

Regulation of Expression and Physiological Function of  
Type VI 3 $\beta$ -Hydroxysteroid Dehydrogenase Isozyme Hsd3b6  
(VI 型 3 $\beta$ -水酸化ステロイド脱水素酵素 Hsd3b6 の  
発現制御と生理機能の解明)

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## Prolegomenon

The enzyme 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase (3 $\beta$ -HSD) is essential for the biosynthesis of all active steroid hormones, such as those secreted from the adrenal gland, testis, ovary, skin, and placenta. The 3 $\beta$ -HSD enzymes exist in multiple isoforms in humans and rodents with different tissue specificity. The mouse has two isoforms in the adrenal: one (*Hsd3b1*) is ubiquitous in the cortex, but the other (*Hsd3b6*) is specific to the zona glomerulosa (ZG), where aldosterone is produced [1]. Both genes are evolutionally conserved in humans, with the genes named *HSD3B1* and *HSD3B2* being a candidate counterpart of the mouse *Hsd3b6* and *Hsd3b1*, respectively. As such, *HSD3B1* corresponds to the ZG-specific 3 $\beta$ -HSD isozyme in human adrenal gland [2]. Angiotensin II (AngII) and potassium (K<sup>+</sup>) are the major physiological regulators of aldosterone synthesis. AngII stimulates expression of *HSD3B1* via the orphan nuclear receptors NGFIB and NURR1 in human adrenocortical H295R cells [3]. However, it remains unknown whether K<sup>+</sup> stimulation similarly activates this NGFIB/NURR1 pathway. Here I examined K<sup>+</sup> sensitivity of *HSD3B1* in H295R cells (**chapter 1**). Furthermore, I extended my research to understanding the roles of this enzyme in other tissues. I found that *Hsd3b6* is expressed in the meibomian glands of mouse eyelid. I explored the physiological function and regulatory mechanisms of this enzyme in the meibomian glands (**chapter 2**).

## **Chapter 1: 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 3 $\beta$ -HSD isoform gene expression in adrenocortical H295R cells**

I show that K<sup>+</sup> stimulation lacks the ability to induce *HSD3B1* expression in human adrenocortical H295R cells. Both AngII and K<sup>+</sup> were able to enhance transcription of the aldosterone synthase gene (*CYP11B2*). Promoter analysis revealed that although both AngII and K<sup>+</sup> activate transcription from the Ca<sup>2+</sup>/cAMP response 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<sup>+</sup>, being only able to enhance transcription after AngII treatment. I 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<sup>+</sup> 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<sup>+</sup>. These data provide evidence that signals from AngII and K<sup>+</sup> can be uncoupled in the regulation of *HSD3B1* in the human adrenocortical H295R cells [4].

### References

- [1] Doi *et al.*, *Nat Med* **16**, 67–74, 2010
- [2] Doi *et al.*, *J Clin Endocrinol Metab* **99**, E257–62, 2014
- [3] Ota *et al.*, *Mol Cell Biol* **34**, 3880–94, 2014
- [4] Yarimizu *et al.*, *Endocrine J* **62**, 765–776, 2015

## **Chapter 1**

**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 3 $\beta$ -HSD isoform gene expression in adrenocortical H295R cells**

## Introduction

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  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase ( $3\beta$ -HSD) is an enzyme that catalyzes the conversion of  $\Delta^5$ -3-hydroxysteroids (e.g. pregnenolone) into hormonally active  $\Delta^4$ -3-ketosteroids (e.g. progesterone) [1, 2], a step required for aldosterone synthesis. Whereas two distinct  $3\beta$ -HSD isoforms (type I  $3\beta$ -HSD encoded by *HSD3B1* and type II  $3\beta$ -HSD encoded by *HSD3B2*) exist in humans, the type II  $3\beta$ -HSD has long been considered the major or sole isoform present in the adrenal gland. However, the recent finding that type I  $3\beta$ -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, I recently showed that AngII could stimulate expression of *HSD3B1* [5]. I also 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). 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<sup>+</sup> 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<sup>+</sup> can both activate transcription of *CYP11B2* through a common *cis*-regulatory element [7]. The *CYP11B2* promoter contains a Ca<sup>2+</sup>/cAMP response element (CRE) to which the CREB/ATF family proteins bind in response to AngII and K<sup>+</sup> [8]. Thus, AngII and K<sup>+</sup> 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<sup>+</sup> in the regulation of other steroidogenic enzyme genes has remained unexplored.

Here I examined K<sup>+</sup> sensitivity of *HSD3B1* in H295R cells. Contrary to my naive expectation, I observed that K<sup>+</sup> stimulation was unable to enhance expression of *HSD3B1*. To understand the underlying mechanism better, I 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 CREB/ATF-mediated transcription pathway that operates in response to both AngII and K<sup>+</sup>.



## **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% CO<sub>2</sub>). 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  $2 \times 10^5$  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<sup>+</sup> 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<sup>+</sup> 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.

