

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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Contents

Prolegomenon.....	2
 Chapter 1: Angiotensin II triggers expression of the adrenal gland zona glomerulosa-specific 3β-HSD isoenzyme through <i>de novo</i> protein synthesis of the orphan nuclear receptors NGFIB and NURR1	4
Introduction	5
Materials and Methods.....	9
Results	23
Discussion.....	43
References	53
 Chapter 2: Differential regulation of the type I 3β-hydroxysteroid dehydrogenase gene expression by angiotensin II and potassium in human adrenocortical H295R cells.....	64
Introduction	65
Materials and Methods.....	68
Results	74
Discussion.....	87
References	93
 Chapter 3: Angiotensin II-regulatable cell-autonomous circadian oscillators in the adrenal zona glomerulosa cells	98
Introduction	99
Materials and Methods.....	102
Results	111
Discussion.....	124
References	128
 Acknowledgements	135

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.

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

Firstly, I show that in both human and mouse, the ZG-specific 3 β -HSD isoform gene expression is increased rapidly upon AngII stimulation. Then, the underlying molecular mechanisms are explored by using the human adrenocortical H295R cells. I show that the ZG isoform *HSD3B1* differs from *HSD3B2* in the ability to respond to AngII. Mechanistically, the induction of *HSD3B1* involves *de novo* protein synthesis of the nuclear orphan receptors, NGFIB and NURR1. The *HSD3B1* promoter contains a functional NGFIB/NURR1-responsive element (NBRE) to which these proteins bind in response to AngII. Knockdown of these proteins and overexpression of a dominant negative NGFIB both reduce the AngII responsiveness of *HSD3B1*. Thus, the AngII-NGFIB/NURR1 pathway controls *HSD3B1*. This work reveals *HSD3B1* as a new regulatory target of AngII [4].

Chapter 2: Differential regulation of the type I 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 [2]. 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.

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 μ m. 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 μ g/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 µg of fragmented chromatin was precleared by incubating with 40 µl of protein A-agarose (Roche) for 2 h at 4°C on a rotating wheel. Precleared chromatin was then incubated with 1 µg 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 µl 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 µl 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 µg of RNase A for 30 min at 37°C and 50 µg 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 µl Nucleofector solution R containing 2 µM (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

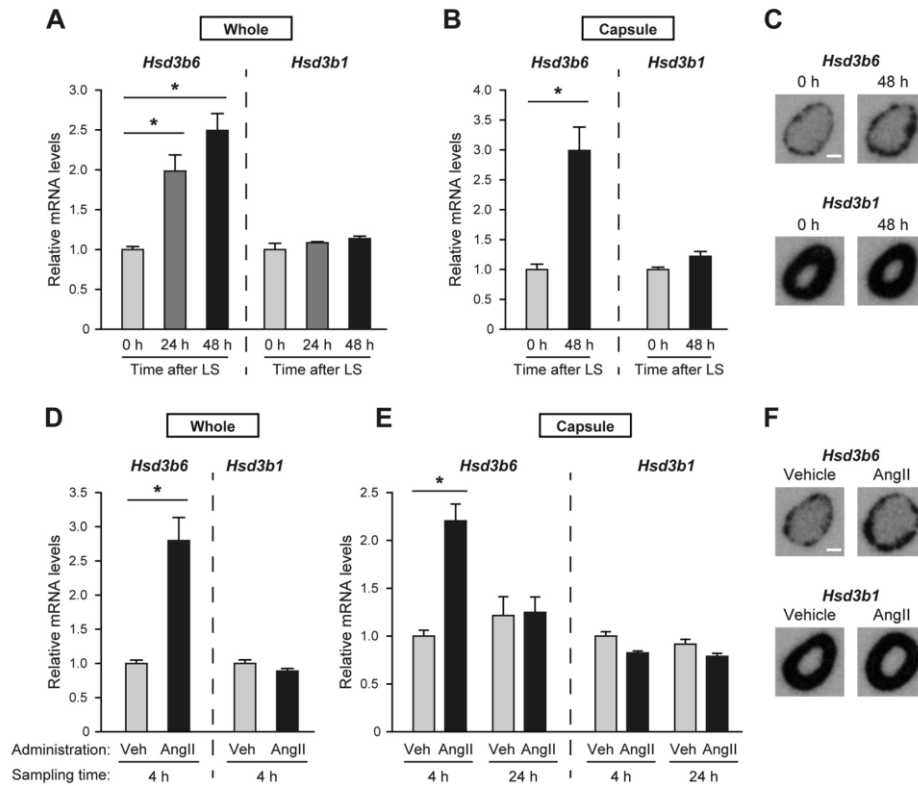


Fig. 1. The mouse adrenal *Hsd3b6* increases in response to AngII treatment and a low-salt diet. (A to C) Adrenal *Hsd3b6* and *Hsd3b1* mRNA levels in mice after a change to a low-salt (LS) diet. The adrenals were removed from animals at 0, 24, and 48 h after sodium restriction. Total RNA was prepared 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).

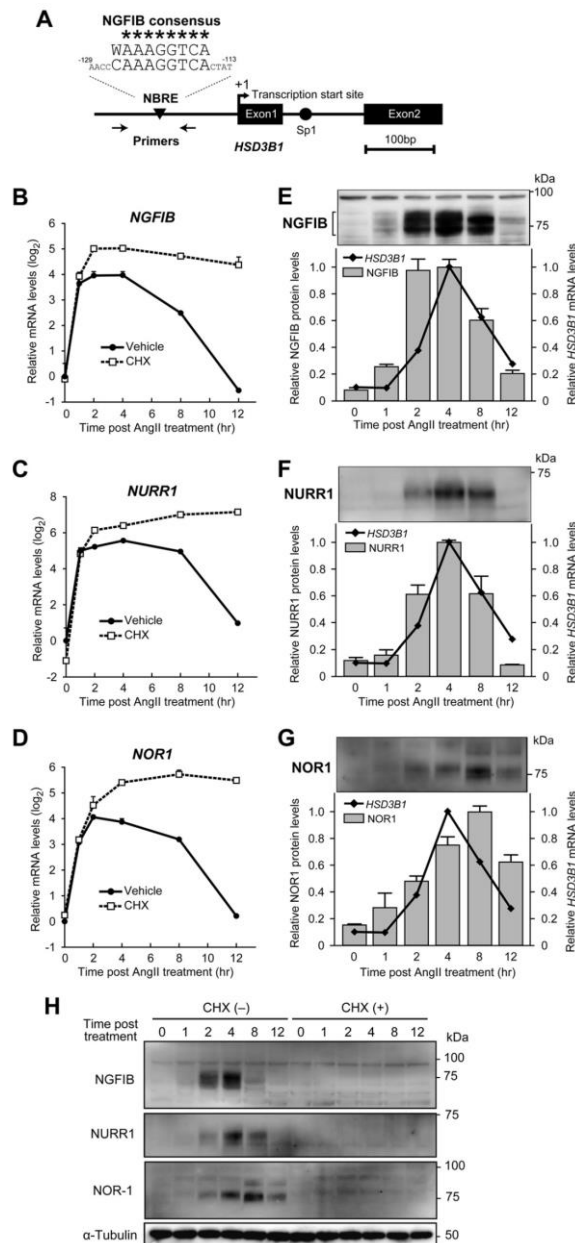


Fig. 2. NGFIB and NURR1 protein levels increase upon AngII stimulation in a pattern similar to that of *HSD3B1* mRNA. (A) Genomic structure upstream of the translation start site of human *HSD3B1*. Numbers indicate the positions relative to the transcription initiation site (position +1). The sequence of the potential NBRE site (positions -129 to -113) is compared with that of the consensus NGFIB-binding motif (W, A or T). *, sequences that conform to the consensus motif; arrows, positions of primers used in the ChIP assay. (B to D) Gene expression profiles of *NGFIB* (B), *NURR1* (C), and *NOR1* (D) after AngII (100 nM) stimulation in H295R cells with or without CHX (10 µg/ml) pretreatment. Relative mRNA levels (means ± SEM, *n* = 3) were determined by qRT-PCR and normalized to those of *RPLP0*. (E to G) Immunoblot analyses showing the protein expression profiles of NGFIB (E), NURR1 (F), and NOR1 (G) after AngII (100 nM) stimulation in H295R cells. Relative band intensities were determined by densitometry and expressed as the means ± variation from two independent experiments. For comparison, *HSD3B1* mRNA expression profiles are displayed in parallel. (H) Western blots showing the protein expression profiles of NGFIB, NURR1, and NOR1 after AngII treatment in the presence or absence of CHX in H295R cells.

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

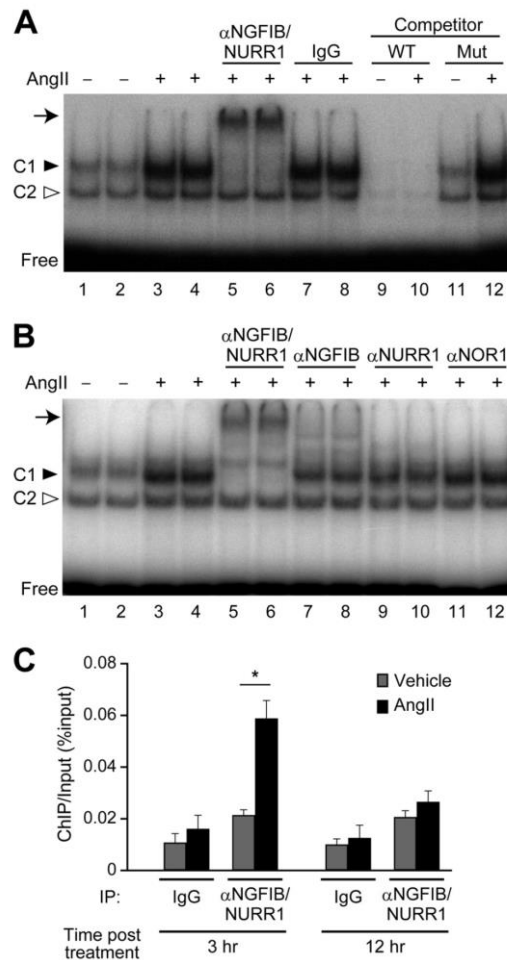


Fig. 3. AngII-induced NGFIB and NURR1 bind to the NBRE site of the *HSD3B1* promoter. (A and B) EMSA analysis for NGFIB binding at the NBRE. A radiolabeled oligonucleotide probe for the *HSD3B1* NBRE (positions -130 to -110) was incubated with the nuclear extracts from AngII-treated or vehicle-treated 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

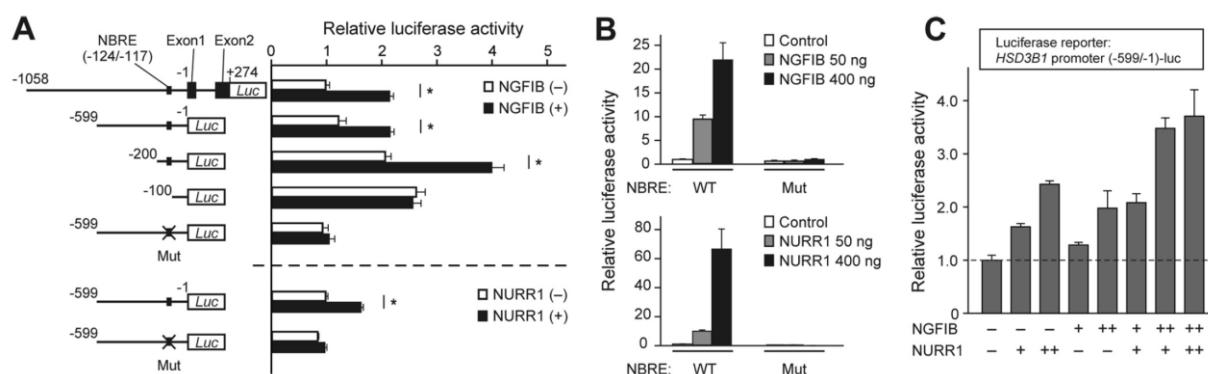


Fig. 4. NGFIB and NURR1 possess the ability to enhance *HSD3B1* promoter activity. (A) Luciferase reporter assays of NGFIB and NURR1 for the *HSD3B1* promoter. Reporter constructs containing serial deletions of the *HSD3B1* promoter (positions -1059 to +274, -599 to -1, -200 to -1, and -100 to -1) as 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

