Stimulus-selective induction of the orphan nuclear receptor NGFIB underlies different influences of angiotensin II and potassium on the human adrenal gland zona glomerulosa-specific 3β-HSD isoform gene expression in adrenocortical H295R cells

Daisuke Yarimizu1)*, Masao Doi1). 2)*, Takumi Ota1) and Hitoshi Okamura1). 2)

1) Department of Systems Biology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8051, Japan
2) Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Saitama 332-0012, Japan

Abstract. In the adrenal, the type I 3β-hydroxysteroid dehydrogenase (HSD3B1) is expressed exclusively in the zona glomerulosa (ZG), where aldosterone is produced. Angiotensin II (AngII) and potassium (K+) are the major physiological regulators of aldosterone synthesis. However, their respective roles in regulation of aldosterone synthesis are not fully defined, particularly in terms of transcriptional regulation of steroidogenic enzyme genes. We previously showed that AngII can stimulate expression of HSD3B1. But, K+ responsiveness of this gene has remained unexplored. Here, we report that K+ stimulation lacks the ability to induce HSD3B1 expression in human adrenocortical H295R cells. Both AngII and K+ were able to enhance transcription of the aldosterone synthase gene (CYP11B2). Promoter analysis revealed that although both AngII and K+ activate transcription from the Ca2+/cAMP-responsive element (CRE) located in the CYP11B2 promoter, the orphan nuclear receptor NGFIB-responsive element (NBRE) located in the HSD3B1 promoter fails to respond to K+, being only able to enhance transcription after AngII treatment. We found that induction of de novo protein synthesis of NGFIB occurs only after AngII treatment. This sharply contrasts with the phosphorylation that occurs in response to both AngII and K+ on the CREB/ATF family transcription factor ATF2. Chromatin immunoprecipitation assay confirmed that the NGFIB protein occupies the HSD3B1 promoter only after AngII, while ATF2 binds to the CYP11B2 promoter in response to both AngII and K+. These data provide evidence that downstream signals from AngII and K+ can be uncoupled in the regulation of HSD3B1 in the human adrenocortical H295R cells.

Key words: 3β-hydroxysteroid dehydrogenase, Aldosterone synthase, Angiotensin II, Potassium, Orphan nuclear receptor NGFIB

AngII and K+ are the major physiological regulators of aldosterone synthesis. However, their respective roles in regulation of aldosterone synthesis are not well defined, particularly in terms of transcriptional regulation of steroidogenic enzyme genes. Aldosterone synthesis occurs exclusively in the adrenal zona glomerulosa (ZG) cells via a series of enzymatic reactions involving a number of enzymes. The 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD) is an enzyme that catalyzes the conversion of Δ5-3β-hydroxysteroids (e.g. pregnenolone) into hormonally active Δ4,3-ketosteroids (e.g. progesterone) [1, 2], a step required for aldosterone synthesis. Whereas two distinct 3β-HSD isoforms (type I 3β-HSD encoded by HSD3B1 and type II 3β-HSD encoded by HSD3B2) exist in humans, the type II 3β-HSD has long been considered the major or sole isoform present in the adrenal gland. However, the recent finding that type I 3β-HSD is expressed in ZG cells [3, 4] has revised the canonical view and raised a question as to whether the expression of this gene (HSD3B1) is under the control of AngII and K+. By using human adrenocortical H295R cells as a model system, we recently showed that AngII could stimulate expression of HSD3B1 [5]. We showed that AngII-induced induction of HSD3B1 requires de novo protein
synthesis of the orphan nuclear receptors nerve growth factor IB (NGFIB) and nuclear receptor related 1 (NURR1) [5]. The HSD3B1 promoter contains a functional NGFIB/NURR1-responsive element (NBRE) to which these proteins bind in response to AngII [5]. However, it remains unknown whether K+ stimulation similarly activates this NGFIB/NURR1 pathway.