### **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 3 $\beta$ -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 [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 3 $\beta$ -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 Step-OnePlus 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 I used linearized plasmid DNA carrying the target amplicon. The data were normalized to the expression levels of the gene encoding ribosomal protein LP0 (*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 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 *CREB*, forward primer 5’-GTC TCC ACA AGT CCA AAC AGT-3’ and reverse primer 5’-ATG GCA GGT GCT GAA GTC-3’; for *ATF1*, forward primer 5’-CAT CCG ACA GCA TAG GCT CC-3’ and reverse primer 5’-CTC CAT CTG TGC CTG GAC TT-3’; for *ATF2*, forward primer 5’-GAG TTG GCG AGT CCA TTT GAG-3’ and reverse primer 5’-GAT GTG GGC TGT GCA GTT TGT G-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% CO<sub>2</sub> 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<sup>+</sup> 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 dish ( $\sim 1 \times 10^7$  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 deoxy- cholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail [Roche]) and sonicated 10 times for 30 sec each time at 4°C using a Bioruptor UCW-201™ 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 sec 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'-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-TAMRA-3'; for *HSD3B2* promoter, forward primer 5'-ACT GAC CAG TGT TCT GTT AAG GCT AAA G-3', reverse primer 5'-CAT TGC TCC CTC 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 TCC ACC CTG TTC-3'.

### **Statistical analysis**

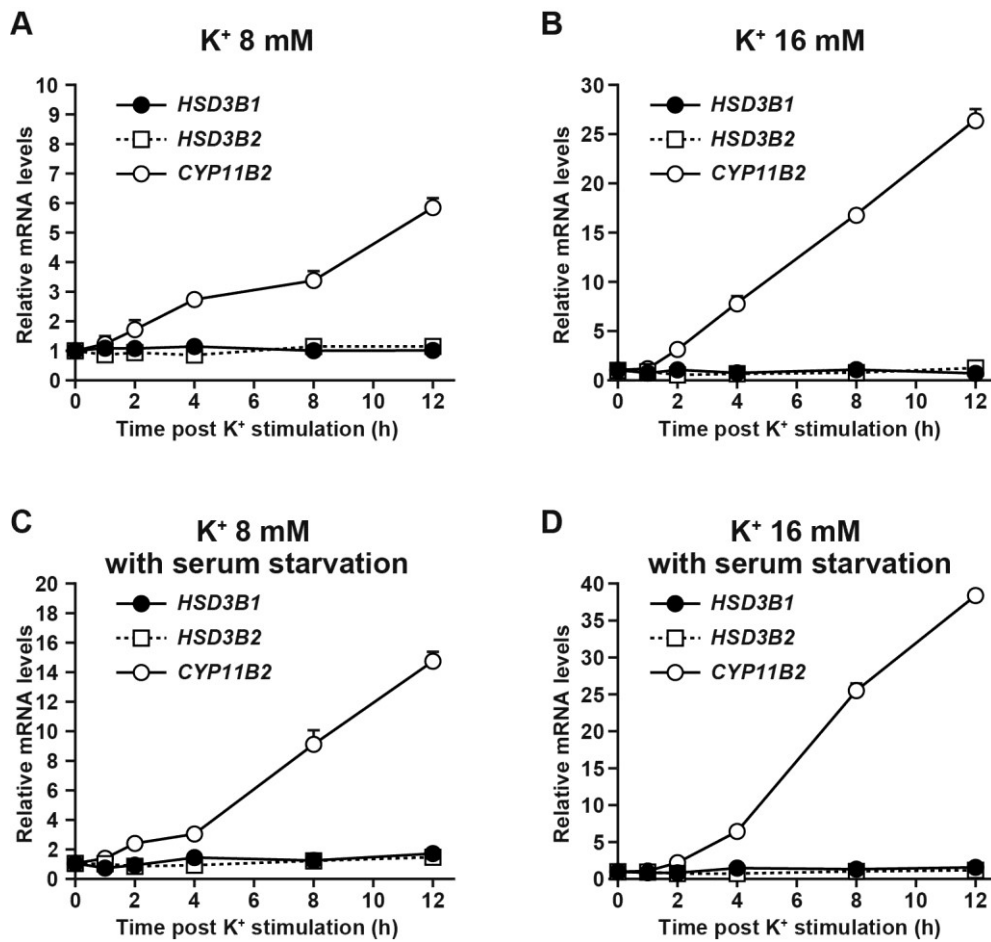
I used two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test to evaluate mean differences between different groups with different treatments in ChIP assay. A value of  $P < 0.01$  was taken as significant.

## Results

### **K<sup>+</sup> does not enhance *HSD3B1* expression.**

The adrenocortical H295R cells were treated with K<sup>+</sup> 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<sup>+</sup>-treated adrenal cells [9-11]. In agreement with previous studies, K<sup>+</sup> treatment increased expression of the aldosterone synthase gene *CYP11B2* [12] (**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<sup>+</sup> treatment, respectively. However, expression levels of *HSD3B1* and *HSD3B2* were almost constant throughout the K<sup>+</sup> treatment even under 16 mM condition (**Fig. 1A, B**). These results demonstrate that K<sup>+</sup>-induced intracellular signaling that leads to *CYP11B2* expression cannot enhance expression of 3 $\beta$ -HSD genes. Importantly, this was also the case when the cells were serum-starved prior to K<sup>+</sup> stimulation (**Fig. 1C, D**).