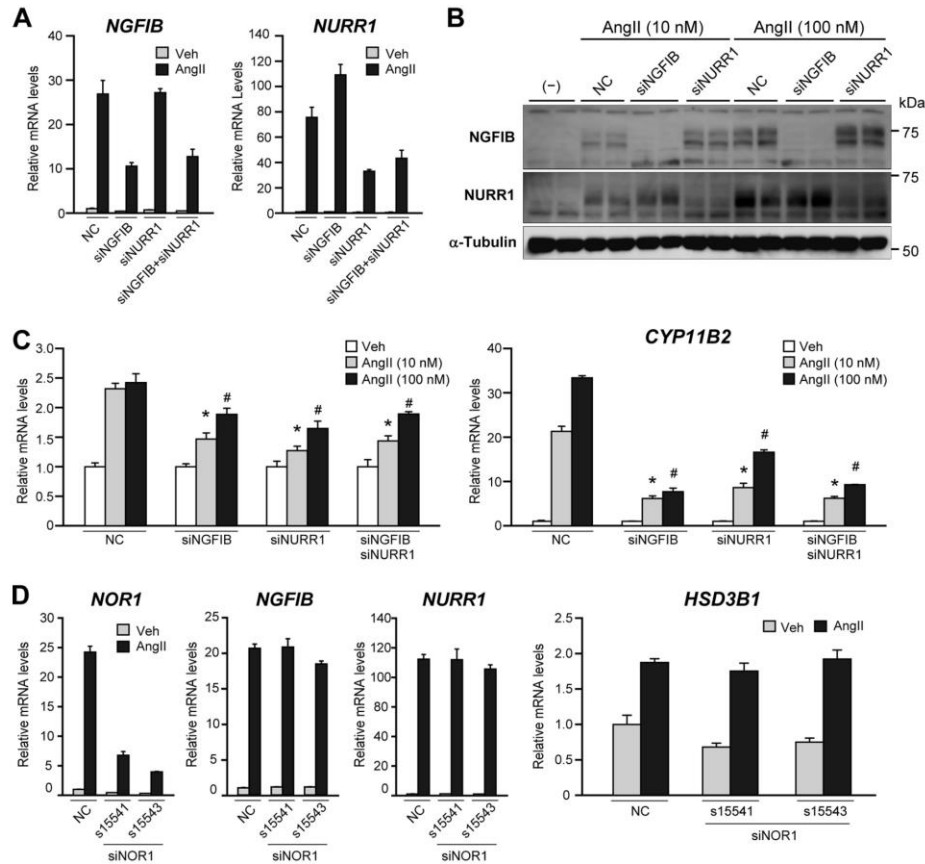


Fig. 5. Knockdown of NGFIB and NURR1 attenuates AngII-stimulated *HSD3B1* induction. (A) Gene-selective knockdown by electroporation of siRNAs against *NGFIB* and/or *NURR1*. H295R cells were transfected by electroporation using the indicated siRNA mixtures, each directed against *NGFIB* (siNGFIB) or *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.

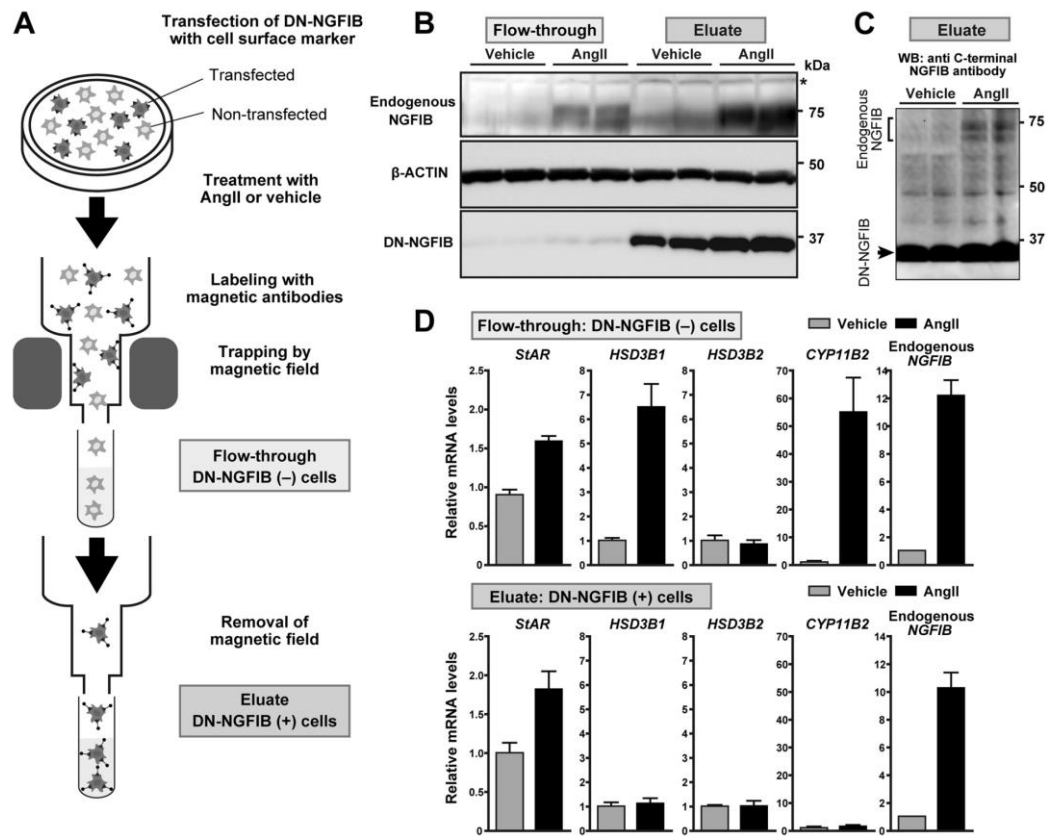


Fig. 6. DN-NGFIB interferes with AngII-induced *HSD3B1* expression in H295R cells. (A) Cell separation scheme for DN-NGFIB-transfected cells (eluate) and control nontransfected cells (flowthrough). The cells were treated with AngII or vehicle and separated with a cell surface marker antibody-based immunomagnetic 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

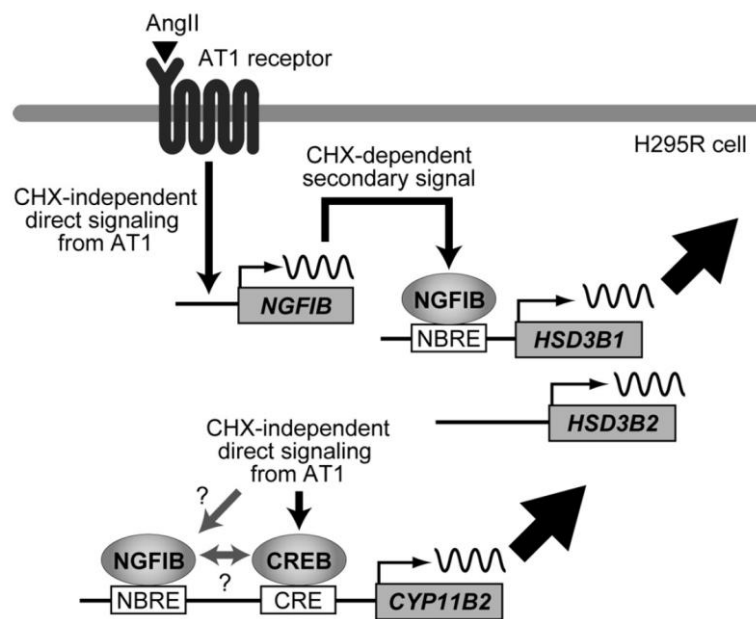


Fig. 7. Schematic model showing AngII regulation of *HSD3B1* in H295R cells. The AngII-AT1 receptor-NGFIB/NURR1 pathway leads to the upregulation of *HSD3B1*. *CYP11B2* differs from *HSD3B1* in relying on a CHX-independent pathway(s) from the AT1 receptor.

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 3 β -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

Introduction

Aldosterone is produced exclusively in the adrenal zona glomerulosa (ZG), and its secretion is regulated primarily by serum levels of angiotensin II (AngII) and potassium (K^+) [1,2]. The 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3β -HSD) is an enzyme that catalyzes the conversion of Δ^5 - 3β -hydroxysteroids (e.g. pregnenolone) into hormonally active Δ^4 -3-ketosteroids (e.g. progesterone) [3], an essential intermediate step for aldosterone synthesis [3,4]. While two different 3β -HSD isoforms (type I 3β -HSD, which is encoded by *HSD3B1*, and type II 3β -HSD, which is encoded by *HSD3B2*) exist in humans, it has long been considered that type II 3β -HSD is the major or sole isoform present in the human adrenal gland [3]. This canonical view was recently revised by the observation that type I 3β -HSD is expressed strongly in the ZG cells [5,6], which are the site where aldosterone is produced. This identification raised the question of whether AngII and K^+ regulate expression of this ZG-specific 3β -HSD gene. By using the human adrenocortical H295R cells as a model system, I showed that AngII led to a profound increase in expression of *HSD3B1* in chapter 1 [7]. This induction of *HSD3B1* involves *de novo* protein synthesis of the orphan nuclear receptors, NGFIB and NURR1 [7]. The *HSD3B1*

promoter contains a functional NGFIB/NURR1- responsive element (NBRE), to which these proteins bind in response to AngII [7]. However, it is currently unknown whether K^+ treatment similarly activates this pathway.

The aldosterone synthase, which is encoded by *CYP11B2*, catalyzes the final step of aldosterone synthesis, i.e. the conversion of 11-deoxycorticosterone to aldosterone [8-12]. Like *HSD3B1*, the expression of *CYP11B2* is confined to the ZG cells. A number of previous studies demonstrate that both AngII and K^+ upregulate the expression of *CYP11B2* [13-20]. Molecular analysis using human adrenocortical H295R cells revealed that AngII- and K^+ -induced *CYP11B2* expression appears to converge on the activator transcription factor (ATF)/CREB pathway: both agonists increase intracellular calcium (Ca^{2+}) levels [21,22] followed by the activation of ATF/CREB family members, which bind to the highly conserved Ca^{2+} /cyclic AMP-responsive element (CRE) in the *CYP11B2* promoter [23]. Thus, AngII and K^+ are thought to share, at least in part, the common Ca^{2+} -CREB/ATF pathway leading to *CYP11B2* expression. Interestingly, recent microarray data suggest that intracellular signaling pathways activated by AngII and K^+ are not completely identical, for example, different genes are acutely induced upon these two stimuli in H295R cells [18]. However, the underlying

molecular mechanisms leading to the difference are currently unclear.

In this study, I show that K^+ -mediated intracellular signaling lacks the ability to induce *HSD3B1*, although it can activate CREB/ATF-mediated pathway leading to *CYP11B2* expression. Western blot analysis revealed that K^+ treatment does not induce *de novo* protein synthesis of the NGFIB family members, although it can evoke phosphorylation of the CREB/ATF family members. In concordance with this, luciferase reporter assay demonstrated that K^+ does not enhance transcription through the NBRE site, while able to activate transcription through the CRE site. Chromatin immunoprecipitation assay confirmed that NGFIB protein occupies the *HSD3B1* promoter only after AngII stimulation, while the CREB/ATF proteins bind to the *CYP11B2* promoter regardless of the type of stimuli. These data indicate that the intracellular signaling cascades activated by AngII and K^+ are different, and that the regulatory mode of *HSD3B1* is distinct from that of *CYP11B2*.

Materials and Methods

Cell culture and treatments

Human adrenocortical H295R cells (ATCC CRL-2128) were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; Nacalai Tesque, Japan) supplemented with 2.5% Nu serum (BD Biosciences) and 1% ITS + Premix (BD Biosciences). Cells were maintained in a 37°C humidified atmosphere (5% CO₂). For stimulation, an aliquot of freshly reconstituted AngII (Peptide Institute, Japan) or KCl (for K⁺ stimulation) was added to the culture medium at the indicated concentrations. Where specified, cycloheximide (CHX; final concentration, 10 µg/ml) was added to the medium 15 min before AngII or K⁺ treatment.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from cells using Sepasol-RNAI Super G (Nacalai Tesque). cDNA was synthesized by reverse transcription using random hexamer primers and SuperScript III first-strand synthesis Super-Mix (Thermo). Quantitative PCR (qPCR) was run in duplicate with the primers and probes shown below. Because of the high sequence similarity between the human 3β-HSD isoform genes (*HSD3B1* and *HSD3B2*, 93.6% identity, including the 5'

and 3' untranslated regions [UTRs]), qPCR analysis of the two genes was performed with a TaqMan PCR reagent (Thermo) using gene-specific TaqMan MGB probes. As our laboratory previously reported [6], these probes distinguish a few nucleotide differences at the region corresponding to the dehydrogenase catalytic Y-X-X-X-K motif [3] 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 THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). As a qPCR device, I used a StepOnePlus real-time PCR monitoring system (Thermo), 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 *CYP11B2*, forward primer 5'-ACT CGC TGG GTC GCA ATG-3' and reverse primer 5'-GTC TCC ACC AGG AAG TGC-3'; for *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 *NURR1*, forward primer 5'-GGC TCC CAG AGG GAA CTG-3' and reverse primer 5'-GAG TCC AGC CTG TCC AAT CTC-3'; for *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 *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 *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 *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' (where VIC is 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein).