The aldosterone synthase, which is encoded by CYP11B2, catalyzes the final step of aldosterone synthesis [6]. Like HSD3B1, expression of CYP11B2 is confined to ZG cells. It has been well established that AngII and K+ both activate transcription of CYP11B2 through a common cis-regulatory element [7]. The CYP11B2 promoter contains a Ca2+/cAMP-response element (CRE) to which the CREB/ATF family proteins bind in response to AngII and K+ [8]. Thus, AngII and K+ are thought to share, at least in part, the CREB/ATF-mediated transcription pathway. However, the extent of difference (or similarity) between AngII and K+ in the regulation of other steroidogenic enzyme genes has remained unexplored.

Here we examined K+ sensitivity of HSD3B1 in H295R cells. Contrary to our naive expectation, we observed that K+ stimulation was unable to enhance expression of HSD3B1. To understand the underlying mechanism better, we compared transcriptional regulation of HSD3B1 with that of CYP11B2. The results presented in this study provide several lines of evidence that the NGFIB/NURR1-HSD3B1 pathway operates only for AngII. This selectivity of action of NGFIB/NURR1 differs from the phospho-CREB/ATF-mediated transcription pathway that operates in response to both AngII and K+.

**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) in a 37°C humidified atmosphere (5% CO2). Unless otherwise noted, cells were cultured in the above-mentioned serum-containing medium. For gene expression analysis, cells were seeded on 24-well plate at a density of 2 × 10^5 cells/well and cultured for 3 days before agonist stimulation. 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. For the analysis of serum-starved cells, cells were cultured as described above except that the medium was replaced with DMEM/F-12 serum-free medium for the last 24 h. Forskolin (cAMP activator) treatment, which has been considered to mimic ACTH stimulation, was also performed to confirm a previously reported response of HSD3B2 to the ACTH signal in H295R cells [9-13].

**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 SuperMix (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 isofoms (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 we previously reported [4], these probes distinguish a few nucleotide differences at the region corresponding to the dehydrogenase catalytic Y-X-X-X-K motif [2] of the human 3β-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, we 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 [5]. The standard curve was generated by amplifying a dilution series of a standard DNA (ranging from 1 to 10,000 zmol), for which we used linearized plasmid DNA carrying the target amplicon. The data were normalized to the expression levels of the gene encoding ribosomal protein L0 (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 TTC CTG GAG CTC TTC ATG-3’ and reverse primer 5’-AGG AGA GCC AGG ATA CTG TCA ATC-3’; for NURR1, forward primer 5’-GCC TTC CAG AGG
GAA CTG-3’ and reverse primer 5’-GAG TCC AGC CTG TCC AAT CTC-3’; for NOR1, forward primer 5’-TCC CCT CCT ACA CTC TT-3’ and reverse primer 5’-CTC CAT CGT TGC CGT TT-3’; for CREB, forward primer 5’-GTC CCT CCT ACA CTC TC-3’ and reverse primer 5’-TGG TTC AGC GCC GTT TT-3’; for ATF1, forward primer 5’-TCC CCG ACC AGT CCA AAC AGT-3’ and reverse primer 5’-GGT GTA TTC CGA GCT GTA TGT CT-3’; for ATF2, forward primer 5’-GGG TGC AGT CCA GTT GAC TT-3’ and reverse primer 5’-GAG TTG GCG AGT CCA TTT GAG-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’-AGA AGA GCC TCT GGA AAA CAC ATG-3’, and probe 5’-GAG TTG GCG AGT CCA TTT GAG-3’.