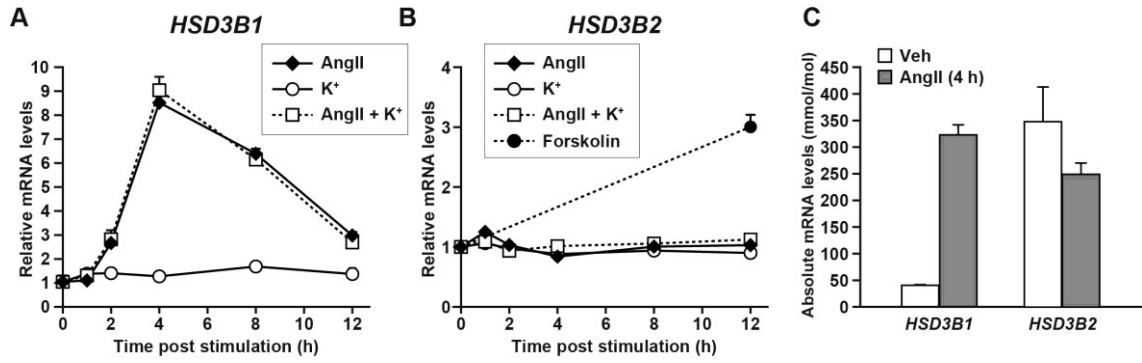




**Figure 1** The effect of K<sup>+</sup> on the expression of *HSD3B1*, *HSD3B2*, and *CYP11B2*. (A–D) Gene expression profiles of *HSD3B1* (filled circle), *HSD3B2* (square), and *CYP11B2* (open circle) after K<sup>+</sup> treatment in H295R cells. KCl was added to the culture medium at a final concentration 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.

K<sup>+</sup> 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<sup>+</sup> (**Fig. 2A**). Thus K<sup>+</sup> 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<sup>+</sup> 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, 13-16]. Thus, the responsiveness of this gene relies on the type of agonists administered to the cells.

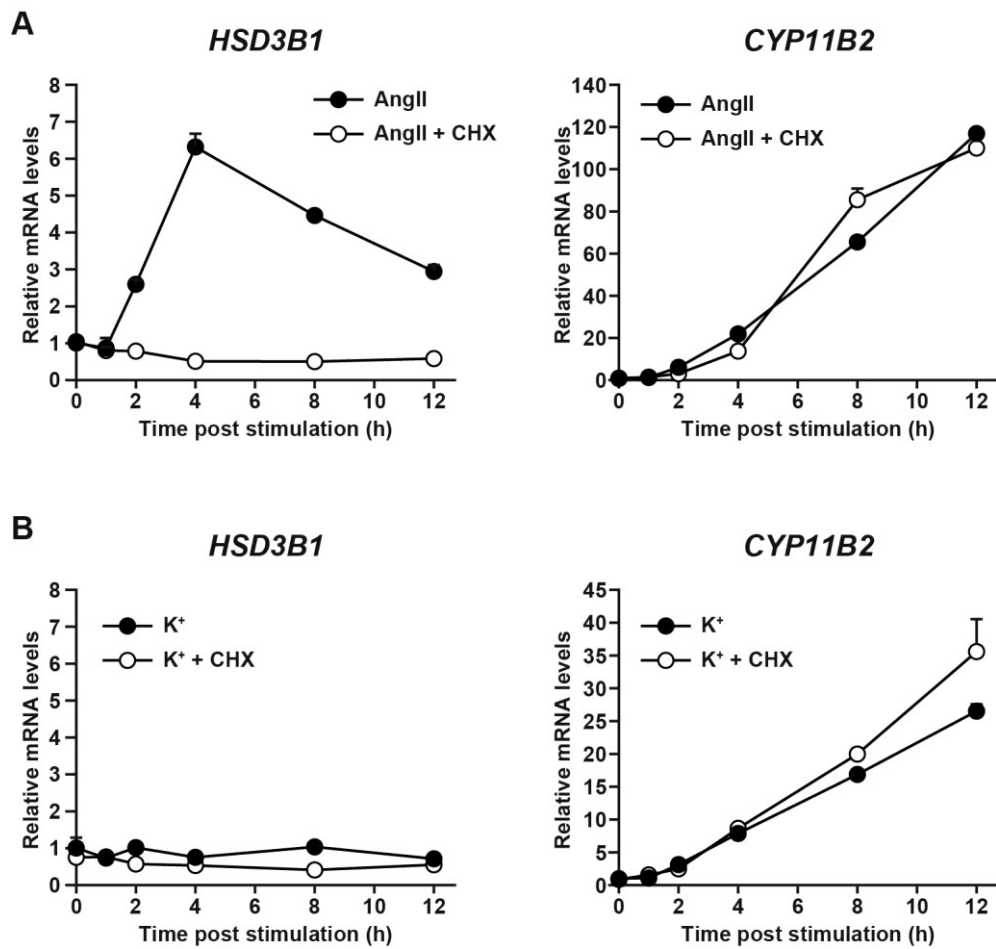
To assess relative expression levels between *HSD3B1* and *HSD3B2* in H295R cells, I determined absolute mRNA levels for each gene (**Fig. 2C**). At the basal levels, *HSD3B2* was approximately 8 times higher than *HSD3B1* ( $40.7 \pm 1.2$  mmol/mol for *HSD3B1* v.s.  $347.8 \pm 65.1$  mmol/mol for *HSD3B2*; the values (means  $\pm$  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* ( $323.2 \pm 18.8$  mmol/mol for *HSD3B1* v.s.  $249.2 \pm 20.8$  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.