Plasmids, transfection, and real-time luciferase monitoring

The following reporter plasmids were used in this study:

(i) *HSD3B1* NBRE-Luc, in which a DNA fragment containing nine tandem copies of the sequence corresponding to the *HSD3B1* NBRE with its flanking sequences (positions -130 to -110) was inserted into the pGL4.23 (luc2/minP) vector (Promega); and (ii) *CYP11B2* CRE-Luc, in which a DNA fragment containing nine tandem copies of the sequence corresponding to the *CYP11B2* CRE with its flanking sequences (positions -82 to -61) was inserted into the pGL4.23 vector. Cells were transfected with 500 ng reporter plasmids using the Lipofectamine LTX/Plus reagent (Thermo) according to the manufacturer's instructions. Six hours after transfection, the medium was replaced with the fresh one. After overnight recovery, the medium was changed to 1 mM D-luciferin-containing medium,

and cells were transferred to a dish-type photon countable luminometer (Kronos Dio, ATTO) under 5% CO₂ atmosphere at 37°C. The luminescence was monitored for 2 min at 20 min intervals. Two days later, cells were treated with either AngII or K⁺ at the indicated concentration. All values were normalized to the means of bioluminescence during 4 hours before stimulation.

Western blotting

Cells were harvested in Laemmli buffer, and immunoblotting was performed as described [7] with the following antibodies toward 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), phospho-ATF2 (1:500 dilution; 9221 antibody; Cell Signaling Technology), ATF2 (1:1,000 dilution; C-19 antibody; Santa Cruz Biotechnology) and α -tubulin (1:2,000 dilution; T6199 antibody; Sigma). Densitometric quantification of phospho-ATF2 bands was performed by using Multi Gauge software (Fuji Film, Japan).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described [7] with modifications. For NGFIB-ChIP, cells were removed from culture dish

and immediately homogenized in phosphate-buffered saline (PBS) containing 2 mM disuccinimidyl glutarate (Thermo), and the homogenates were kept at room temperature for 20 min. Formaldehyde was then added at 1% of final concentration and incubated for a further 5 min, Cross-linking reactions were stopped by glycine (final concentration, 150 mM) on ice. The homogenates were centrifuged at 700× *g* for 10 min, and the resultant nuclear pellets were washed twice with ice-cold PBS. The nuclei were resuspended in immunoprecipitation (IP) 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 [Roche]) and sonicated 10 times for 30 s each time at 4°C using a Bioruptor UCW-201TM apparatus (Tosho Denki, Japan). Approximately 1.8 µg fragmented chromatin was immunoprecipitated with 1 µg of anti-NGFIB/NURR1 antibody (E-20 antibody; Santa Cruz Biotechnology). For ATF2-ChIP, cells were homogenized in PBS containing 1% formaldehyde and kept at room temperature for 10 min. Following addition of glycine and wash, the nuclei were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, 1 mM PMSF, PhosSTOP phosphatase inhibitors [Roche], protease inhibitor cocktail) and sonicated 5 times for 30 s each time at 4°C using a Bioruptor

apparatus. The lysates were diluted ten times with IP buffer, and 6 μ g fragmented chromatin was immunoprecipitated with 1 μ g of anti-ATF2 antibody (C-19 antibody; Santa Cruz Biotechnology). Immunoprecipitated DNA fragments were quantified by either TaqMan qPCR for *HSD3B1* promoter or SYBR green-based qPCR for *CYP11B2* promoter with the following primers and probe: for *HSD3B1* promoter, 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-MGB-3'; for *CYP11B2* promoter, forward primer 5'-CCC ACG CCT TTT CTC AGC ATC-3' and reverse primer 5'-AAT GCT CCC TCC ACC CTG TTC-3'.

Statistical analysis

I used two-way analysis of variance (ANOVA) with Bonferroni post hoc test to evaluate mean differences between different groups with different treatments in ChIP assay. A value of $P < 0.01$ was taken as significant.

Results

K⁺ does not enhance *HSD3B1* expression.

The adrenocortical H295R cells were treated with K⁺ over a range of periods (1, 2, 4, 8, and 12 h) at the concentration of 8 mM (Fig. 1A) or 16 mM (Fig. 1B), and total RNA was extracted from the cells and analyzed by qRT-PCR using specific TaqMan MGB probes and a set of primers for *HSD3B1* and *HSD3B2*. Due to the high degree of cDNA sequence similarity between *HSD3B1* and *HSD3B2* (93.6% identity, including the 5' and 3' untranslated regions), mRNA expression profiles of *HSD3B1* and *HSD3B2* have never been strictly characterized in K⁺-treated adrenal cells [14]. In agreement with previous studies, K⁺ treatment increased expression of the aldosterone synthase gene *CYP11B2* (Fig. 1). The levels of *CYP11B2* mRNA in H295R cells were dose-dependently increased to about 6- and 25-fold over basal levels after 8 mM and 16 mM K⁺ treatment, respectively. However, the expression levels of *HSD3B1* and *HSD3B2* were almost constant throughout the K⁺ treatments even under 16 mM condition (Fig. 1). These results demonstrate that K⁺-induced intracellular signaling that leads to *CYP11B2* expression cannot activate expression of the 3 β -HSD genes.

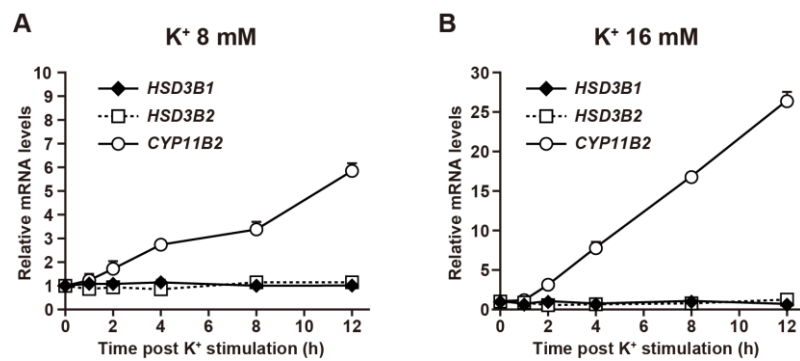


Fig. 1. The effect of K⁺ on the expression of *HSD3B1*, *HSD3B2* and *CYP11B2*. (A and B) Gene expression profiles of *HSD3B1* (diamond), *HSD3B2* (square), and *CYP11B2* (circle) after K⁺ treatment in H295R cells. KCl was added to the culture medium at a final concentration of 8 mM (A) or 16 mM (B). After the indicated periods of time, total RNA was isolated from the cells and the levels of *HSD3B1*, *HSD3B2*, and *CYP11B2* mRNA were determined by qRT-PCR. Values (means $\pm$ SEM, $n = 3$) are normalized to the levels of *RPLP0* and plotted relative to those at time 0.

Different effects of K⁺ and AngII on expression of *HSD3B1* and *CYP11B2*

H295R cells were treated with AngII (100 nM) or K⁺ (16 mM) in the presence or absence of the protein synthesis inhibitor, cycloheximide (CHX) (Fig. 2). Both AngII and K⁺ evoked induction of *CYP11B2* even in the presence of CHX. In contrast, *HSD3B1* increased only after AngII stimulation, and the increase was abolished by the CHX treatment. Thus, the increment of *HSD3B1* expression relies on *de novo* protein synthesis [7]. The induction kinetics also differs between *HSD3B1* and *CYP11B2*. The level of *HSD3B1* mRNA began to increase 2 h after AngII treatment, rapidly peaked at 4 h with approximately 6-fold over basal levels, and gradually decreased. In contrast, the levels of *CYP11B2* mRNA continued to increase over the course of 12 h treatment with AngII and K⁺. These data suggest that the underlying mechanisms that control expression of *HSD3B1* and *CYP11B2* are different.

As I reported in chapter 1 [7], AngII did not increase *HSD3B2* (Fig. 2): no appreciable induction was observed for *HSD3B2* over a 12-h course of AngII treatment. These data indicate that *HSD3B1* and *HSD3B2* are different in the ability to respond to AngII.

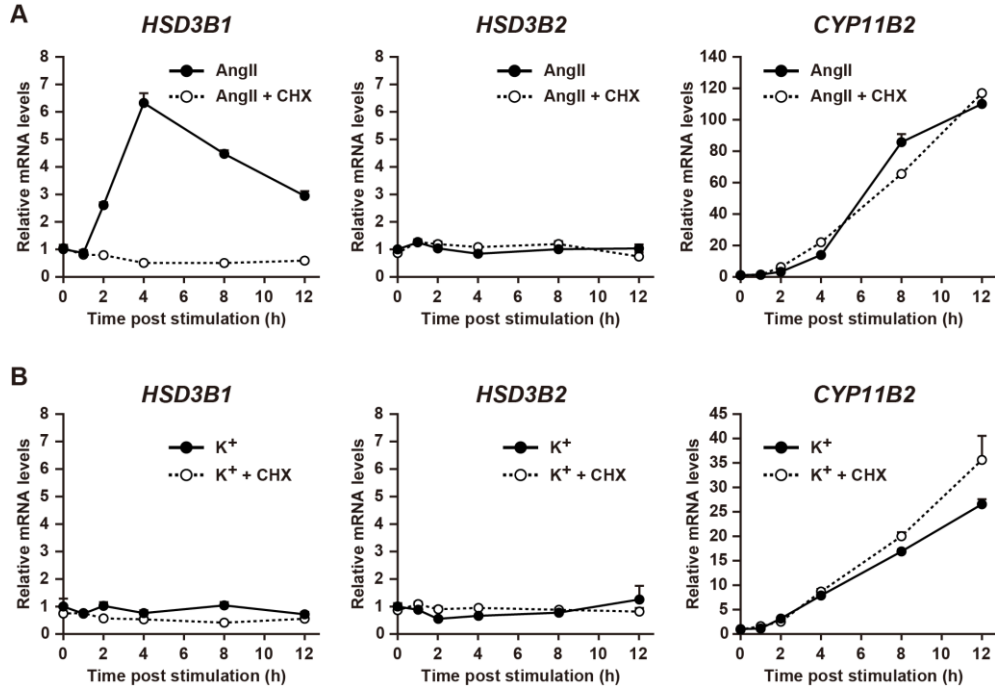


Fig. 2. *HSD3B1* mRNA does not increase in response to K⁺, despite displaying an overt increase in response to AngII, a situation different from *CYP11B2*. (A and B) Responsiveness of *HSD3B1* (left), *HSD3B2* (middle), and *CYP11B2* (right) to treatment with AngII (100 nM, A) or K⁺ (16 mM, B) in the presence (open circle) or absence (filled circle) of cycloheximide (CHX, 10 µg/ml) in H295R cells. After the indicated periods of time, total RNA was isolated from the cells and the levels of *HSD3B1*, *HSD3B2*, and *CYP11B2* mRNA were determined by qRT-PCR. Values (means ± SEM, *n* = 3) are normalized to the levels of *RPLP0* and plotted relative to those at time 0 without CHX treatment.

Different effects of K⁺ and AngII on *HSD3B1* promoter NBRE enhancer activity

Why does K⁺-induced intracellular signaling lack the ability to induce *HSD3B1* expression? In chapter 1, I demonstrated that AngII induces *HSD3B1* expression through *de novo* protein synthesis of the orphan nuclear receptors, NGFIB and NURR1 [7]. These proteins bind to the cognate NBRE sequence of the *HSD3B1* promoter in response to AngII and enhance transcription [7]. K⁺ signaling, therefore, might not be able to enhance transcription through the NBRE site. To test this hypothesis, a luciferase reporter vector containing multiple copies of the NBRE sequence of *HSD3B1* (*HSD3B1* NBRE-luc) was transfected into H295R cells, and bioluminescence was monitored in culture over a course of treatment with AngII or K⁺ (Fig. 3A). Interestingly, robust induction of reporter activity was observed only after AngII treatment (Fig. 3A). K⁺ treatment failed to induce reporter gene expression through the NBRE site (Fig. 3A, C, and D).

