Short term regulation of aldosterone secretion after stimulation and suppression experiments in mice

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Abstract

Aldosterone is synthesized acutely from the zona glomerulosa cells upon stimulation by the renin–angiotensin–aldosterone system. Several enzymes are involved in this steroidogenic process including the steroidogenic acute regulatory protein (*StAR*), P450 side chain cleavage enzyme (*Cyp11a1*), and aldosterone synthase (*Cyp11b2*) which has been demonstrated to be transcriptionally regulated by the nuclear transcription factors *NGF1-B* and *Nurr1*. We investigated the short time transcriptional regulation of these genes in wild-type mice at 10 min intervals for 1 h following application of 0.2 nmol angiotensin II (ANGII) or sodium chloride in comparison sham injections. Using real-time PCR a fast upregulation of adrenal *Cyp11b2* expression ($53 \pm 5\%$ increase over baseline) could be observed 10 min after sham injection which was accompanied by a transient increase in aldosterone secretion while *StAR* and *Cyp11a1* upregulation was delayed and more sustained. ANGII caused an increase of *StAR* and *Cyp11a1* expression similar to that observed after sham injection while *Cyp11b2* upregulation was more pronounced (10 min, $236 \pm 39\%$) and reflected ANGII induced stimulation of aldosterone output. Sodium challenge was followed by a sustained reduction of all three genes examined (*Cyp11b2*, 20 min, $-63 \pm 6\%$) which was accompanied by a significant suppression of aldosterone secretion detectable after 60 min. While increases in *NGF1-B* mRNA levels were similar between the treatment groups, *Nurr1* expression levels were induced only upon ANGII administration. These data suggest that acute regulation of aldosterone synthesis is accompanied by fast transcriptional modulation of steroidogenic enzymes and transcription factors that are likely to be involved in aldosterone secretion.

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Introduction

Owing to their highly lipophilic structure, steroids including aldosterone cannot be stored in substantial quantities within the adrenal cortex but have to be synthesized on demand. Thus, rapid regulation of steroid synthesis is of particular importance to maintain homeostasis upon acute stimuli. Early on it had been demonstrated that the acute stimulation of glucocorticoid output from the adrenal gland by ACTH is sensitive to protein synthesis inhibitors (Ferguson 1963). Further studies identified steroidogenic acute regulatory protein (*StAR*) to initiate the cascade of steroid biosynthesis with the translocation of cholesterol from the outer to the inner mitochondrial membrane (Black *et al.* 1994, Clark *et al.* 1994, Spat & Hunyady 2004). *StAR* expression is enhanced by ACTH and Ca^{2+} mediated stimuli such as angiotensin II (ANGII) and potassium (Spat & Hunyady 2004). Upon stimulation *StAR* mRNA and protein levels are acutely induced via cAMP mediated mechanisms that result in the increase of *StAR* transcription and mRNA

stability (Caron *et al.* 1997). Other transcription factors regulating long term *StAR* expression include steroidogenic factor 1 (SF-1), C/EBPs and putatively Sp1 (Reinhart *et al.* 1999). Furthermore, the transcriptional effect of ANGII involves the activation of ERK1/2 which represses DAX-1, a transcription factor known to inhibit SF-1 dependent transcription of *StAR* (Gummow *et al.* 2006). Moreover, post-translational modification including *StAR* phosphorylation on a threonine residue has been demonstrated to be required for acute induction of steroidogenesis (Stocco & Clark 1993, Stocco *et al.* 2005).

Conversion of cholesterol to pregnenolone is catalyzed by the cholesterol side-chain cleavage enzyme (*Cyp11a1*) and takes place at the matrix side of the inner mitochondrial membrane (Hum & Miller 1993). Pregnenolone exits the mitochondria and is converted to progesterone by 3- β -hydroxysteroid dehydrogenase in the microsomal compartment. Progesterone is then further hydroxylated to form 11-desoxycorticosterone by 21 hydroxylation (Otis & Gallo-Payet 2007). The aldosterone synthase (*Cyp11b2*) is required for the final

steps of aldosterone synthesis through hydroxylation and 18-oxidation of 11-desoxycorticosterone that ultimately results in the formation of aldosterone (Curnow *et al.* 1991, Muller 1998).