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 transfected 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 [5] 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), and ATF2 (1:1,000 dilution; C-19 antibody; Santa Cruz Biotechnology). 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 [5] with modifications. H295R cells grown to confluence on 10 cm dishes (~1 × 107 cells) were treated with AngII (100 nM), K+ (16 mM), or vehicle (PBS) for 4 h. 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, which recognizes both NGFIB and NURR1 (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 µg fragmented chromatin was immunoprecipitated with 1 µg of anti-ATF2 antibody (C-19 antibody; Santa Cruz Biotechnology). Immunoprecipitated DNA fragments were quantified by either TaqMan qPCR (for HSD3B1 and HSD3B2) or SYBR green-based qPCR (for CYP11B2) with the following primers and probe: for HSD3B1 promoter, forward primer 5'-CAT TGC TCT CTC CTA TGG G-3', and TaqMan probe 5'-VIC-TGC CAC ACT GCA TGT CCT GTC AAG GCT AAA CCC AAG AC-3', reverse primer 5'-CAT TGC TCT CTC CTA TGG G-TAMRA-3'; for HSD3B2 promoter, forward primer 5'-ACT GAC TGT TCT GTT AAG GCT AAA G-3', reverse primer 5'-CAT TGC TCC CTC CTC CAG A-3', and TaqMan probe 5'-VIC-CTT TAT CAC ACT GTG GCC TTA AGA TCC CTC CTC CAG A-3', and TaqMan probe 5'-VIC-CTT TAT CAC ACT GTG GCC TTA AGA TTG GAT TTC TC-TAMRA-3'; for CYP11B2 promoter, forward primer 5'-CCC ACG CCT TTT CTC AGC ATC-3' and reverse primer 5'-AAT GCT CCC TCT CTC CTA TGG G-3', TaqMan probe 5'-VIC-TGC CAC ACT GCA GCA AAG GCT AAA CCC AAG AC-3', reverse primer 5'-CAT TGC TCT CTC CTA TGG G-TAMRA-3'; for 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 [9, 14, 15]. In agreement with previous studies, K+ treatment increased mRNA in H295R CYP11B2 [16] (Fig. 1). The levels of CYP11B2 mRNA in H295R cells were dose-dependently increased to about 6-fold or 25-fold over basal levels after 8 mM or 16 mM K+ treatment, respectively. However, expression levels of HSD3B1 and HSD3B2 were almost constant throughout the K+ treatment even under 16 mM condition (Fig. 1A, B). These results demonstrate that K+-induced intracellular signaling that leads to CYP11B2 expression cannot enhance expression of 3β-HSD genes. Importantly, this was also the case when the cells were serum-starved prior to K+ stimulation (Fig. 1C, D).

K+ treatment was next performed in conjunction with AngII (Fig. 2). As reported, AngII treatment increased expression of HSD3B1 [5] (Fig. 2A). Importantly, however, expression levels of HSD3B1 were not further increased by a simultaneous treatment with AngII and K+ (Fig. 2A). Thus, K+ treatment does not enhance HSD3B1 expression even in the presence of AngII. As shown in Fig. 2B, AngII was unable to induce HSD3B2 [5]. Again, K+ stimulation had no effect on HSD3B2 expression, regardless of whether administered alone or in combination with AngII (Fig. 2B). Forskolin treatment, on the other hand, could increase expression of HSD3B2 (Fig. 2B) as previously suggested [9-13]. Thus, the responsiveness of this gene relies on the type of agonists administered to the cells.

To assess relative expression levels between HSD3B1 and HSD3B2 in H295R cells, we determined absolute mRNA levels for each gene (Fig. 2C). At the basal levels, HSD3B2 was approximately 8 times higher than HSD3B1 (40.7 ± 1.2 mmol/mol for HSD3B1 v.s. 347.8 ± 65.1 mmol/mol for HSD3B2; the values (means ± SEM, n=3) were normalized with RPLP0). On the other hand, after AngII stimulation, HSD3B1 mRNA was dramatically increased to the levels almost equal to or higher than those of HSD3B2 (323.2 ± 18.8 mmol/mol for HSD3B1 v.s. 249.2 ± 20.8 mmol/mol for HSD3B2). Thus, in addition to the responsiveness to AngII, HSD3B1 and HSD3B2 are different in the steady-state basal levels in H295R cells.

Statistical analysis

We 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 a 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 gene-specific TaqMan MGB probes and primers for HSD3B1 and HSD3B2. Due to a 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 [9, 14, 15]. In agreement with previous studies, K+ treatment increased expression of the aldosterone synthase gene CYP11B2 [16] (Fig. 1). The levels of CYP11B2 mRNA in H295R cells were dose-dependently increased to about 6-fold or 25-fold over basal levels after 8 mM or 16 mM K+ treatment, respectively. However, expression levels of HSD3B1 and HSD3B2 were almost constant throughout the K+ treatment even under 16 mM condition (Fig. 1A, B). These results demonstrate that K+-induced intracellular signaling that leads to CYP11B2 expression cannot enhance expression of 3β-HSD genes. Importantly, this was also the case when the cells were serum-starved prior to K+ stimulation (Fig. 1C, D).