Equivalent effects of K⁺ and AngII on *CYP11B2* promoter CRE enhancer activity

The *CYP11B2* promoter contains a consensus Ca²⁺/cAMP-responsive element (CRE) [15,23,24]. To compare the

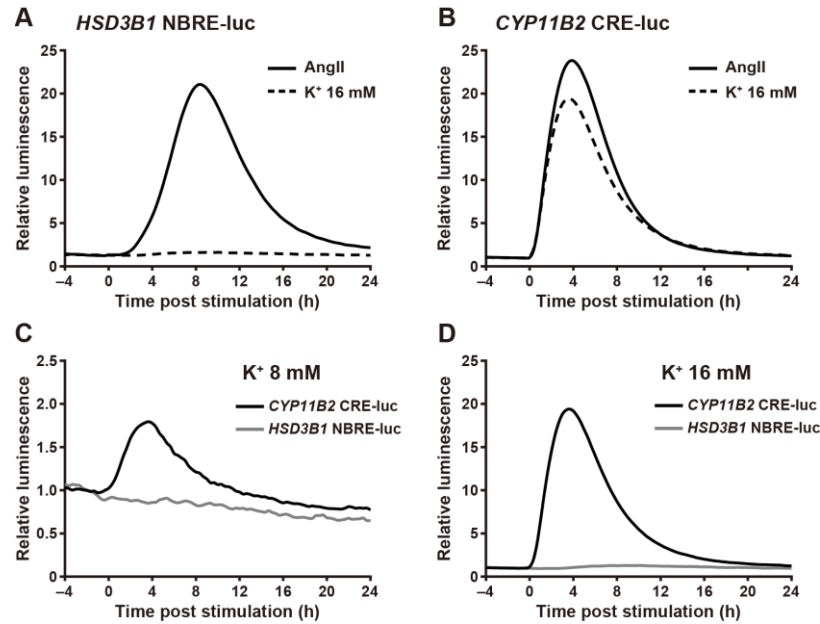


Fig. 3. Transcriptional enhancer activity of the NBRE site of *HSD3B1* does not increase in response to K^+ , despite displaying an overt increase in response to AngII, a profile different from that of the CRE site of *CYP11B2*. (A and B) Luciferase reporter gene expression from the CRE site of *CYP11B2* (A) and the NBRE site of *HSD3B1* (B) after stimulation with 100 nM AngII (black line) or 16 mM K^+ (dashed line) in H295R cells. (C and D) Luciferase reporter gene expression from the CRE site of *CYP11B2* (black) and the NBRE site of *HSD3B1* (gray) after stimulation with 8 mM K^+ (C) or 16 mM K^+ (D) in H295R cells. Cells were transfected with a reporter vector containing either multiple copies of the NBRE sequence of *HSD3B1* (*HSD3B1* NBRE-luc) or the CRE sequence of *CYP11B2* (*CYP11B2* CRE-luc). Traces show representative bioluminescence monitored before and after each treatment. Similar traces were obtained in three independent experiments.

responsiveness of the CRE with that of the NBRE, a luciferase reporter vector containing multiple copies of the isolated CRE sequence of *CYP11B2* (*CYP11B2* CRE-luc) was transfected into H295R cells and monitored for the response to AngII or K^+ (Fig. 3B to D). In agreement with the endogenous *CYP11B2* mRNA expression profiles (Figs. 1 and 2), both AngII and K^+ caused a significant enhancement of the CRE-luc reporter activity with an amplitude of ~25-fold for AngII (Fig. 3B), ~2-fold for 8 mM K^+ (Fig. 3C), and ~20-fold for 16 mM K^+ (Fig. 3B and D). These results indicate that the CRE is a common response element for AngII and K^+ .

The orphan nuclear receptor NGFIB family members do not increase in response to K^+ .

The orphan nuclear receptor NGFIB family consists of three members, NGFIB, NURR1, and NOR1. It has been well established that AngII can stimulate mRNA expression of *NGFIB*, *NURR1*, and *NOR1* [7,25] (also see chapter 1). However, K^+ responsiveness of this gene family remains unclear [17,18]. Moreover, to date, there have been no studies that assess the protein levels of NGFIB, NURR1, and NOR1 in K^+ -stimulated H295R cells. Therefore, to gain a better picture of this family, I assessed mRNA and protein expression profiles of all the NGFIB members in

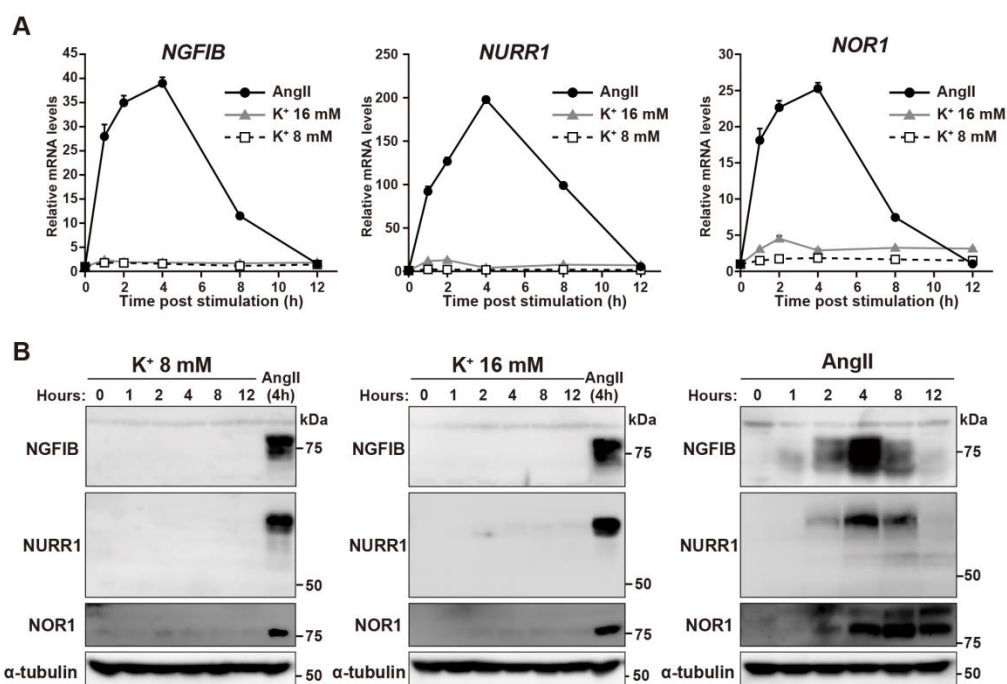


Fig. 4. The orphan nuclear receptor NGFIB family members do not increase in response to K⁺, despite displaying an overt increase in response to AngII. (A) Gene expression profiles of *NGFIB* (left), *NURR1* (middle), and *NOR1* (right) after stimulation with 100 nM AngII (circle), 8 mM K⁺ (square), or 16 mM K⁺ (triangle) in H295R cells. After the indicated periods of time, total RNA is isolated from the cells and the levels of *NGFIB*, *NURR1*, and *NOR1* mRNA were determined by qRT-PCR. Values (means ± SEM, *n* = 3) are normalized to the levels of *RPLP0* and plotted relative to those at time 0. (B) Western blots showing the protein expression profiles of NGFIB (upper), NURR1 (middle), and NOR1 (lower) after AngII (100 nM) or K⁺ (8 mM or 16 mM) stimulation in H295R cells. α-tubulin was used as a loading control.

H295R cells after treatment with either AngII or K⁺ (Fig. 4). The mRNA levels of *NGFIB*, *NURR1*, and *NOR1* were all rapidly increased after AngII treatment by approximately 40-, 200-, and 25-fold, respectively, over basal levels. However, K⁺ treatment produced only a faint peak mRNA expression of *NGFIB* (1.8-fold at 1 h, for 8 mM K⁺; 2.4-fold at 1 h, for 16 mM K⁺), *NURR1* (2.2-fold at 1 h, for 8 mM; 13-fold at 2 h, for 16 mM), and *NOR1* (1.8-fold at 4 h, for 8 mM; 4.5-fold at 2 h, for 16 mM) (Fig. 4A). Furthermore, to test the consequence of this faint mRNA expression, I performed Western blot analysis (Fig. 4B) and found that K⁺ treatment failed to induce detectable protein expression of NGFIB, NURR1, and NOR1. After AngII treatment, remarkably increased protein expression of NGFIB, NURR1, and NOR1 was observed (Fig. 4B). Thus, substantial protein induction of the members of the NGFIB family occurs only after AngII.

Both K⁺ and AngII induce phosphorylation of the CREB/ATF family member ATF-2.

In contrast to the stimulus-selective induction of the NGFIB family proteins, I found that phosphorylation of ATF-2 occurs in response to both AngII and K⁺ in H295R cells (Fig. 5). Since it has been reported [23] that in H295R cells, the mRNA levels of ATF-2 are relatively high, ATF-2 was selected for the subsequent analysis. As

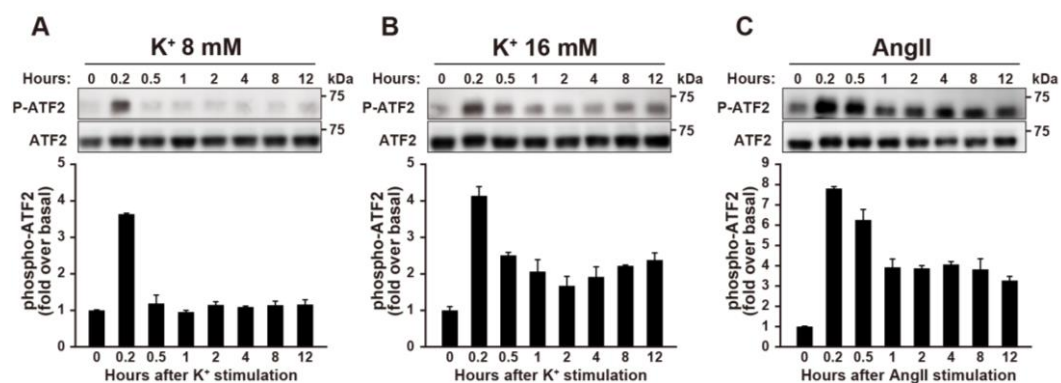


Fig. 5. Both K⁺ and AngII induce phosphorylation of ATF2 in H295R cells. (A to C) Western blots showing time-dependent phosphorylation profiles of ATF2 after stimulation with 8 mM K⁺ (A), 16 mM K⁺ (B), or 100 nM AngII (C) in H295R cells. Whole cell lysates were immunoblotted with the antibodies indicated. Relative band intensities of phospho-ATF2 were determined by densitometry. Data are the means $\pm$ variation from two independent experiments.

shown in Fig. 5, Western blot analysis revealed that the levels of phosphorylated ATF-2 were increased to ~7.5-, ~3.5-, and ~4.0-fold above basal values within 0.2 h after treatment with AngII, 8 mM K⁺, and 16 mM K⁺, respectively. Total ATF-2 contents remained invariable throughout the treatments.

AngII, but not K⁺, induces NGFIB recruitment to the *HSD3B1* promoter.

Finally, I performed chromatin immunoprecipitation (ChIP) assays to validate the impacts of AngII and K⁺ on NGFIB/NURR1 protein recruitment to the promoter region of *HSD3B1*. H295R cells were incubated with AngII (100 nM), K⁺ (16 mM), or control vehicle for 4 h. Then, cross-linked, sheared chromatin fragments were immunoprecipitated with either normal IgG or anti-NGFIB/NURR1 antibody, which recognizes both NGFIB and NURR1. DNA fragments from the immunoprecipitates were examined by qPCR with a sequence-specific TaqMan probe and primers for the promoter region of *HSD3B1* (Fig. 6A). I found that AngII significantly increased the levels of NGFIB/NURR1 binding to the promoter of *HSD3B1* (~2.3-fold over vehicle control, $P < 0.01$, Fig. 6A). However, ChIP values did not increase in response to K⁺ compared with the vehicle control (Fig. 6A). Thus, these data

demonstrate that detectable recruitment of NGFIB/NURR1 to the *HSD3B1* promoter region occurs only after AngII treatment. To compare the responsiveness of NGFIB/NURR1 with that of ATF-2, I then performed anti-ATF-2 ChIP assays for the promoter of *CYP11B2* (Fig. 6B). As reported previously by Nogueira & Rainey [23], both AngII and K⁺ significantly increased the levels of ATF-2 recruitment to the promoter region of *CYP11B2* (~3.2- and ~2.7-fold over vehicle control, respectively, Fig. 6B).