**Figure 2** K<sup>+</sup> 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<sup>+</sup> (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<sup>+</sup> (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).

### **Different effects of K<sup>+</sup> and AngII on expression of *HSD3B1* and *CYP11B2***

H295R cells were treated with AngII (100 nM) or K<sup>+</sup> (16 mM) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) (**Fig. 3**). Both AngII and K<sup>+</sup> evoked induction of *CYP11B2* in a CHX-independent manner. In contrast, *HSD3B1* increased only after AngII treatment, and this induction was completely blocked by CHX. These profiles, observed here, are basically the same as what I reported previously [5] and confirmed that the increment of *HSD3B1* expression relies on *de novo* protein synthesis. The induction kinetics also differs between *HSD3B1* and *CYP11B2*. *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<sup>+</sup>. These data suggest that the underlying mechanisms that control expression of *HSD3B1* and *CYP11B2* are different.



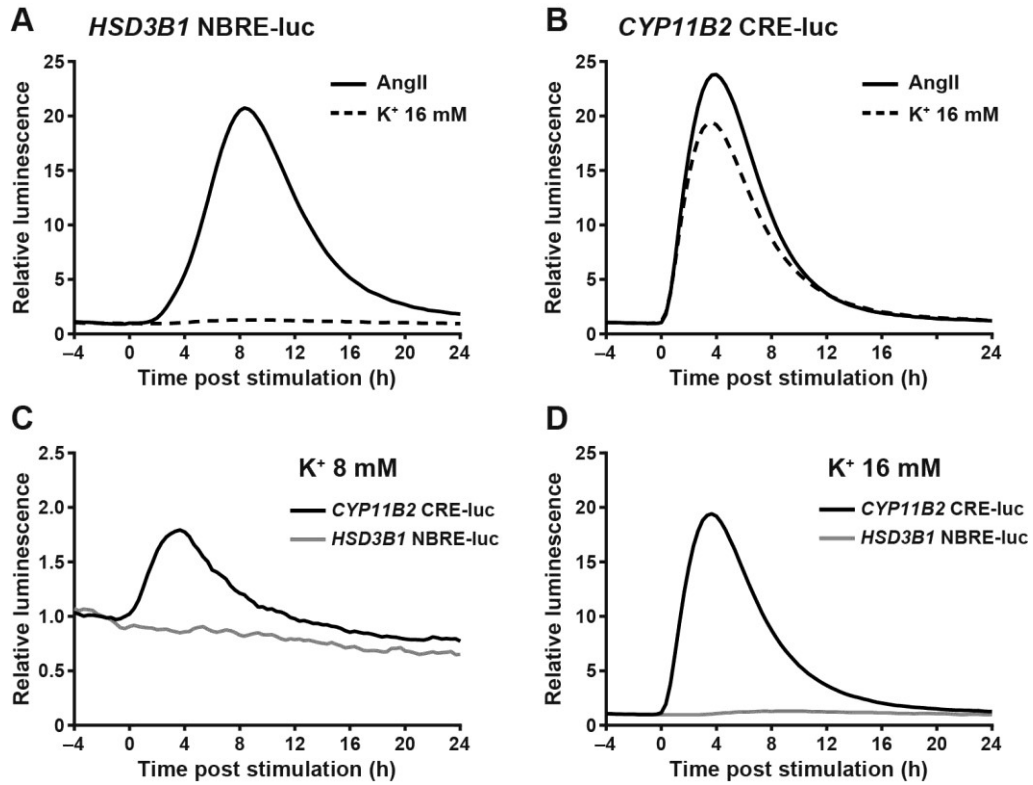
**Figure 3** *HSD3B1* does not increase in response to  $K^+$ , despite displaying an overt increase in response to AngII, a situation different from *CYP11B2*. (A)(B) Responsiveness of *HSD3B1* and *CYP11B2* to treatment with AngII (100 nM, A) or  $K^+$  (16 mM, B) in the presence (open circle) or absence (filled circle) of cycloheximide (CHX, 10  $\mu$ g/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  $\pm$  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<sup>+</sup> and AngII on *HSD3B1* promoter NBRE enhancer activity**

Why does K<sup>+</sup>-induced intracellular signaling lack the ability to induce *HSD3B1*? Our laboratory 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<sup>+</sup> 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<sup>+</sup> (**Fig. 4A**). Interestingly, robust induction of reporter activity was observed only after AngII treatment (**Fig. 4A**). K<sup>+</sup> treatment failed to induce reporter gene expression through the NBRE site (**Fig. 4A, C, D**).