K+ treatment was next performed in conjunction with AngII (Fig. 2). As reported, AngII treatment increased expression of HSD3B1 [5] (Fig. 2A). Importantly, however, expression levels of HSD3B1 were not further increased by a simultaneous treatment with AngII and K+ (Fig. 2A). Thus, K+ treatment does not enhance HSD3B1 expression even in the presence of AngII. As shown in Fig. 2B, AngII was unable to induce HSD3B2 [5]. Again, K+ stimulation had no effect on HSD3B2 expression, regardless of whether administered alone or in combination with AngII (Fig. 2B). Forskolin treatment, on the other hand, could increase expression of HSD3B2 (Fig. 2B) as previously suggested [9-13]. Thus, the responsiveness of this gene relies on the type of agonists administered to the cells.

To assess relative expression levels between HSD3B1 and HSD3B2 in H295R cells, we determined absolute mRNA levels for each gene (Fig. 2C). At the basal levels, HSD3B2 was approximately 8 times higher than HSD3B1 (40.7 ± 1.2 mmol/mol for HSD3B1 v.s. 347.8 ± 65.1 mmol/mol for HSD3B2; the values (means ± SEM, n=3) were normalized with RPLP0). On the other hand, after AngII stimulation, HSD3B1 mRNA was dramatically increased to the levels almost equal to or higher than those of HSD3B2 (323.2 ± 18.8 mmol/mol for HSD3B1 v.s. 249.2 ± 20.8 mmol/mol for HSD3B2). Thus, in addition to the responsiveness to AngII, HSD3B1 and HSD3B2 are different in the steady-state basal levels in H295R cells.

**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. 3). Both AngII and K+ evoked induction of CYP11B2 in a CHX-independent manner. In contrast, HSD3B1 increased only after AngII, and this induction was completely blocked by CHX. These profiles confirmed that the increment of HSD3B1 expression relies on de novo protein synthesis as reported previously [5]. The induction kinetics also differs between HSD3B1 and CYP11B2.
Response of HSD3B1 to AngII and K⁺

Fig. 1  The effect of K⁺ on the expression of HSD3B1, HSD3B2, and CYP11B2. (A–D) 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, C) or 16 mM (B, D). 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. For (A) and (B), cells were cultured in a standard medium containing 2.5% Nu serum. For (C) and (D), cells were serum-starved prior to stimulation. Values (means ± SEM, n = 3) are normalized to the levels of RPLP0 and plotted relative to those at time 0.

Fig. 2  K⁺ does not increase expression of HSD3B1 or HSD3B2 even in the presence of AngII. (A) Expression profiles of HSD3B1 in H295R cells after treatment with K⁺ (16 mM) alone or in combination with AngII (100 nM). Values (means ± SEM, n = 3) were determined as described in Fig. 1. (B) Expression profiles of HSD3B2 in H295R cells after treatment with K⁺ (16 mM) alone or in combination with AngII (100 nM). For control, cells were treated with forskolin (10 µM) for 12 h. Values are means ± SEM (n = 3). (C) Absolute mRNA levels of HSD3B1 and HSD3B2 in H295R cells after treatment with AngII (100 nM) or vehicle (Veh) for 4 h. qRT-PCR was performed with a standard curve method, and the values were normalized with the expression levels of RPLP0 (means ± SEM, n = 3).
HSD3B1 began to increase 2 h after AngII treatment, and it increased rapidly to about 6-fold over basal level by 4 h. Following the peak at 4 h, the levels of HSD3B1 mRNA decreased gradually. 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.