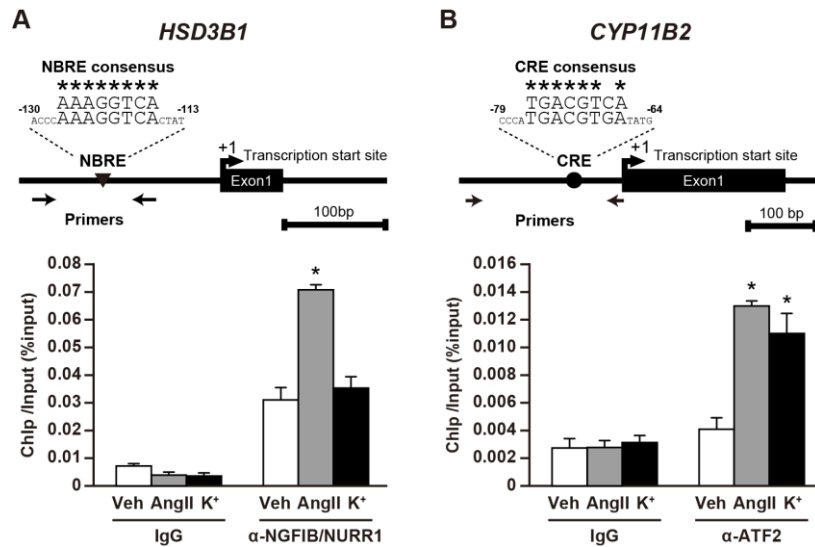


Fig. 6. Binding of NGFIB/NURR1 to the *HSD3B1* NBRE site does not increase in response to K⁺, despite displaying an increase in response to AngII, a situation different from ATF2 binding to the *CYP11B2* CRE site. (A and B) Upper: schematic genomic structures of *HSD3B1* (A) and *CYP11B2* (B). Numbers, the positions relative to the transcription initiation site (position +1); *, nucleotide residues that conform to the consensus sequences of NBRE (A) and CRE (B); arrows, positions of primers used in ChIP assays. Lower: ChIP of *HSD3B1* (A) and *CYP11B2* (B). After treatment with vehicle, AngII (100 nM), or K⁺ (16 mM), cross-linked nuclear extract from H295R cells were subject to ChIP assay with anti-NGFIB/NURR1 (A) or anti-ATF2 (B). Normal rabbit IgG was used as a control for immunoprecipitation. ChIP values are expressed as percentages of the input amount of chromatin. Values are the means $\pm$ SEM of three independent samples. Veh, vehicle control treatment. *, $P < 0.01$ compared with vehicle control, Bonferroni test.

Discussion

AngII and K^+ are known to stimulate aldosterone production in adrenal ZG cells through the common calcium messenger system [17]. However, recent microarray data using H295R cells show that transcription factors induced by AngII and K^+ are not identical [18], suggesting that the signaling pathways downstream of AngII and K^+ are different. In this study, I showed that these two aldosterone stimulators use different intracellular signaling cascades that result in different effects on the expression of *HSD3B1*. As I reported in chapter 1 [7], AngII triggers *HSD3B1* expression through *de novo* protein synthesis of NGFIB family genes. In contrast, K^+ did not cause any appreciable changes in *HSD3B1* expression, and the levels of the NGFIB family proteins showed little or no change in response to K^+ . Moreover, I did not observe any transcriptional activation from the *HSD3B1* NBRE after K^+ treatment in luciferase reporter assays. Thus, AngII and K^+ differ in their ability to activate the NGFIB-*HSD3B1* pathway (Fig. 7).

In this study, I found that K^+ does not activate the NGFIB pathway. At mRNA level, AngII dramatically increased the expression of *NGFIB* family genes, while each member was marginally upregulated by the K^+ treatment, which is consistent with previously

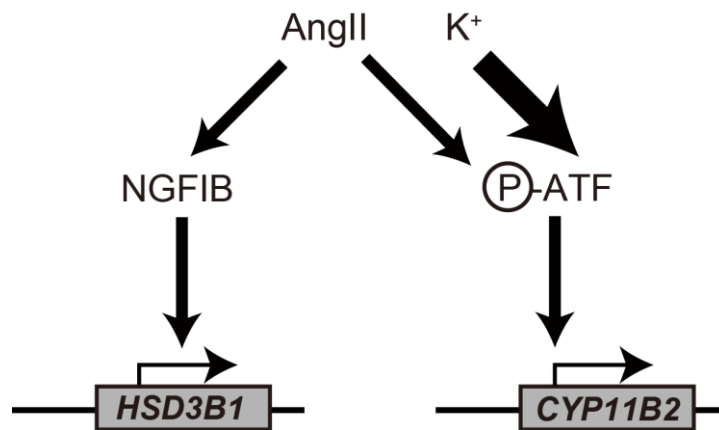


Fig. 7. A model showing different responsiveness of *HSD3B1* and *CYP11B2* to AngII and K^+ . *HSD3B1* responds to AngII, but not to K^+ , due to AngII-selective induction of the NGFIB nuclear receptor family proteins. In contrast, *CYP11B2* responds to both stimuli through a common mechanism involving activation (phosphorylation) of the CREB/ATF family members.

published data [17,18]. This stimulus selectivity was far more obvious when compared by Western blot analysis. The induction of any NGFIB family protein was almost undetectable throughout the experimental period after K^+ treatment. Furthermore, I confirmed by ChIP assay that K^+ treatment does not increase NGFIB binding to the *HSD3B1* NBRE. Moreover, NBRE-luc reporter assays confirmed that transcriptional enhancer activity of the NBRE site does not increase in response to K^+ treatment. Then, what makes the activation of the NGFIB family selective to AngII? It has been well demonstrated that, although K^+ increases the intracellular Ca^{2+} level through activation of voltage-sensitive Ca^{2+} channels in adrenal ZG cells [26], AngII activates Src signaling and protein kinase C as well as increases intracellular Ca^{2+} levels [27]. Pharmacologically, these signaling cascades are shown to be related to AngII induction of NGFIB family members [28]. Thus, K^+ -induced elevation of the intracellular Ca^{2+} level alone is not sufficient for activation (the protein induction) of the members of the NGFIB family.

I here showed that the mechanism by which AngII mediates its positive effects of *HSD3B1* expression differs from that by which AngII increases *CYP11B2* expression. Although AngII-induced *HSD3B1* expression was completely abolished by treatment with CHX, AngII effect on *CYP11B2* induction was not blocked by

CHX. The difference in the sensitivity to CHX between *HSD3B1* and *CYP11B2* is consistent with the idea that while the induction of *HSD3B1* relies on *de novo* protein synthesis of NGFIB family members, *CYP11B2* is induced by activation (phosphorylation) of preexisting CREB/ATF family proteins. This idea is supported by the fact that AngII induces *HSD3B1* and *CYP11B2* with distinct time courses. Mechanistic differences between the induction of these two genes could contribute to the differential sensitivity of expression of these genes to K⁺ treatment (Fig. 7).

I found that the CREB/ATF family is activated by AngII and K⁺ with different kinetics from endogenous *CYP11B2* mRNA induction by these two stimuli. Notably, expression of the CRE reporter gene and phosphorylation of ATF2 were acutely and transiently increased after treatment with either AngII or K⁺ in H295R cells, whereas endogenous *CYP11B2* mRNA exhibited a continuous increase even 12 h after stimulation. The mode of *CYP11B2* induction would be somewhat interesting, given that *CYP11B2* expression is controlled by activated CREB/ATF family proteins. This timing dissociation between the CRE reporter gene activation and endogenous *CYP11B2* mRNA induction might be explained by chromatin modification by CREB-binding proteins, CBP and p300. It is well known that activated CREB/ATF family

transcription factors recruit CBP and p300 to the promoter regions, where CBP and p300 acetylate N-terminal residues of histone, leading to chromatin relaxation [29]. Relaxed chromatin conformation in turn favor access of other transcription factors [30]. Therefore, the CRE might serves as a “trigger” for continuous upregulation of *CYP11B2*.

3 β -HSD is necessary for biosynthesis of all active steroid hormones [1,2]. HSD3B1 is exclusively expressed within ZG cells, and its overexpression is closely associated with enhanced aldosterone production of idiopathic hyperaldosteronism (IHA) [5,6]. In this study, I revealed that *HSD3B1* does not respond to K⁺ stimulation. Although it is currently unknown whether the unresponsiveness of *HSD3B1* to K⁺ treatment is physiologically relevant, it could be related to overexpression of HSD3B1 in IHA. Insensitivity of *HSD3B1* expression toward K⁺ stimulation suggests that *HSD3B1* expression might not be suppressed in low serum K⁺ concentration. In IHA patients, increased plasma aldosterone levels cause hypokalemia. Due to the K⁺-insensitivity of *HSD3B1*, HSD3B1 expression might remain high in IHA. Further studies will be required to clarify the pathophysiological relevance of unresponsiveness of *HSD3B1* to K⁺ treatment.

In summary, this work provides evidence that AngII and

K⁺ activate distinct intracellular signaling cascades in H295R cells. Treatment of H295R cells with AngII, but not K⁺, activates NGFIB family members, and in turn enhances expression of HSD3B1, whereas both treatments upregulate expression of *CYP11B2* through, at least in part, activating CREB/ATF family proteins. Future studies aiming at deciphering the mechanism underlying the differential regulation of expression of *HSD3B1* and *CYP11B2* will help to provide a better understanding of pathogenesis of IHA.

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Chapter 3

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

INTRODUCTION

Most organisms living on the earth have an endogenous circadian clock system to survive 24 h rhythmic environmental changes. Disturbance of the circadian clock links to the pathogenesis of a variety of diseases [1,2]. Indeed, our laboratory previously showed that circadian clock-deficient *Cry*-null (*Cry1*^{-/-}; *Cry2*^{-/-}) mice showed salt-sensitive hypertension due to abnormally high synthesis of aldosterone [3,4]. Hyperaldosteronism in *Cry*-null mice is caused by constantly high expression of adrenal *Hsd3b6*, the expression of which is under the control of the circadian oscillator. This oscillator is based on interacting transcriptional-translational feedback loops composed of clock genes: core loop and accessory loop [5]. In the core loop, the positive transcription factors, *Clock* and *Bmal1*, drive expression of the period genes (*Per1*, *Per2* and *Per3*) and cryptochrome genes (*Cry1* and *Cry2*). The period and cryptochrome proteins in turn repress the *Clock/Bmal1*-dependent induction of their own transcription [6]. The accessory loop involving *Rev-erb* and *Ror* represses or induces, respectively, *Bmal1* gene expression to help the maintenance of the near 24 h cyclicity [7]. These loops control the expression of a wide variety of clock-controlled genes (including genes encoding the circadian

transactivator *Dbp* and transrepressor *E4bp4*) [8,9].

In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus act as a master pacemaker of the circadian clock system; the SCN receives environmental timing information and synchronizes the organism to the outside world. Not only SCN but also most peripheral organs, such as liver, kidney, and adrenal gland, possess circadian oscillator driven by molecular clock mechanisms [10-12]. The circadian oscillator controls expression of up to 10% of the transcriptome in a given tissue, including rate-limiting enzymes, giving circadian variation to many physiological events [13]. Peripheral oscillators are synchronized by SCN via systemic signals: hormonal signals, nervous signals, body temperature, feeding, and/or activity rhythms [14].

The renin-angiotensin-aldosterone system has long been demonstrated to be under the control of circadian clock system; the plasma renin activity and plasma aldosterone level have diurnal variation [15], contributing to a diurnal change in blood pressure. Although abnormally high aldosterone production in the *Cry*-null adrenal gland suggests that aldosterone production is directly regulated by circadian oscillator in adrenal-aldosterone producing cells, it remains unknown whether they have functional clock or not because of the multilayered intricate structure of the

adrenal cortex. The adrenal cortex consists of functionally distinct three layers: mineralocorticoid producing zona glomerulosa (ZG), glucocorticoid producing zona fasciculata (ZF), and sex steroid producing zona reticularis (ZR) [16]. Among them, ZG is a thin layer and comprises only 15% of the cortex volume [17].