### **Equivalent effects of K<sup>+</sup> and AngII on *CYP11B2* promoter CRE enhancer activity**

The *CYP11B2* promoter contains a consensus 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*



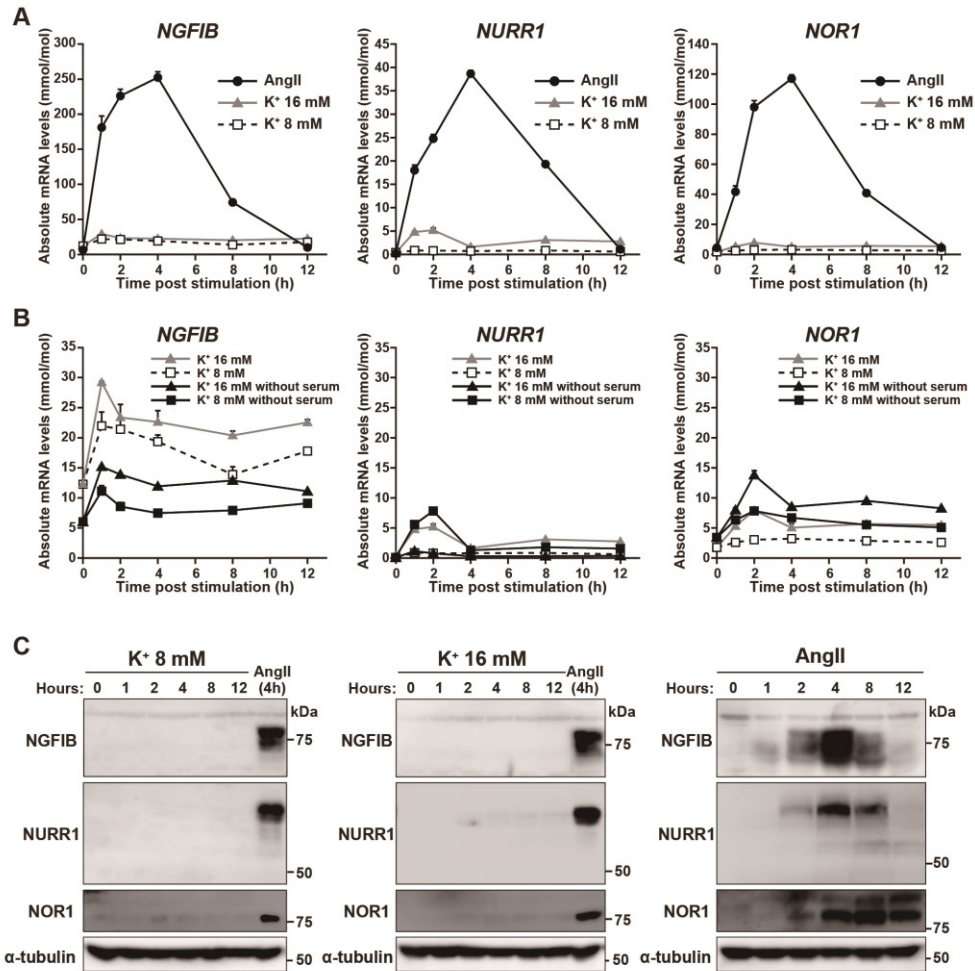
**Figure 4** Transcriptional enhancer activity of the NBRE site of *HSD3B1* does not increase in response to K<sup>+</sup>, despite displaying an overt increase in response to AngII, a profile different from that of the CRE site of *CYP11B2*. (A)(B) Luciferase reporter gene expression from the NBRE site of *HSD3B1* (A) and the CRE site of *CYP11B2* (B) after stimulation with 100 nM AngII (black line) or 16 mM K<sup>+</sup> (dashed line) in H295R cells. (C)(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<sup>+</sup> (C) or 16 mM K<sup>+</sup> (D) in H295R cells. Cells were transfected with a reporter vector containing either multiple copies of the NBRE site of *HSD3B1* (*HSD3B1* NBRE-luc) or the CRE site of *CYP11B2* (*CYP11B2* CRE-luc). Traces show representative bioluminescence monitored before and after each treatment. Similar traces were obtained in three independent experiments.

(*CYP11B2* CRE-luc) was transfected into H295R cells and monitored for the response to AngII and K<sup>+</sup> (**Fig. 4B–D**). In agreement with the endogenous *CYP11B2* mRNA expression profiles (**Figs. 1 and 3**), both AngII and K<sup>+</sup> caused a significant enhancement of the CRE-luc reporter activity with an amplitude of ~25-fold for AngII (**Fig. 4B**), ~2-fold for 8 mM K<sup>+</sup> (**Fig. 4C**), and ~20-fold for 16 mM K<sup>+</sup> (**Fig. 4B, D**). These results indicate that the CRE is a common element for AngII and K<sup>+</sup> activity [6, 7].