Upon binding of ANGII the ANGII receptor type 1 (AT₁) couples to the G_q protein, which in turn stimulates phospholipase C to generate the second messengers diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG and IP₃ then activate protein kinase C (PKC) and liberate intracellular stores of calcium (de Gasparo *et al.* 2000). The immediate phase of aldosterone stimulation is followed by sustained release, which is dependent on depolarization and activation of T- and L-type Ca²⁺ channels (Rossier *et al.* 1996). Increased intracellular calcium levels are followed by activation of the calcium binding protein calmodulin (CaM) by CaM kinase (CaMK) I and/or CaMK IV (Condon *et al.* 2002). Furthermore, several lines of evidence suggest additional activation of CaMK directly through cAMP dependent effects (Gambaryan *et al.* 2006). CaMK induced phosphorylation of ATF-1 and CREB and transcriptional activation of NGF1-B and Nurr1 in turn induces promoter activation of *Cyp11b2* (Bassett *et al.* 2004a). An alternate signaling pathway engaged by the AT₁ receptor is the phosphorylation and activation of MAPK family members including ERK1/ERK2. Although ANGII induced ERK1/ERK2 activation had been shown to require activation of PKC, it has been recently revealed that these intracellular events can also occur independently (Daniels *et al.* 2005).

Taken together, transcriptional, translational, and posttranslational regulation of *StAR* that defines steroid output in steroidogenic tissues and molecular mechanisms that ultimately dictate expression levels of *Cyp11b2* as the final step responsible for aldosterone secretion have been characterized in great detail. However, so far, no study has investigated the transcriptional regulation coincident with aldosterone secretion within minutes of acute stimuli. With the present study, we set out to define immediate transcriptional changes that take place in the adrenal gland upon specific stimulation and suppression experiments modulating aldosterone secretion *in vivo*.

Materials and methods

Animals and housing conditions

All animal studies were performed according to protocols examined and approved by the Regierung von Oberbayern and according to the German Animal Protection Law. Mice were kept in a non-specific pathogen free animal facility at an ambient temperature of 22 ± 2 °C, (relative humidity 60 ± 5%) and on a

12 h light:12 h darkness circle. The animals were fed with standard breeding chow (sodium 0.24%, potassium 0.91%; Ssniff R/M-H, Soest, Germany) *ad libitum* with free access to tap water. The experiments were performed on 12-week-old female C3HeB/FeJ wild-type mice (Jackson Laboratory, Bar Harbour, ME, USA), which were maintained in groups of 6–8 individuals.

Stimulation and suppression tests

Steroidogenic response and aldosterone secretion was investigated upon specific functional tests. As stimuli, ANGII injection and i.p. sodium load were chosen. Moreover, a control group received a sham injection, in order to investigate potential effects of injection itself on adrenocortical function. For each one of the ANGII, sodium load and sham injection, seven groups of five animals were established. The mice of the first group for each stimulus were euthanized by isoflurane inhalation and decapitation for collection of trunk blood and adrenals under baseline conditions. The mice from the remaining six groups received an i.p. injection of 0.2 nmol ANGII (Fluka, Taufkirchen, Germany; Davisson *et al.* 2000, Cao *et al.* 2006), 50 µl/g NaCl 0.9% (B Braun, Melsungen, Germany), or a sham injection without anesthesia. After 10, 20, 30, 40, 60, and 120 min five mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection. To avoid an increase of the examined parameters induced by blood sampling, this procedure was kept to an interval of <60 s. All adrenals were immediately cleaned from adjacent tissue using a stereo microscope, snap frozen in liquid nitrogen and stored at –80 °C until further processing.