While a number of studies have demonstrated that functional clock exist in the adrenal gland [18,19], functional properties of local clock in the ZG cells have yet to be explored. Here I provide evidence that a functional local clock system exists in adrenal gland ZG cells of human and rodent. Using live-cell bioluminescence imaging and mRNA expression profiling with laser-captured cells, I illustrated robust circadian expression profiles of canonical clock genes in ZG cells. Genetic perturbation studies confirmed a requirement of clock genes for autonomous oscillation of 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 ZG cells.

METHODS

Plasmids and Chemicals

For construction of *mPer2:luc*-PB, the 5'-flanking region (1.7 kb) of the mouse *Per2* gene was cloned into a luciferase reporter plasmid pGL4.10 (Promega), and a 235-bp internal *piggyBac* 3'-terminal sequence (3'TS) and a 310-bp 5'-terminal sequence (5'TS) were PCR amplified from p3E1.2 plasmid [20] and inserted into a *KpnI* site upstream of the *Per2* promoter and a *BamHI* site downstream of the luciferase cDNA, respectively. Construction of *FerH*-PBTP was previously described [21]. AngII was purchased from the Peptide Institute, Inc. PD123,319 was purchased from Sigma. CV11974 was donated by Takeda Chemical Industries, Ltd, Osaka, Japan.

Animals

I used 2- to 5-month-old male C57BL6 wild-type, *Cry1*^{-/-}; *Cry2*^{-/-} [22] and *Clock*^{Δ19/Δ19} [23] mice. Before experiments, all animals were acclimated for at least 2 weeks in an environment with a 12-h light 12-h dark (LD) cycle, maintained at 22 ± 2°C, with food and water provided *ad libitum*. For laser microdissection analysis, I transferred the mice into constant darkness (DD) and

collected the adrenal glands by surgery at selected time points on the first day in DD. For adrenal slice culture, I used *Per2^{Luc}* knock-in mice [24]. *Per2^{Luc/+}: Clock^{Δ19/Δ19}* mice were generated by intercrossing *Per2^{Luc}* knock-in and *Clock^{Δ19/Δ19}* mice. For a primary culture of rat glomerulosa cells, I used Sprague-Dawley rats or *Per2*-dluc transgenic rats [25]. All the studies were approved by the Animal Experimentation Committee of Kyoto University.

Laser Microdissection

I conducted laser microdissection as described with slight modification [3]. In brief, cryosections (7 μm thickness) of fresh frozen adrenal gland were treated with ice-cold mixture of ethanol and acetic acid (19:1) and stained with 0.1% Cresyl Echt Violet. Cells in ZG were microdissected using a LMD7000 device (Leica; x40 magnification) and lysed into TRIzol reagent (Invitrogen). Total RNA was purified using RNeasy micro kit (Qiagen).

Quantitative RT-PCR

Total RNA was converted to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative PCR analysis of individual cDNAs was achieved using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) with StepOnePlus real-time

PCR monitoring system (Applied Biosystems). Data were normalized to a non-oscillatory housekeeping gene, *RPLP0* mRNA levels for human and *Rplp0* for rat and mouse experiments. The sequences for the primers and probes are as follows: for mouse *Per2*, forward primer 5'-CAT CCA CAA GAA GAT CCT AC-3' and reverse primer 5'-GCT CCA CGG GTT GAT GAA GC-3'; for mouse *Bmal1*, forward primer 5'-CCT AAT TCT CAG GGC AGC AGA T-3' and reverse primer 5'-TCC AGT CTT GGC ATC AAT GAG T-3'; for mouse *Dbp*, forward primer 5'-AAT GAC CTT TGA ACC TGA TCC CGC T-3' and reverse primer 5'-GCT CCA GTA CTT CTC ATC CTT CTG T-3'; for mouse *Rplp0*, forward primer 5'- CGA AAA TCT CCA GAG GCA CCA TTG-3' and reverse primer 5'-CTC CCA CCT TGT CTC CAG TC-3'; for rat *Cyp11b2*, forward primer 5'-GAA CCC TGG TAG CCT GAA GTT CAT C-3', reverse primer 5'-TCT GAG AGC TGC CGA GTC TG-3' ; rat *Rplp0*, forward primer 5'-GTC ACA GTA CCT GCT CAG AAC AC-3', reverse primer 5'-CTC CCA CCT TGT CTC CAG TC-3'; for human *PER1*, forward primer 5'-GCA TCT CAG CGG AGC TCA CA-3' and reverse primer 5'-GAG GCT GTA GGC AAT GGA ACT G-3'; for human *PER2*, forward primer 5'-GTG CAG CTC CAC CCT AGT GA-3' and reverse primer 5'-GAT TTT CCT GCT CCA TGG GTT GAT G-3'; for human *CRY1*, forward primer 5'-AGC AAA CTC ACC TGT TGA AGC AAG G-3' and reverse primer 5'-GCT GCA ACA GTA TTC CTC CTG AAT G-3'; for human *BMAL1*,

forward primer 5'-AGT CTG TCT TCA AGA TCC TCA ACT AC-3' and reverse primer 5'-CTG GAA GTC CAG TTT TTG CAT CTA TG-3'; for human *DBP* forward primer 5'-GAA CCC GAC CCA GCT GAT CT-3' and reverse primer 5'-CTT GGC TGC CTC GTT GTT CTT GT-3'; for human *E4BP4*, forward primer 5'-CCC GAG AGC AGG AAC ACG AT-3' and reverse primer 5'-ACC CTA TCT ATG TGT GTA GGA GAA C-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'; and 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).

Adrenal slice culture

For adrenal slice preparation, I sacrificed *Per2^{Luc}* knock-in mice at ZT5–7 in LD cycle (5–7 h post light onset). Immediately after surgical isolation, I freed adrenal glands of adherent fat and cut into slices (300 µm thickness) with a tissue chopper (McIlwain Laboratory Engineering Co., Ltd., Surrey, UK). The slices were placed on a membrane (Millicell-CM, Millipore) dipped into serum-free minimum essential medium (MEM) containing 20 mM HEPES, 36 mM glucose, 100 units/ml penicillin, and 100 µg/ml

streptomycin with 1% ITS + Premix (BD Bioscience) at 35°C. Following incubation for 3 hours, the slices were transferred into a sealed 35 mm dish with 1 ml of the culture medium containing 1 mM D-luciferin. The bioluminescence from the cultured adrenal slice was measured as described [26] with a highly sensitive cryogenic CCD camera (Spectra Video SV16KV/CT: Pixel Vision) coupled to a microscope (Axiovert 135TV: Carl Zeiss). Successive images were acquired every 20 minutes, each with an exposure time of 20 min. Background noise was removed from the images by applying a median filter (Metamorph Software, Molecular devices, Tokyo, Japan) to the whole stack of pictures, before measuring luminescence intensity from the area of interest. Luminescence intensity data were filtered by a low-pass filter with a cutoff threshold of 72 cycles/day to eliminate stochastic ultradian oscillations. For phase-shift experiments, 4 µl of prewarmed HEPES buffer with or without AngII was administrated in the culture medium (final AngII concentration, 100 nM) at the time indicated. When indicated, slices were treated with 1 µM CV11974 an hour before AngII administration. Phase shifts were calculated as the time-interval between the second and third peaks minus the time-interval between the first and second peak. By convention, phase delay and advance are expressed as negative and positive, respectively.

Primary culture of rat glomerulosa cells

Male Sprague-Dawley rats (350–450 g) or *Per2*-dluc transgenic rats were sacrificed at ZT5–7 and adrenal glands were immediately removed and dissected free of fat. The capsules were separated from fasciculata-reticularis by manual compression. The capsules were incubated for 20 min at 37°C in oxygenized MEM (4 capsules/ml) containing 2 mg/mL collagenase and 125 units/ml deoxyribonuclease. After incubation, the cells were disrupted mechanically, filtered through Cell Strainer (100 µm, BD Falcon), and centrifuged for 10 min at 300x *g*. The cell pellet was resuspended in OPTI-MEM medium supplemented with 2% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. For gene expression analysis, the cells were plated in 24 well plates (1 capsule/well) and the culture medium was replaced at 24 h intervals for 3 days. Then, the cells were cultured with or without 100 nM AngII for 12 h and lysed in TRIzol reagent (Invitrogen). Total RNA was purified using RNeasy mini kit (Qiagen). For tracing *Per2*-dluc expression by real-time luminometry, the cells from *Per2*-dluc transgenic rats were plated in 35 mm dish (4 capsules/dish). The culture medium was subsequently replaced at 24-hr intervals for 3 days followed by addition of D-luciferin (final 1 mM). Then, the cells were transferred to a photomultiplier tube detector assembly with a

37°C, 5% CO₂ atmosphere control (Kronos Dio, Atto) for luminometric measurements. Photons were counted during 4 min bins at 20 min intervals.

Immunocytochemistry

Isolated glomerulosa cells were seeded on poly-D-lysine-coated coverslips and cultured with or without 100 nM AngII for 12 h. Then, the cells were fixed with acetone for 10 min at –20°C, immediately rehydrated in 0.1 M phosphate buffered saline (PBS) for 5 min, and incubated with primary antibody (anti-CYP11B2 antibody (MAB6021, Millipore), 1:100 dilution, in 0.1 M PBS containing 0.3% Triton X-100 (PBX)) for 5 days at 4°C. After washing with PBX, the cells were incubated with secondary antibody (Alexa594 labeled donkey anti mouse IgG (Invitrogen), 1:5,000 dilution in PBX) for 12 h at 4°C. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) before microscopic observation.

H295R cell culture, RNA extraction and real-time luminescence measurements

Human adrenocortical H295R cells were obtained from the American Type Culture Collection (CRL-2128). For gene

expression analysis, cells grown in a culture medium (DMEM/F12 supplemented with 2.5% Nu serum (BD Bioscience), 1% ITS + Premix, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin) were plated in 24 well plates at a density of $\sim 2.0 \times 10^5$ cells/well. After 3 cycles of temperature entrainment (12-h 37°C /12-h 33°C), cells were released into constant 37°C conditions and lysed in TRIzol reagent (Invitrogen) every 4-hour for 2 days. Total RNA was purified using RNeasy mini kit (Qiagen). For tracing *mPer2*-luc expression by real-time luminometry, cells plated in 35 mm dish were transfected with a plasmid mixture containing *mPer2*-luc-PB and *FerH*-PBTP at the indicated concentrations using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Six hours after the transfection, the medium was replaced with 1 mM D-luciferin-containing medium and cells were pre-incubated for 4 days in a 5% CO₂ incubator at 37°C. Then, cells were transferred to a photomultiplier tube detector assembly with a 37°C, 5% CO₂ atmosphere control (Kronos Dio, Atto) for luminometric measurements. Photons were counted during 2 min bins at 20 min intervals. At time 0, AngII (final concentration: 100 nM) was added to the medium. When indicated, cells were treated with 1 µM CV11974, 1 µM PD123,319 or vehicle (0.1 M Na₂CO₃) 1 hour before AngII administration.

Statistical analysis

I analyzed rat *Cyp11b2* expression levels with two-tailed Student's t-test. I used one-way analysis of variance (ANOVA) with Bonferroni's post hoc test for phase shift analysis.