**The orphan nuclear receptor NGFIB family members do not increase in response to K<sup>+</sup>.**

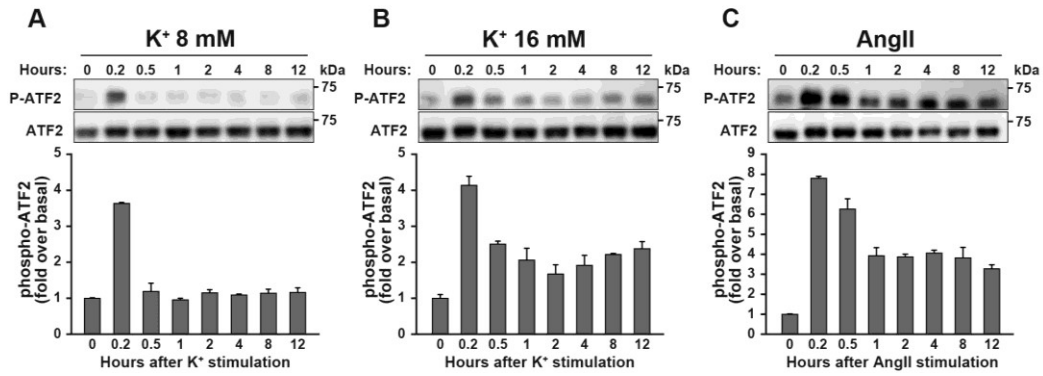
The orphan nuclear receptor NGFIB family contains three members, NGFIB, NURR1, and NOR1. It has been well established that AngII can stimulate mRNA expression of *NGFIB*, *NURR1*, and *NOR1* [5, 18, 19]. However, K<sup>+</sup> responsiveness of this gene family remains unclear [20, 21]. Moreover, to date, there have been no studies that assess the protein levels of NGFIB, NURR1, and NOR1 in K<sup>+</sup>-stimulated H295R cells. Therefore, to gain a better picture of this family, I 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<sup>+</sup>. The mRNA levels of *NGFIB*, *NURR1*, and *NOR1* were all rapidly increased





**Figure 5** The orphan nuclear receptor NGFIB family members do not increase in response to K<sup>+</sup>, 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), 16 mM K<sup>+</sup> (triangle), or 8 mM K<sup>+</sup> (square) in H295R cells. Culture medium contains 2.5 % Nu serum. After the indicated periods, total RNA was isolated from the cells, and absolute mRNA levels of *NGFIB*, *NURR1*, and *NOR1* were determined by qRT-PCR. Values (means ± SEM, n = 3) are normalized to the levels of *RPLP0*. (B) Absolute mRNA levels of *NGFIB* (left), *NURR1* (middle), and *NOR1* (right) in serum-starved H295R cells after stimulation with 8 mM K<sup>+</sup> or 16 mM K<sup>+</sup>. For comparison, the data shown in (A) were replotted in parallel. (C) Western blots showing the protein expression profiles of NGFIB (upper), NURR1 (middle), and NOR1 (lower) after AngII (100 nM) or K<sup>+</sup> (8 mM or 16 mM) stimulation in H295R cells. The culture medium used here contains 2.5 % Nu serum.