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

Why does K⁺-induced intracellular signaling lack the ability to induce HSD3B1? We previously demonstrated that AngII leads to induction of HSD3B1 through de novo protein synthesis of the orphan nuclear receptors NGFIB and NURR1 [5]. These proteins bind to the cognate NBRE sequence of the HSD3B1 promoter and enhance transcription [5]. 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. 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.

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

The CYP11B2 promoter contains a consensus Ca²⁺/cAMP response element (CRE) [7, 17]. To compare the responsiveness of the CRE with that of the NBRE, a luciferase vector containing multiple copies of the isolated CRE sequence of CYP11B2 (CYP11B2 CRE-luc)
Response of HSD3B1 to AngII and K^+

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, we assessed absolute mRNA levels (Fig. 5A, B) and protein expression profiles (Fig. 5C) of all the NGFIB members in H295R cells after treatment with either AngII or K^+. The mRNA levels of NGFIB, NURR1, and NOR1 were all rapidly increased after AngII treatment by approximately 40-, 200-, and 25-fold over basal levels, respectively, and their peak values were: 252.4 ± 8.1 mmol/mol for NGFIB, 38.7 ± 0.7 mmol/mol for NURR1, and 117.0 ± 2.5 mmol/mol for NOR1 (Fig. 5A) [all the values (means ± SEM, n=3) were normalized with RPLP0]. On the other hand, however, K^+ treatment produced only a faint peak mRNA expression for NGFIB (22.0 ± 2.3 mmol/mol for 8 mM K^+; 29.2 ± 0.2 mmol/mol for 16 mM K^+), NURR1 (0.9 ± 0.1 mmol/mol for 8 mM; 5.2 ± 0.4 mmol/mol for 16 mM K^+). The orphan nuclear receptor NGFIB family members do not increase in response to K^+

The orphan nuclear receptor NGFIB family contains three members, NGFIB, NURR1, and NOR1. It has been well established that AngII can stimulate mRNA expression of NGFIB, NURR1, and NOR1 [5, 18, 19]. However, K^+ responsiveness of this gene family remains unclear [20, 21]. 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, we assessed absolute mRNA levels (Fig. 5A, B) and protein expression profiles (Fig. 5C) of all the NGFIB members in H295R cells after treatment with either AngII or K^+. The mRNA levels of NGFIB, NURR1, and NOR1 were all rapidly increased after AngII treatment by approximately 40-, 200-, and 25-fold over basal levels, respectively, and their peak values were: 252.4 ± 8.1 mmol/mol for NGFIB, 38.7 ± 0.7 mmol/mol for NURR1, and 117.0 ± 2.5 mmol/mol for NOR1 (Fig. 5A) [all the values (means ± SEM, n=3) were normalized with RPLP0]. On the other hand, however, K^+ treatment produced only a faint peak mRNA expression for NGFIB (22.0 ± 2.3 mmol/mol for 8 mM K^+; 29.2 ± 0.2 mmol/mol for 16 mM K^+), NURR1 (0.9 ± 0.1 mmol/mol for 8 mM; 5.2 ± 0.4 mmol/mol for 16 mM K^+).
mM), and NOR1 (3.2 ± 0.2 mmol/mol for 8 mM; 7.9 ± 0.2 mmol/mol for 16 mM) (Fig. 5A). As shown in Fig. 5B, serum-starved H295R cells also show similar peak expression values for NGFIB (11.1 ± 0.9 mmol/mol for 8 mM K⁺; 15.2 ± 0.4 mmol/mol for 16 mM K⁺), NURR1 (1.2 ± 0.1 mmol/mol for 8 mM; 7.8 ± 0.2 mmol/mol for 16 mM), and NOR1 (7.8 ± 0.5 mmol/mol for 8 mM; 13.8 ± 0.4 mmol/mol for 16 mM). We therefore conclude that while K⁺ appears to be able to enhance mRNA expression for each gene to some extent (Fig. 5A, B), the respective peak absolute mRNA levels are considerably low, compared to that seen for AngII treatment. Furthermore, to test the consequence of this modest mRNA expression, we performed Western blot
Finally, we performed chromatin immunoprecipitation (ChIP) assays to validate the impacts of AngII and K⁺ on NGFIB/NURR1 protein recruitment to the promoter region of *HSD3B1*. 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. DNA fragments from the immunoprecipitates were examined by qPCR with a sequence-specific TaqMan probe and primers for the promoter region of *HSD3B1* (Fig. 7A). AngII significantly increased the levels of NGFIB/NURR1 binding to the promoter of *HSD3B1* (~3.9-fold over the vehicle control, *P* < 0.01). Importantly, however, K⁺ treatment did not cause any observable effect on the ChIP values, relative to the vehicle control (Fig. 7A), revealing that significant recruitment of NGFIB/NURR1 occurs only after AngII treatment. AngII-specific recruitment of NGFIB/NURR1 was also observed for *HSD3B2* promoter (Fig. 7B) that bears a consensus NBRE sequence reported to be important for its response to ACTH [14].