RESULTS

Clock gene expression within adrenal ZG cells

I first characterized expression of clock genes within mouse adrenal ZG cells *in vivo*. ZG cells, lying in the outermost adrenocortical layer, were selectively isolated from the whole adrenal sections by using laser capture microdissection (Fig. 1A). To pursue circadian changes driven by the endogenous clock, I used animals kept in constant darkness and sampled the adrenals from them at 4-hour intervals starting from circadian time (CT) 0 (the onset of the subjective morning). Quantitative RT-PCR analysis (Fig. 1B, left) revealed that the expression levels of the key clock components, *Period2* (*Per2*) and *Bmal1*—representatives of two major feedback loops within the clockwork—showed high-amplitude circadian oscillations with peak-to-trough ratios of ~4.4 for *Per2* and ~18.8 for *Bmal1*. Overt circadian oscillations in ZG cells were further illustrated by a pronounced circadian expression of *Dbp*—a representative of the clock-controlled output genes—in the laser-captured ZG cells. The expression peaked around CT16 for *Per2*, CT20–0 for *Bmal1*, and CT8 for *Dbp*—a phase-relationship similar to what has been observed in a number of peripheral clock tissues, such as liver, kidney, and heart. In

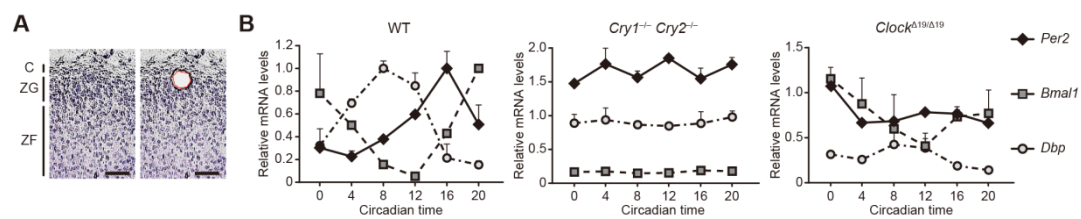


Fig. 1. Clock gene expression profiles in adrenal ZG cells. (A) A representative tissue section before and after laser microdissection. ZG cells are ovoid in shape and are arranged in globular clusters, lying directly beneath the capsular layer. C, capsule; ZF, zona fasciculata. Cresyl Echt Violet staining. Scale bars, 50 μ m. (B) Circadian expression profiles of *Per2*, *Bmal1* and *Dbp* mRNAs in adrenal ZG cells from WT (left), *Cry*-null (middle), and *Clock^{Δ19/Δ19}* (right) mice. The peak values of each gene in WT adrenal ZG cells were set to 1. I collected the adrenal samples from the animals kept under constant darkness (DD) (i.e. without photic time cue) at 4-hour intervals starting at circadian time (CT) 0 (the onset of the subjective day). Values are the means $\pm$ variation ($n = 2$ mice for each data point) normalized to expression of *Rplp0*.

Cry1^{-/-}; *Cry2*^{-/-} (Fig. 1B, middle) and *Clock*^{Δ19/Δ19} (Fig. 1B, right) mutant mice, ZG cells displayed compromised circadian rhythmicity of both clock and clock-controlled genes. These data provide evidence that the circadian oscillations in wild-type (WT) ZG cells are driven by the endogenous (hence, genetically encoded) biological clock.

Cultured adrenal ZG cells display self-sustained circadian oscillation.

The molecular clock components exist and fluctuate *in vivo* locally within the ZG cells (Fig. 1 A and B); however, it remains unclear whether they constitute an autonomous clockwork able to operate locally without the aid of exogenous systemic cues. To address this point, I used cultured adrenal explants from *Per2*^{Luc} knock-in mice [24], an animal model in which a luciferase-coding sequence is inserted in-frame into the 3' end of the endogenous *Per2* gene (the *Per2*:Luc knock-in product recapitulates the endogenous *Per2* protein cycling). Real-time bioluminescence imaging with high resolution video microscopy [26] was employed to monitor circadian dynamics within the ZG layer in cultured adrenal slices. As shown in Fig. 2A, bright luminescence was observed only in the outermost ZG region of cultured adrenal slices.

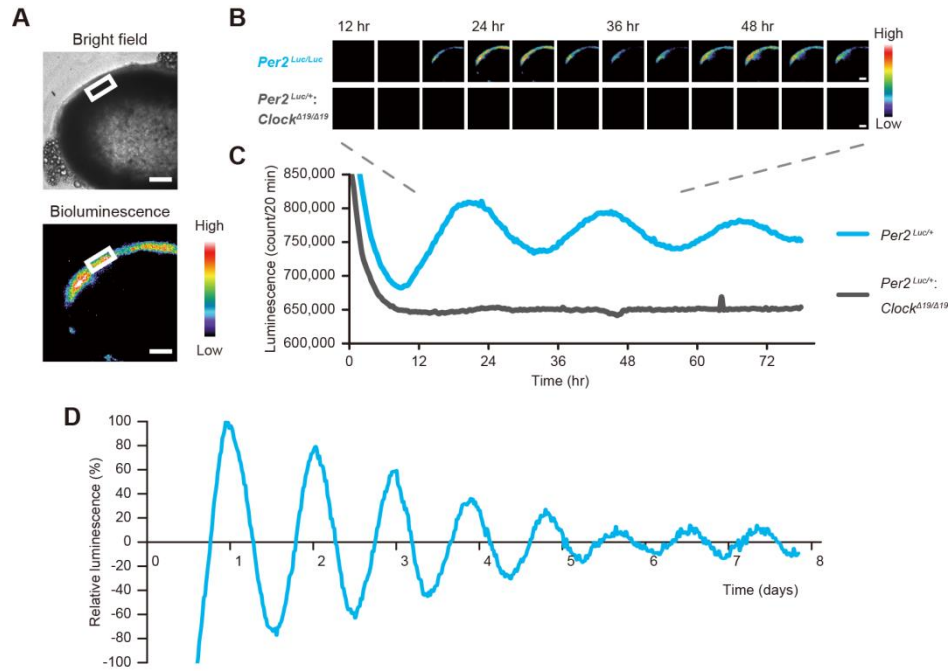


Fig. 2. Autonomous circadian oscillator in mouse adrenal ZG. (A) Optical (upper) and bioluminescence (lower) image of *Per2^{Luc/Luc}* adrenal slice. Bioluminescence intensity was indicated pseudocolour scale. White boxes within the images indicate the standard region of interest used for quantification of bioluminescence in the ZG. Scale bars, 200 μ m. (B) Time-lapse images of bioluminescence from *Per2^{Luc/Luc}* (upper) and *Per2^{Luc/+}; Clock ^{Δ 19/ Δ 19}* (lower) adrenal slices. The images are lined every 12 images (every 4 h) for 2 days. Bioluminescence intensity was indicated pseudocolour scale. Scale bars, 200 μ m. (C) Bioluminescence time-series from ZG region in adrenal slices obtained from *Per2^{Luc/+}* (blue) and *Per2^{Luc/+}; Clock ^{Δ 19/ Δ 19}* (gray) mice. Shown are the representative data without any smoothing. (D) Circadian rhythms in ZG were persisted for more than 1 week ex vivo. Organotypic adrenal slices obtained from *Per2^{Luc/Luc}* mice were monitored for 200 h. Shown is the detrended time series of bioluminescence from ZG cells. Detrending was performed by subtraction of the 24-h moving average from the raw data. The maximum bioluminescence value was set to 100%.

Continuous monitoring of light emission (Fig. 2B and C) revealed that ZG cells exhibit persistent circadian oscillation for more than a week (Fig. 2D) with a period length of 23.67 ± 0.68 h ($n = 4$, means $\pm$ SEM). The adrenal slices from *Clock* ^{$\Delta 19/\Delta 19$} mutant mice lacked a circadian rhythm of bioluminescence (Fig. 2B and C), revealing that the oscillations in the WT adrenal slices arise from intrinsic clockworks.

In the organotypic slice culture system, I found circadian bioluminescence in the outermost adrenal area where ZG cells exist; however, I cannot exclude that the observed circadian rhythms were solely derived from connective tissues or capsular portions. To rule out this possibility, I isolated glomerulosa cells from the rat adrenal gland and measured their rhythmicity in culture. At first, I confirmed that collected cells still possess a characteristic feature of ZG cells; Cyp11b2 expression and AngII responsiveness. Immunocytochemistry with anti-Cyp11b2 antibody showed that approximately 90% of the cells were immunopositive for Cyp11b2 (Fig. 3A); the rest seems to be unavoidable inner cell contamination. Moreover, their *Cyp11b2* mRNA expression levels were increased upon AngII stimulation (Fig. 3A and B). These results indicate that glomerulosa cells were successfully collected.

To test whether isolated glomerulosa cells possess a

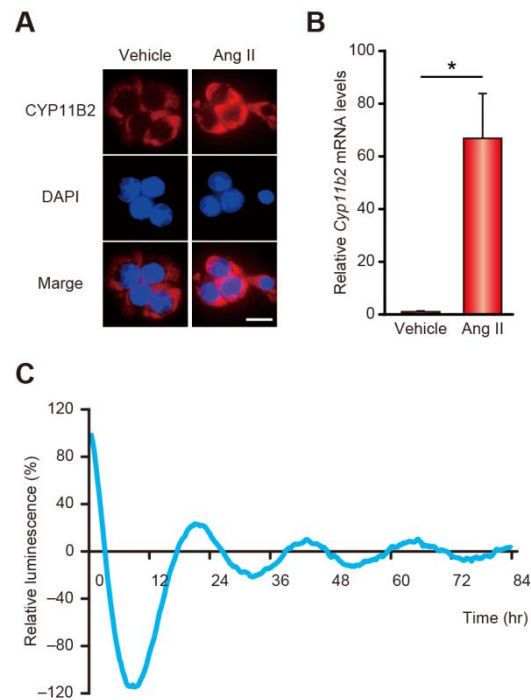


Fig. 3. Autonomous circadian oscillator in rat primary ZG cells. (A) Representative immunocytochemical images depicting CYP11B2 expression levels in rat glomerulosa cells with or without AngII-stimulation. Scale bars, 10 μ m. (B) AngII-induced *Cyp11b2* mRNA expression in isolated glomerulosa cells. The mean of vehicle treatment was set to 1. Values are the means $\pm$ SEM ($n = 3$ for each group) normalized to expression of *Rplp0*. *, $P < 0.05$, Student's t-test. (C) Bioluminescence time-series from *Per2:dluc* glomerulosa cells. Isolated glomerulosa cells from *Per2:dluc* transgenic rats were entrained by 24 h interval medium changes and then their bioluminescence was measured continuously for 4 days. Data are detrended by subtraction of the 24-h moving average from the raw data. The maximum bioluminescence value was set to 100%.

functional circadian oscillator, I used *Per2*-dluc transgenic rats that carry a destabilized luciferase reporter gene under the control of the *mPer2* promoter [25]. Previous works have shown that peripheral oscillators, such as fibroblasts, are sensitive to medium changes [10,27], which can synchronize the circadian rhythms of cells in culture. I therefore synchronized *Per2*-dluc glomerulosa cells by 24-hr interval of medium changes followed by real-time monitoring of their bioluminescence. The synchronized cells showed distinct circadian oscillation of bioluminescence, albeit with a continuous decrease in the amplitude (Fig. 3C). Although the amplitude of the circadian bioluminescence was gradually damped, the rhythm persisted more than 3 days after entrainment. Essentially the same circadian profile was observed in *Per1*-luc glomerulosa cells [28]. These results indicate that, in adrenal ZG cells, a circadian clock is able to operate autonomously without requirement of systemic drivers.

Human adrenocortical H295R cells possess temperature-entrainable clock.

Human adrenocortical H295R cells have been widely used to study adrenal cell physiology, especially aldosterone production. I asked whether, in these cells, canonical clock genes show rhythmic transcriptional profiles. Because circadian regulation

of body temperature has been proposed to synchronize peripheral oscillators *in vivo* and *in vitro* [29], I subjected H295R cells to a 12-hour 37°C /12-hour 33°C temperature cycle. Thereafter, I harvested them every 4 hours during 2 consecutive days and analyzed expression of *PER2*, *BMAL1*, and *DBP* (Fig. 4A). I found that these genes displayed overt circadian expression and the relative phases observed *in vivo* ZG cells are reproduced in temperature-entrained H295R cells: *DBP* mRNA oscillations precede *PER2* mRNA oscillations, which in turn precede *BMAL1* mRNA oscillations (Fig. 4A). Further analysis revealed that all clock genes examined displayed overt circadian expression in H295R cells (Fig. 4B).

I next addressed whether this oscillator functionally drives cyclic expression of aldosterone-producing enzyme in H295R cells. I therefore examined the expression profile of *HSD3B1*, the human counterpart of mouse *Hsd3b6*, after temperature cycles. Unexpectedly, the expression profile of *HSD3B1* mRNA showed no obvious circadian rhythms (T. Ota, M. Doi, and H. Okamura, unpublished data).

AngII acts as a phase modulator for adrenal ZG cells.