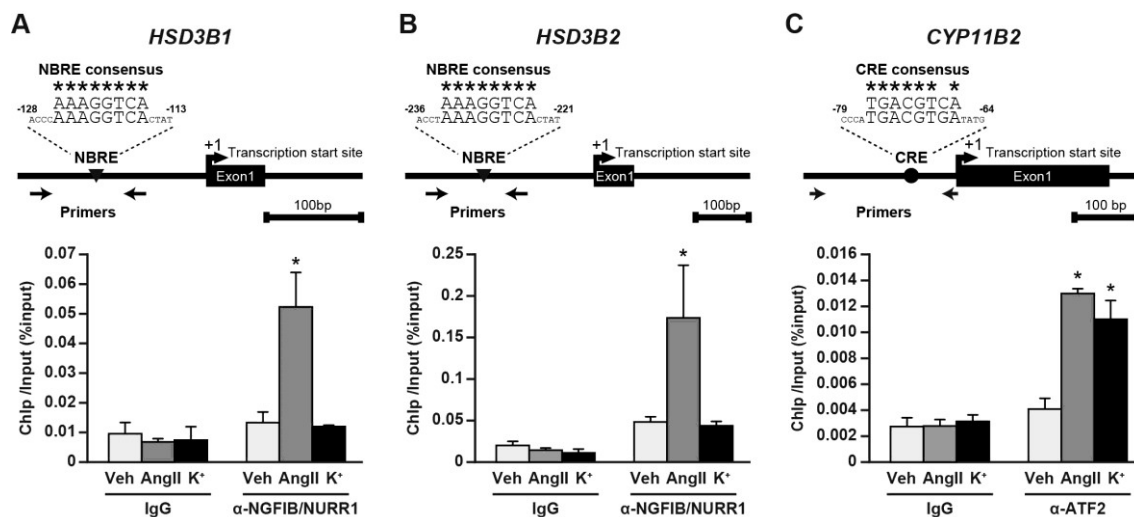
after AngII treatment by approximately 40-, 200-, and 25-fold over basal levels, respectively, and their peak values were:  $252.4 \pm 8.1$  mmol/mol for *NGFIB*,  $38.7 \pm 0.7$  mmol/mol for *NURR1*, and  $117.0 \pm 2.5$  mmol/mol for *NOR1* (**Fig. 5A**). On the other hand, however,  $K^+$  treatment produced only a faint peak mRNA expression for *NGFIB* ( $22.0 \pm 2.3$  mmol/mol for 8 mM  $K^+$ ;  $29.2 \pm 0.2$  mmol/mol for 16 mM  $K^+$ ), *NURR1* ( $0.9 \pm 0.1$  mmol/mol for 8 mM;  $5.2 \pm 0.4$  mmol/mol for 16 mM), and *NOR1* ( $3.2 \pm 0.2$  mmol/mol for 8 mM;  $7.9 \pm 0.2$  mmol/mol for 16 mM) (**Fig. 5A**). As shown in **Fig. 5B**, serum-starved H295R cells also show similar peak expression values for *NGFIB* ( $11.1 \pm 0.9$  mmol/mol for 8 mM  $K^+$ ;  $15.2 \pm 0.4$  mmol/mol for 16 mM  $K^+$ ), *NURR1* ( $1.2 \pm 0.1$  mmol/mol for 8 mM;  $7.8 \pm 0.2$  mmol/mol for 16 mM), and *NOR1* ( $7.8 \pm 0.5$  mmol/mol for 8 mM;  $13.8 \pm 0.4$  mmol/mol for 16 mM). I 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, I performed Western blot analysis (**Fig. 5B**) and found that  $K^+$  treatment failed to induce detectable protein expression of the *NGFIB*, *NURR1*, and *NOR1* proteins. After AngII treatment, expression of these proteins was remarkably increased (**Fig. 5A**). Thus, substantial protein induction of the members of the *NGFIB* family occurs only after AngII treatment.



**Figure 6 Both K<sup>+</sup> and AngII induce phosphorylation of ATF2 in H295R cells.** (A)(B)(C) Western blots showing time-dependent phosphorylation profiles of ATF2 after stimulation with 8 mM K<sup>+</sup> (A), 16 mM K<sup>+</sup> (B), or 100 nM AngII (C) in H295R cells. Whole cell lysates were blotted and probed with the antibodies indicated. Relative band intensities of phospho-ATF2 were determined by densitometry. Data are the means  $\pm$  variation from two independent experiments.

**Both K<sup>+</sup> and AngII induce phosphorylation of the CREB/ATF family member ATF2.**

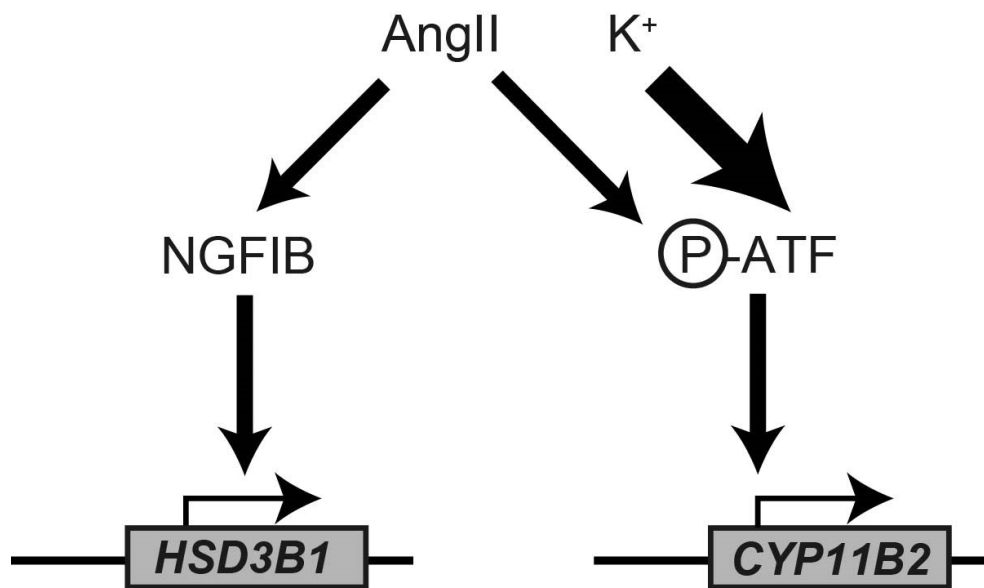
In contrast to the stimulus-selective induction of the NGFIB family proteins, I found that phosphorylation of ATF2 occurs in response to both AngII and K<sup>+</sup> (**Fig. 6**). ATF2 was analyzed here because it has been reported [8] that in H295R cells mRNA levels of ATF2 are relatively high (7.8 nmol/mol) compared to those of the other members, such as CREB ( $3.2 \times 10^{-3}$  nmol/mol) and ATF1 (3.2 nmol/mol) [values in parentheses indicate absolute mRNA concentrations relative to *RPLP0*; see **Materials and Methods**]. As shown in **Fig. 6**, Western blot analysis revealed that phosphorylated protein levels of ATF2 were increased to ~7.5-, ~3.5-, or ~4.0-fold above basal values within 0.2 h after treatment with AngII, 8 mM K<sup>+</sup> or 16 mM K<sup>+</sup>, respectively. Total ATF2 contents remained invariable throughout the treatments. There have been no studies that determined K<sup>+</sup>-induced phosphorylation profiles of the CREB/ATF family in H295R cells. My data therefore demonstrate for the first time that K<sup>+</sup> treatment can dose-dependently induce ATF2 phosphorylation in H295R cells.