Aldosterone measurement

Aldosterone was determined with an in-house time-resolved fluorescent immunoassay as recently described in detail (Manolopoulou *et al.* 2008). In brief, 50 µl of each plasma sample were extracted using 2 ml dichloromethane/polyethylene glycol 10 000 (50 mg/l). The mixture was vortexed and after the two phases separated, the organic phase was removed, transferred to another tube, and left to evaporate overnight. On the following day, samples were reconstituted with pure methanol and assay buffer. After reconstitution, 50 µl of each of the samples was pipetted in duplicate onto microtiter plates previously coated with the monoclonal mouse anti-aldosterone capture antibody (Gomez-Sanchez *et al.* 1987), along with aldosterone calibrators and controls. Subsequently, biotinylated aldosterone tracer was added into each well (5 pg/well) and the plate was incubated

Table 1 Primer sequences and annealing temperatures used for real-time PCR

	Sequence	Annealing temperature (°C)
Amplification product		
<i>β-actin</i> (fwd)	TCATGAAGTGACGTGGACATCC	56
<i>β-actin</i> (rev)	CCTAGAAGCATTTGCGGTGGACGATG	
<i>Cyp11b2</i> (fwd)	CAGGGCCAAGAAAACCTACA	63
<i>Cyp11b2</i> (rev)	ACGAGCATTTTGAAGCACCT	
<i>Star</i> (fwd)	GACCTTGAAAGGCTCAGGAAGAAC	60
<i>Star</i> (rev)	TAGCTGAAGATGGACAGACTTGC	
<i>Cyp11a1</i> (fwd)	AGGACTTTCCTGCGCT	53
<i>Cyp11a1</i> (rev)	GCATCTCGGTAATGTTGG	
<i>NGF1-B</i> (fwd)	ATGCCTCCCCTACCAATCTTC	60
<i>NGF1-B</i> (rev)	CACCAGTTCTGGAACCTGGA	
<i>Nurr1</i> (fwd)	TCAGAGCCACGTCGATT	60
<i>Nurr1</i> (rev)	TAGTCAGGGTTTGCCTGGAA	

overnight at 4 °C. The following day, plates were washed and 200 µl per well of Europium labeled streptavidin were added. After 30-min incubation at room temperature, the amount of biotinylated aldosterone bound was measured using a fluorometer (VICTOR3, Perkin-Elmer, Waltham, MA, USA). Analytical sensitivity of the assay was 8 pg/ml, intra-assay coefficients of variation were at concentrations of 18, 34, and 139 pg/ml (7.3, 6.3, and 4.4% respectively). Inter-assay coefficients were 15.2, 15.1, and 8.0% respectively.

Real-time PCR

Both adrenals from each individual animal were combined and homogenized in extraction buffer while still frozen. RNA extraction was performed using the SV Total RNA Isolation System according to the manufacturer's instructions (Promega). For cDNA synthesis 1 µg total RNA was reverse transcribed utilizing the reverse transcription system (Promega). Quantification of investigated genes was accomplished using the FastStart DNA MasterPlus SYBR Green I reaction mix in the LightCycler 1.5 (Roche). Real-time PCR conditions were pre-incubation at 95 °C for 10 min followed by amplification of 40 cycles at 95 °C for 10 s, the annealing temperature (primer dependent as given in Table 1) for 5 s, and extension at 72 °C, at which the time is calculated by the product length in bps divided by 25 (Roche). The melting curve analysis was performed between 65 and 95 °C (0.1 °C/s) to determine the melting temperature of the amplified product and to exclude undesired primer dimers. Furthermore, the products were run on a 1% agarose gel to verify the amplified product. Quantification was adjusted using the housekeeping gene *β-actin*. To facilitate overall comparison of individual real-time experiments, expression levels of the particular genes

were set as 100% for wild-type animals and for each time point expression levels were expressed as % increase or decrease from baseline levels respectively.

Statistical analysis

All results are expressed as mean ± s.e.m. Statistical significance was determined using the Mann-Whitney *U*-test. Statistical significance was defined as $P < 0.05$ indicated by asterisks in Figs 1–3.

Results

Short term stimulation and suppression of aldosterone secretion

In the control group, aldosterone secretion in animals 10 min after sham injection was significantly higher than under baseline conditions (baseline, 88 ± 10 pg/ml versus 10 min; 228 ± 23 pg/ml, $P < 0.005$). This increase

Figure 1 Aldosterone values in control animals after sham injection, after ANGII stimulation and upon NaCl suppression. Stars denote significant changes over baseline values (i.e., 0 min).