AngII is a potent physiological regulator of ZG cell functions. Finally, I examined effect of AngII on the ZG clock using

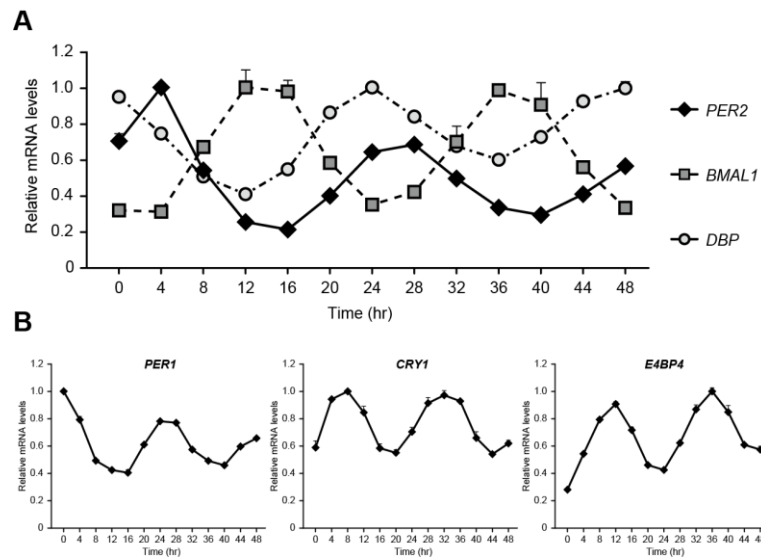


Fig. 4. Human adrenocortical H295R cells exhibit circadian oscillation. (A and B) Circadian expression of various clock genes in human adrenocortical H295R cells. H295R cells were exposed to temperature cycles (12-h 37°C/12-h 33°C, 3 cycles) and then harvested every 4 h for 2 days. The maximum values were set to 1. Values are the means $\pm$ SEM ($n = 3$ for each time points) normalized to expression of *RPLP0*.

adrenal slice culture. When AngII was administered at 12 h after the trough point of the luminescence (at the peak point of the luminescence), the next peak was significantly phase-delayed in comparison to vehicle administration (AngII, -3.50 ± 0.62 h, $n = 4$; vehicle, 0.17 ± 0.22 h, $n = 4$, means $\pm$ SEM; negative and positive values represent phase delay and advance, respectively; $P < 0.01$; Fig. 5A and D). In contrast, AngII administration at 6 h after the peak point of the luminescence (in the decreasing phase) significantly induced phase advance of the next peak compared with vehicle administration (AngII, 2.56 ± 0.78 h, $n = 3$; vehicle, -0.89 ± 0.91 h, $n = 3$; $P < 0.05$; Fig. 5B and D). The phase shift was abolished by pretreatment of the slices with an AT1 antagonist (AngII, -3.50 ± 0.62 h, $n = 4$; AngII + CV11974, 0.44 ± 0.29 h, $n = 3$; $P < 0.01$; Fig. 5C and D). These results indicate that AngII induces a phase-dependent phase-shift in the ZG region of the adrenal slice.

To further estimate effects of AngII on the ZG clock, I tested whether AngII induces phase reset in adrenocortical H295R cells. To observe the internal circadian clock oscillator in living cells, I used transposon-based real-time bioluminescence assay with mouse *Per2* promoter-driven firefly luciferase as a reporter [30]. I took advantage of the *piggyBac* transposon-based system because *piggyBac* transposase was shown to transpose a DNA fragment more

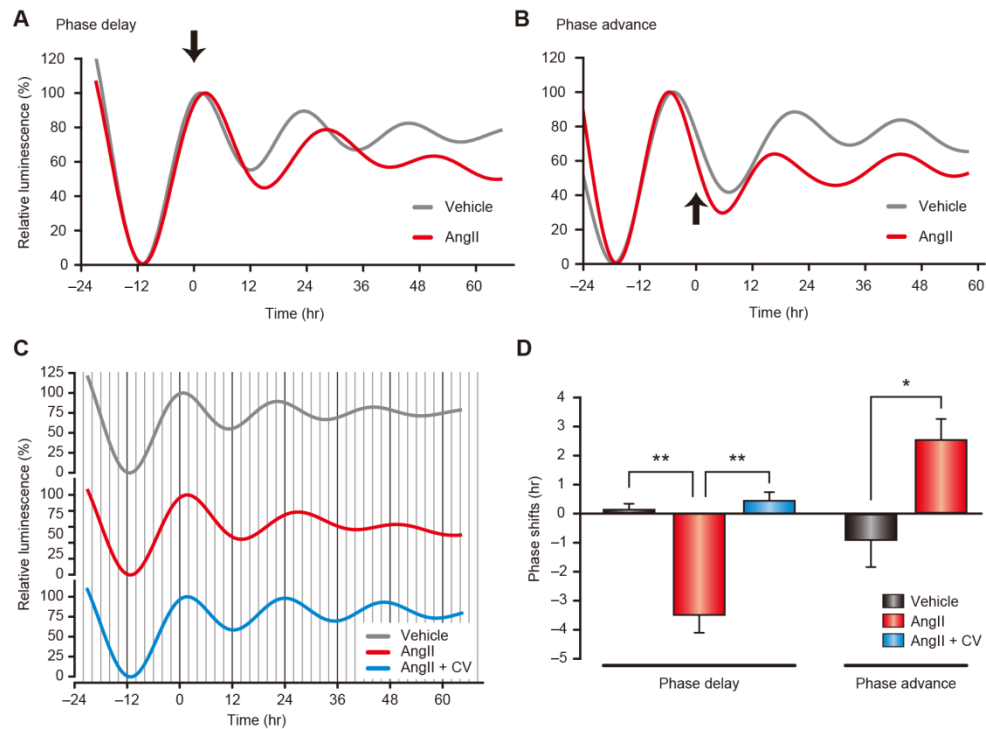


Fig. 5. AngII elicits phase-dependent phase shifts in adrenal ZG cells. (A to B) AngII was administered to cultured adrenal slices at either 12 h after the first trough (A) or 6 h after the first peak (B). Shown are representative records of bioluminescence from ZG. Arrows indicate the time of AngII or vehicle administration. The values of the first peak and trough were adjusted to 100 and 0, respectively. Note that the phase responses varied depending on the time (phase) of the AngII administration. (C) AngII was administered to the culture medium as in A. CV11974 (final concentration: 1 μ M) was added to the medium 1 h before AngII administration. The values of the first peak and trough were adjusted to 100 and 0, respectively. Vehicle and AngII are the replots of A. (D) Summary of phase shift induced by AngII administration with or without AT1 blocker. By convention, delays are negative and advances are positive. Values are the means $\pm$ SEM ($n = 3-4$ slices). *, $P < 0.05$, **, $P < 0.01$, Bonferroni test.

efficiently than other transposase in mammalian cells [31-33]. I cotransfected H295R cells with *piggyBac* transposase expression plasmid (*FerH*-PBTP) and *piggyBac* transposon-based *mPer2*-luc vectors (*mPer2*-luc-PB) (Fig. 6A) and luminescence was measured in real-time mode with PMT detectors. The transfected cells showed nearly constant bioluminescence before AngII stimulation. AngII elicited circadian bioluminescence even when H295R cells pretreated with vehicle or PD123,319, an AT2 antagonist. In contrast, when the cells were pretreated with CV11974, bioluminescence remained nearly constant after AngII stimulation (Fig. 6B), indicating that the AngII-AT1 pathway evoked circadian bioluminescence in H295R cells.

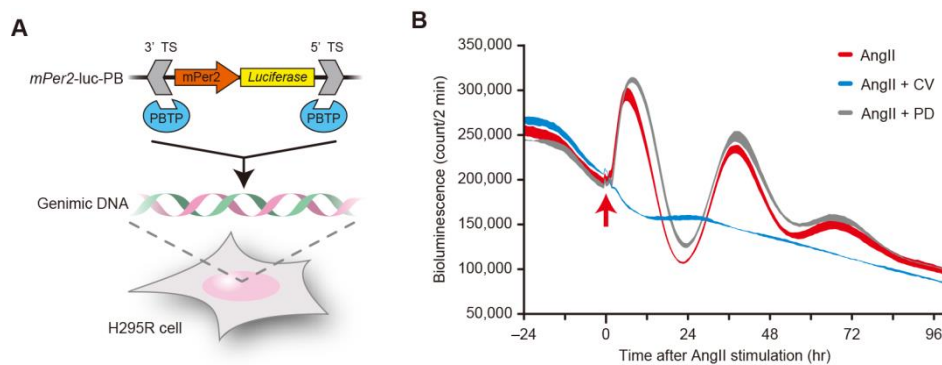


Fig. 6. AngII elicits circadian oscillation in H295R cells. (A) A schematic diagram of transposon-based real-time bioluminescence assay. A DNA fragment between 3'TS and 5'TS will be transposed by *piggyBac* transposase. (B) Time-series of *mPer2* promoter-driven bioluminescence from H295R cells, following successive treatments with selective AngII antagonist and agonist. H295R cells grown to confluence in 35 mm dishes were transfected with 1.3 μg *mPer2*:luc-PB and 2.7 μg *FerH*-PBTP, and treated with vehicle (red), CV11974 (blue; final concentration 1 μM), or PD123,319 (gray; final concentration 1 μM) 1 h prior to AngII administration. Values are the means $\pm$ variation ($n = 2$ for each group).

Discussion

In this study, I demonstrated that molecular clock components exist and fluctuate in adrenal zona glomerulosa cells. The phase relations of these clock gene mRNA oscillations were as expected from the molecular makeup of the circadian circuitry [34]. Moreover, oscillations of these clock genes are completely abolished in *Cry*-null mice lacking molecular clocks and diminished in *Clock* mutant mice. Taken together, the circadian oscillations in wild-type (WT) ZG cells are driven by the endogenous (hence, genetically encoded) biological clock.

The above findings demonstrate the presence of a genetically encoded molecular clock in ZG cells. To determine whether this clock is autonomous, I observed circadian rhythm in ZG cells in culture. In all culture systems I used, I observed distinct circadian rhythms, but their oscillation gradually damped. The oscillation damping is probably because of a gradual desynchronization of the individual cells in the population known for non-SCN mammalian cells and tissues in culture [35].

In the organotypic slice culture system presented here, bioluminescence and overt circadian rhythms were observed in the outermost ZG region. Unexpectedly, however, the levels of

luminescence from inner cortex (zona fasciculata-reticularis) and medulla were constantly low in these slices. Oster *et al.* [18, 36] reported that clock genes displayed distinct expression differences between two time points in zone fasciculata and medulla *in vivo*. These observations perhaps suggest that these regions require exogenous factors, such as ACTH, or sympathetic nerve activity (or culture supplements), for maintenance of their circadian rhythms.

In different microarray analyses, up to 10% of transcripts were judged to be rhythmically expressed [13], and many of them have roles in biosynthetic and metabolic processes [1]. In the murine adrenal, around 5% of all transcripts are expressed under the control of the circadian pacemaker and some of them are related to steroidogenesis [36]. Whereas mouse *Hsd3b6* expression in the adrenal gland shows circadian fluctuation, I failed to observe circadian expression of *HSD3B1*, the human counterpart of mouse *Hsd3b6*, in temperature-entrained H295R cells. Considering that the 5'-flanking genomic sequence of *HSD3B1* contains a consensus Dbp-binding motif, which is also present in the mouse *Hsd3b6* promoter region (T. Ota, M. Doi, and H. Okamura, unpublished data), it is possibly that regulation of *HSD3B1* expression may be uncoupled from molecular clock mechanisms in H295R cells. An alternative explanation is also possible. For example,

H295R cells may not fully recapitulate characteristics of ZG cells; the level of *HSD3B1* mRNA in H295R cells is significantly lower than that in human ZG cells, but rather similar to that in human ZF cells (T. Ota, M. Doi, and H. Okamura, unpublished data). To elucidate the relationship between the circadian clock system and pathogenesis of primary hyperaldosteronism, it is required to develop new model systems, such as a cell line in which the molecular clock system functionally regulates aldosterone production.

This study also demonstrated that AngII can shift and reset the phase of the circadian oscillator in mouse ZG and human H295R cells. I also showed that the phase shift completely depended on the AT1 signaling. AngII is known to activate many signaling pathways, including CREB activation [37]. It is well known that CREB activation is closely associated with induction of the *Per1* gene and mediates phase shift *in vivo* or in organotypic culture and phase reset in cultured cells [38,39]. The *Per1* induction seems to contribute to the phase-dependent phase shift in the ZG clock.

In conclusion, I have provided evidence that ZG cells in both the human and rodent adrenal have a cell-autonomous clock. However, it is currently unknown whether the circadian clock-gene oscillations in ZG cells are important for their function. It has also yet to be examined whether malfunction of this local clock causes

hyperaldosteronism observed in *Cry*-null mice. The analysis of ZG-specific *Cry*-null mice will help to address these questions. I also showed that the ZG clock is modulated by AngII. On the basis of the observations presented in this study, it is tempting to speculate that AngII may be one of the factors that coordinate biological functions by modulating the phase of circadian gene expression, and that abnormal AngII regulation, like pre-eclampsia [40], might induce alteration in circadian expression of clock genes and/or clock-controlled genes in ZG cells.

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