In order to compare the responsiveness of NGFIB/NURR1 with that of ATF2, we then performed anti-ATF2 ChIP assay for the promoter of *CYP11B2* (Fig. 7C). As previously shown by Nogueira & Rainey [8], both AngII and K⁺ significantly increased the levels of ATF2 recruitment to the promoter region of *CYP11B2* (~3.2- and ~2.7-fold over vehicle control, respectively), confirming that the *CYP11B2* CRE sequence
signals from AngII and K$^+$ are not identical; a different set of genes was reported to be induced in response to AngII and K$^+$ in H295R cells [21], although the underlying mechanism(s) remains unknown. In the present study, we showed that the NGFIB/NURR1 pathway leading to $HSD3B1$ expression is only operative for AngII. The current data therefore suggest that the pathways involving the orphan nuclear receptor NGFIB family members would be one of the key elements that diversify the downstream signals from AngII and K$^+$. There are a number of studies that describe AngII serves as a common element for AngII and K$^+$ [6, 7].

**Discussion**

It has been well established that both AngII and K$^+$ activate transcription of $CYP11B2$ in H295R cells. Based on this observation, AngII and K$^+$ are thought to share their downstream signals, at least in part, for the regulation of $CYP11B2$. In the present study, we revealed that signals from AngII and K$^+$ could be uncoupled in the regulation of $HSD3B1$ in H295R cells. As depicted in Fig. 8, $HSD3B1$ 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 the activation (phosphorylation) of the CREB/ATF family members.

AngII and K$^+$ are the major physiological regulators of aldosterone synthesis. However, their respective roles in the regulation of steroidogenic genes are not fully understood. The CREB/ATF pathway is operative for both AngII and K$^+$, and so this pathway cannot account for a possible difference between the two stimuli. Microarray studies suggest that downstream signals from AngII and K$^+$ are not identical; a different set of genes was reported to be induced in response to AngII and K$^+$ in H295R cells [21], although the underlying mechanism(s) remains unknown. In the present study, we showed that the NGFIB/NURR1 pathway leading to $HSD3B1$ expression is only operative for AngII. The current data therefore suggest that the pathways involving the orphan nuclear receptor NGFIB family members would be one of the key elements that diversify the downstream signals from AngII and K$^+$.

Fig. 7 NGFIB binding 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–C) Upper: schematic genomic structure of $HSD3B1$ (A), $HSD3B2$ (B), and $CYP11B2$ (C). Numbers, the positions relative to the transcription initiation site (position +1); *, sequences that conform to the consensus sequences of NBRE (A, B) and CRE (C); arrows, positions of primers used in ChIP assay. Lower: ChIP of $HSD3B1$ (A), $HSD3B2$ (B), and $CYP11B2$ (C). 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, B) or anti-ATF2 (C). anti-NGFIB/NURR1 recognizes both NGFIB and NURR1. Normal rabbit IgG was used as a control for immunoprecipitation. ChIP values are expressed as a percentage of the input amount of chromatin. Values are means ± SEM of three independent samples. Veh, vehicle control treatment. *, P < 0.01 compared with vehicle control, Bonferroni test.