Figure 2 (A) Increase or decrease of the mRNA expression levels of *StAR*, *Cyp11a1*, and *Cyp11b2* genes in the control group upon sham injection, (B) after ANGII stimulation, and (C) after NaCl challenge. Stars denote significant changes for each gene over baseline expression levels which are set as 100% for each gene.

was still present but less pronounced in the 20 min sampling (154 ± 18 pg/ml, $P < 0.05$) while at 30 min and thereafter aldosterone values of sham-treated mice dropped back to levels comparable with baseline values.

Ten minutes after the injection of ANGII, the aldosterone concentration were significantly elevated in comparison with baseline (baseline, 88 ± 10 pg/ml versus 10 min, 858 ± 126 pg/ml, $P < 0.001$). The following measurements at 20, 30, and 40 min showed a stepwise reduction while aldosterone secretion still remained significantly higher in comparison with the

baseline values (20 min, 585 ± 133 pg/ml; 30 min, 485 ± 69 pg/ml; 40 min, 297 ± 48 pg/ml, $P < 0.01$).

After i.p. injection of 1 ml NaCl 0.9%, the aldosterone secretion showed a short term increase (10 min, 192 ± 20 pg/ml, $P < 0.005$) comparable with that seen after sham injection. This initial peak was followed by a slow decrease with the lowest aldosterone levels 60 min after the injection, which were significantly lower in comparison with baseline values (38 ± 4 pg/ml, $P < 0.005$).

Short term transcriptional regulation of *StAR*, *Cyp11a1*, and aldosterone synthase

Real-time analysis revealed an increase of *StAR* expression only after 20 min, the sham injection (expression over baseline: $233 \pm 47\%$, $P < 0.05$). This increase was sustained until 60 min later ($93 \pm 29\%$, $P < 0.05$) and returned to normal levels after 120 min ($20 \pm 15\%$, $P = 0.31$). Similarly, *Cyp11a1* expression started to increase 20 min after the sham injection ($363 \pm 31\%$, $P < 0.001$) and did not normalize within the observation period (120 min, $117 \pm 36\%$, $P < 0.05$). By contrast, *Cyp11b2* expression for the animals that received a sham injection revealed only a slight increase over the baseline values 10 min after the injection ($53 \pm 5\%$, $P < 0.05$) which came back to baseline expression levels in the 20 min group ($-2 \pm 7\%$, $P = 0.93$) and thereafter.

ANGII injection induced an increase in *StAR* expression already after 10 min ($111 \pm 22\%$, $P < 0.05$) and after a peak at 20 min ($264 \pm 95\%$, $P < 0.05$) expression remained elevated until 120 min later ($51 \pm 14\%$, $P < 0.05$). Similarly, *Cyp11a1* expression displayed a significant increase already 10 min after the injection ($474 \pm 121\%$, $P < 0.01$) and the levels remained significantly elevated over baseline up to 120 min later ($446 \pm 81\%$, $P < 0.05$). Furthermore, ANGII injection resulted in a significant increase in *Cyp11b2* expression after 10 min ($236 \pm 39\%$, $P < 0.005$) which was sustained up to 60 min later ($244 \pm 65\%$, $P < 0.01$). Overall, induction of both *StAR* and *Cyp11a1* expression levels were within a similar range after sham injection and after ANGII stimulation. By contrast, ANGII induced upregulation of *Cyp11b2* expression was significantly higher in comparison with that followed by sham injection ($P < 0.01$ for time points 10–60 min).

