NGFIB family proteins, but K^+ does not. In contrast, both AngII and K^+ increase phosphorylation of the CREB/ATF family CRE-binding proteins. Chromatin immunoprecipitation assays confirm that NGFIB protein occupies the *HSD3B1* promoter only after AngII treatment, while CREB/ATF protein binds to the *CYP11B2* promoter regardless of the type of stimuli. Thus, regulatory modes of *HSD3B1* and *CYP11B2* are different. This work reveals that *HSD3B1*'s different reactivities to AngII and K^+ are likely due to stimulus-selective induction of NGFIB.

Chapter 3: Angiotensin II-regulatable cell-autonomous circadian oscillators in the adrenal zona glomerulosa cells

The mouse *Hsd3b6* shows circadian expression in the adrenal gland, and its expression becomes abnormal when clock genes are deleted. Thus, this gene is under the control of the internal circadian clock. However, little is known about a molecular clock system within the ZG cells. At least partly because of the multilayered complex structure of the adrenal cortex, functional properties of the local clock in the ZG cells have never been studied. Here I provide evidence that a functional local clock system exists in human and rodent adrenal gland ZG cells. Using live-cell bioluminescence imaging and mRNA expression profiling with laser-captured cells, I illustrate robust circadian expression profiles of canonical clock genes in the ZG cells. Genetic perturbation studies confirm a requirement of clock genes for the autonomous oscillation of the ZG cells. I show that the clocks in the ZG cells exhibit phase-dependent phase shifts in response to AngII stimulation. Thus, AngII stimulation-responsive cell-autonomous circadian oscillators are present in the ZG cells.

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