Fig. 8 A model showing different responsiveness of $HSD3B1$ and $CYP11B2$ to AngII and K$^+$. 
treatment-induced activation of NGFIB, NURR1, and NOR1 in H295R cells [5, 18, 19]. However, K+ responsiveness of this gene family has remained unclear [20, 21]. For example, microarray data presented by Romero et al. indicate that although K+ stimulation tends to increase expression of NGFIB, NURR1, and NOR1 in H295R cells, these increases did not reach significance [21]. To gain a complete picture, we therefore assessed mRNA and protein expression profiles of all the NGFIB family members in K+-treated H295R cells. Interestingly, K+ treatment could increase expression of this gene family, but the incremental increase of each member was modest, with the peak absolute mRNA levels of each gene remarkably less than those for AngII treatment. This modest mRNA induction was not accompanied by detectable protein expression. Moreover, in combination with the data from the ChIP and luciferase reporter-based promoter assays, we conclude that K+ cannot evoke functional activation of the NGFIB family members.

HSD3B2 did not respond to AngII or K+. Based on the measurement of absolute mRNA levels of HSD3B1 and HSD3B2, we noticed that the steady-state basal levels of HSD3B2 are relatively high, compared to those of HSD3B1. ChIP assay indicates that AngII could induce binding of NGFIB/NURR1 to the promoter of HSD3B2. Currently, we do not know why this binding did not bring about any obvious induction of HSD3B2 mRNA after treatment with AngII. Although the underlying mechanism needs to be clarified, one possible explanation might be that because of the relatively high steady-state mRNA expression levels of HSD3B2, the recruitment of NGFIB/NURR1 alone may not suffice to produce overt changes in the net amount of this transcript in H295R cells.

The current study also advanced our understanding of CYP11B2. It has been previously demonstrated that the CRE enhancer element of the CYP11B2 promoter is required for the normal transcriptional response of CYP11B2 to AngII and K+ [6, 7]. However, the previous studies were based on “single time point” luciferase assays, thus leaving a question as to “time course” of the response. In the present study, based on real-time bioluminescence measurement, we monitored the dynamics of CYP11B2 CRE activity and found that its response is very rapid and transient, peaking at around 4 h after stimulation with AngII and K+. This dynamics, interestingly, differs from that of the endogenous CYP11B2 mRNA, which continued to increase with time during the course of a 12 h treatment with AngII and K+. Therefore, it is interesting to speculate that the CRE might serve as a “trigger” for the subsequent continuous elevation of CYP11B2. There might be a number of possible explanations for this model. For example, continuous chromatin remodeling and/or stabilization of transcripts of CYP11B2 might be involved. CHX treatment suggests that this process should not depend on de novo protein synthesis. Future studies will be required to understand the mechanism of continuous upregulation of CYP11B2.

In summary, we showed that AngII and K+ could evoke distinct intracellular signaling in H295R cells. Treatment of the cells with AngII, but not with K+, resulted in activation of NGFIB family members and in turn enhanced expression of HSD3B1, whereas both treatments evoked expression of CYP11B2 through activation of the CREB/ATF member(s). The human H295R adrenocortical cell is one of the most characterized cellular models for the analysis of adrenal cell biology [22]. The relevance of our finding in physiology and diseases such as for better understanding of gene regulatory mechanism in aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) will need further exploration. Aldosterone synthesis is a complex process potentially subject to many levels of regulation by AngII and K+. Thus, a comprehension of differential regulation of HSD3B1 and CYP11B2 would help to understand the multi-modal regulation of steroidogenesis by AngII and K+.

Acknowledgements

We thank Jean-Michel Fustin and Yuuki Nakagawa for technical assistance and scientific discussion. This work was supported in part by CREST Program of the Japan Science and Technology Agency (H.O.), and by the Ministry of Education, Culture, Sports, Science and Technology of Japan through a Funding Program for Next Generation World-Leading Researchers (M.D.). This work was also supported by the Nakatomi Foundation, the Inoue Foundation for Science, the Kanzawa Medical Research Foundation, and the Japan Research Foundation for Clinical Pharmacology (M.D.).

Disclosure

The authors have no competing interests to disclose.
References