Both *StAR* and *Cyp11a1* expression was reduced upon sodium challenge between 20 min (*StAR*, $-77 \pm 7\%$; $P < 0.001$, *Cyp11a1*, $-74 \pm 7\%$; $P < 0.001$) and 60 min (*StAR*, $-68 \pm 9\%$; $P < 0.05$, *Cyp11a1*, $-72 \pm 7\%$, $P < 0.05$) and *Cyp11b2* levels remained low even after 120 min ($-55 \pm 12\%$, $P < 0.05$). Furthermore, sodium load had significant effects on *Cyp11b2* expression levels that remained suppressed between 20 min ($-63 \pm 6\%$, $P < 0.05$) and 60 min ($-58 \pm 6\%$, $P < 0.01$).

Figure 3 (A) Increase or decrease of the mRNA expression levels of *Nurr1* and *NGF1-B* in the control group upon sham injection, (B) after ANGII stimulation, and (C) after NaCl challenge. Stars denote significant changes for each gene over baseline expression levels which are set as 100% for each gene.

Short term transcriptional regulation of NGF1-B and Nurr1

After sham injection, NGF1-B expression was significantly upregulated already after 10 min ($512 \pm 87\%$; $P < 0.001$) whereas Nurr1 expression showed a transient decrease over baseline up to 20 min ($-48 \pm 13\%$; $P < 0.05$) and subsequently a slight increase (60 min, $195 \pm 65\%$; $P < 0.05$). By contrast, ANGII injection caused a pronounced increase in the expression of both NGF1-B and Nurr1 already 20 min after injection

(Nurr1, $682 \pm 170\%$; $P < 0.01$, NGF1-B, $1020 \pm 71\%$; $P < 0.001$) which lasted up to 60 min (Nurr1, $740 \pm 136\%$; $P < 0.005$, NGF1-B, $485 \pm 63\%$; $P < 0.001$). By contrast, 30 min after the sodium load, Nurr1 mRNA displayed a transient increase ($185 \pm 60\%$; $P < 0.05$) and later a progressive decrease with a significant drop under baseline parameters after 120 min ($-70 \pm 7\%$; $P < 0.005$). NGF1-B expression, however, displayed a constant and significant increase during the first hour (10 min: $641 \pm 192\%$; $P < 0.05$, 40 min: $703 \pm 209\%$; $P = 0.01$) similar to those observed after sham and ANGII injection.

Discussion

Transcriptional activation as well as post-transcriptional modification of steroidogenic enzymes is the major regulatory mechanisms for acute and chronic modulation of adrenocortical steroid production and release (Christenson & Strauss 2001). In the present study, we investigated aldosterone secretion and concomitant transcriptional regulation of key steroidogenic enzymes and nuclear transcription factors upon short term stimulation and suppression experiments *in vivo*. Using an in-house developed time-resolved fluorescent immunoassay (Manolopoulou *et al.* 2008), we demonstrate the expected acute stimulation of aldosterone secretion upon ANGII stimulation and delayed suppression of aldosterone output following sodium challenge. Of note, the handling procedure including i.p. injection was followed by a significant increase in aldosterone levels starting already 10 min after the procedure. A concomitant increase in expression levels of *StAR*, *Cyp11a1*, and of *Cyp11b2* was detectable in the adrenals of sham-treated animals. As ACTH is a known stimulator of *StAR* transcription (Lehoux *et al.* 1998) and stress-induced activation of the hypothalamus–pituitary–adrenal (HPA) axis has been demonstrated to stimulate zona glomerulosa steroidogenesis (Stier *et al.* 2004), the observed short term increases in injection-induced aldosterone synthesis are likely to result from endogenous ACTH stimulation. In general, *in vivo* experiments are prone to unintended side effects induced by stress or by pharmacological inhibition of HPA axis activity which limit the accuracy of experimental endpoints. However, molecular mechanisms that are modulated by complex regulatory interactions between the renin–angiotensin–aldosterone (RAA) system and HPA axis that are also present under physiological conditions can only be studied in the *in vivo* setting. Notably, stress induced changes in adrenal transcriptional profiles were more pronounced and sustained for *StAR* and *Cyp11a1* while *Cyp11b2* expression levels were only significantly elevated over baseline at very early time points.