**Figure 7** NGFIB binding to the *HSD3B1* NBRE site does not increase in response to K<sup>+</sup>, 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<sup>+</sup> (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.

**AngII, but not K<sup>+</sup>, induces NGFIB recruitment to the *HSD3B1* promoter.**

Finally, I performed chromatin immunoprecipitation (ChIP) assays to validate the impacts of AngII and K<sup>+</sup> on NGFIB/NURR1 protein recruitment to the promoter region of *HSD3B1*. Cells were incubated with AngII (100 nM), K<sup>+</sup> (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<sup>+</sup> 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 [10]. In order to compare the responsiveness of NGFIB/NURR1 with that of ATF2, I then performed anti-ATF2 ChIP assay for the promoter of *CYP11B2* (**Fig. 7C**). As previously shown by Nogueira & Rainey [8], both AngII and K<sup>+</sup> significantly increased the levels of ATF2 recruitment to the promoter



**Figure 8 A model showing different responsiveness of *HSD3B1* and *CYP11B2* to AngII and K<sup>+</sup>.** *HSD3B1* responds to AngII, but not to K<sup>+</sup>, 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.

region of *CYP11B2* (~3.2- and ~2.7-fold over vehicle control, respectively), confirming that the *CYP11B2* CRE sequence serves as a common element for AngII and K<sup>+</sup> [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, I 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  $K^+$ , 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 [8].

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  $K^+$  [6], 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, I 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<sup>+</sup>.

There are a number of studies that describe AngII treatment-induced activation of *NGFIB*, *NURR1*, and *NOR1* in H295R cells [5, 18, 19]. However, K<sup>+</sup> responsiveness of this gene family has remained unclear [20, 21]. For example, microarray data presented by Romero *et al.* indicate that although K<sup>+</sup> 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, I therefore assessed mRNA and protein expression profiles of all the NGFIB family members in K<sup>+</sup>-treated H295R cells. Interestingly, K<sup>+</sup> 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, I conclude that K<sup>+</sup> cannot evoke functional activation of the NGFIB family members.

*HSD3B2* did not respond to AngII or K<sup>+</sup>. Based on the measurement of absolute mRNA levels of *HSD3B1* and *HSD3B2*, I 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, I 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 my understanding of *CYP11B2*. It was previously demonstrated that the CRE enhancer element of the *CYP11B2* promoter is required for the normal transcriptional response of *CYP11B2* to AngII and K<sup>+</sup> [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, I 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<sup>+</sup>. 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<sup>+</sup>. Therefore, it is interesting to speculate that the CRE might serve as a “trigger” for the subsequent continuous elevation of *CYP11B2*. There could be many plausible explanations for this model. For example, continuous chromatin remodeling and/or stabilization of transcripts of *CYP11B2* may be involved. CHX treatment also 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, I showed that AngII and K<sup>+</sup> could evoke distinct intracellular signaling in H295R cells. Treatment of the cells with AngII, but not with K<sup>+</sup>, 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 family 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 my 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<sup>+</sup>. Thus, a comprehension of differential regulation of *HSD3B1* and *CYP11B2* would help to understand the multi-modal regulation of steroidogenesis by AngII and K<sup>+</sup>.

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## Chapter 2

### **Circadian clock-regulated 3 $\beta$ -HSD activity in the meibomian glands**

It is well known that malfunction of the circadian clock is linked to the pathogenesis of a wide variety of diseases. Meibomian glands are holocrine glands embedded in the eyelid and secrete lipid onto the ocular surface tear film. However, the molecular mechanisms by which circadian clock contributes to the meibomian gland function are poorly understood. By performing immunohistochemistry using Hsd3b6 subtype-specific antibody, I found Hsd3b6 is specifically expressed in acinar epithelial cells of the meibomian gland. Notably, consistent with the expression of this enzyme, I found that the meibomian glands exhibit intra-tissue 3 $\beta$ -HSD enzymatic activity, suggesting that Hsd3b6 contributes to the local steroid production within the meibomian glands. Moreover, I provide evidence that 3 $\beta$ -HSD enzymatic activity oscillates in a circadian fashion in the meibomian gland. These findings suggest that malfunction of circadian clock-regulated 3 $\beta$ -HSD activity may underlie meibomian gland dysfunction.

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