ANGII as one of the main stimulator of glomerulosa aldosterone output expectedly induced a fast and pronounced increase in aldosterone levels also in our stimulation experiment. The observed elevation of aldosterone release was accompanied by fast changes in adrenal mRNA levels which were evident already 10 min after the stimulatory event. In contrast to ANGII dependent changes of *StAR* and *Cyp11a1* mRNA levels which were in a similar range than those of sham-treated animals, ANGII-induced upregulation of *Cyp11b2* was much more pronounced as compared with sham injection related changes. To our knowledge, regulation of the expression of *Cyp11b2* within such a short period of time has not been reported to date. As a general elongation rate of mRNAs has been determined in the range of 18 nucleotides per second (Schafer *et al.* 1991) detectable upregulation of the investigated steroidogenic enzymes with a transcript size in the range of 855 (*StAR*), 1503 (*Cyp11b2*), and 1581 (*Cyp11a1*) bp is conceivable. However, other mechanisms contributing to the observed changes in mRNA levels such as regulation of RNA stability cannot be excluded.

Intra-peritoneal sodium challenge was followed by an immediate slight increase of aldosterone response similar to that observed after sham injection. While at this earliest time point both *StAR* and *Cyp11a1* showed a trend towards increased expression levels, the steroidogenic enzymes including *CYP11b2* dropped below baseline levels at 20 min and thereafter. This suppression in expression was followed by a delayed reduction in aldosterone secretion at 60 min. It has been demonstrated that *StAR* is subject to down-regulation by the nuclear factor *DAX-1* (Stocco 2001) but whether this mechanism is involved in a short term negative regulation as observed in our *in vivo* model requires further investigations.

In situ hybridization studies in rats have suggested that chronic ANGII stimulation does not increase *StAR* expression within zona glomerulosa cells, whereas expression of *Cyp11b2* was induced (Peters *et al.* 1998). Thus, regulatory mechanisms are likely to be in place that specifically modify steroidogenic capacity towards mineralocorticoid output. In this context, *NGFI-B* and *Nurr1* have been identified as two transcription factors involved in CaMK dependent transcriptional regulation of *Cyp11b2* expression. Interestingly, we observed that time-dependent increases of mRNA levels of *NGFI-B* are comparable between the treatment groups of sham injection, or ANGII and NaCl administration respectively and, thus, likely to be the result of stress related alterations. By contrast, *Nurr1* expression levels only slightly changed during the course of sham injection and sodium load, but were robustly induced upon ANGII administration. Thus, these results provide indirect evidence that among the

two transcription factors which have been proposed to be involved in aldosterone secretion, only *Nurr1* is likely to have potential impact in short term aldosterone regulation. However, as *Nurr1* expression levels are not clearly suppressed upon sodium load, additional regulatory mechanisms must be in place to explain the observed sodium dependent suppression of steroidogenic enzymes.

In accordance with these findings, *in vitro* studies in H295R human adrenocortical cells have demonstrated an ANGII dependent regulation of *Nurr1* (Romero *et al.* 2004). By contrast, *NGFI-B* has been suggested to preferentially regulate steroid enzyme genes related to cortisol production (Kelly *et al.* 2004). Furthermore, expression studies in adrenal adenomas have demonstrated higher *Nurr1* immunoreactivity in aldosteronomas in comparison with Cushing adenomas, whereas *NGFI-B* immunoreactivity showed no significant differences between the two groups. In addition, an association between the mRNA expression of *Nurr1* and *Cyp11b2* could be observed (Bassett *et al.* 2004b, Lu *et al.* 2004).

Taken together, we describe regulation of aldosterone secretion following specific stimulation and suppression paradigms *in vivo* that are accompanied by short term transcriptional changes of key steroidogenic enzymes and nuclear transcription factors. From the investigated parameters on the mRNA level *StAR*, *Cyp11a1* and *NGFI-B* appear to respond more to unspecific stress-related effects, whereas *Cyp11b2* and *Nurr1* are preferentially regulated by specific stimulation of the renin-angiotensin-aldosterone system. In all instances, changes in adrenal mRNA levels were detectable within a short time frame. Overall, the determination of specific pattern of these transcriptional changes will provide the framework for elucidation of molecular mechanisms underlying these physiological changes.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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