Identification of adrenal genes regulated in a potassium-dependent manner

Alexander Dierks^{1*}, Urs D Lichtenauer^{1,2*}, Simone Sackmann^{2*},
Ariadni Spyroglou¹, Igor Shapiro^{1,2}, Marcel Geyer³, Jenny Manonopoulou¹,
Martin Reincke¹, Constanze Hantel¹ and Felix Beuschlein^{1,2}

¹Endocrine Research Unit, Medizinische Klinik-Innenstadt, Ludwig-Maximilians-University, Ziemssenstr. 1, D-80336 Munich, Germany

²Institute of Molecular Medicine and Cell Research, Albert-Ludwigs-University Freiburg, D-79085 Freiburg, Germany

³Department of Internal Medicine IV, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany

(Correspondence should be addressed to F Beuschlein who is now at Department of Medicine, Endocrine Research, University Hospital Innenstadt, Medizinische Klinik-Innenstadt, Ziemssenstr. 1, D-80336 Munich, Germany; Email: felix.beuschlein@med.uni-muenchen.de)

*(A Dierks, U D Lichtenauer and S Sackmann contributed equally to this work)

Abstract

Potassium and angiotensin II are the main stimulators of aldosterone secretion from the adrenal cortex. As potassium-induced *in vivo* gene regulation in the adrenal cortex has not been studied in detail, we applied a stepwise screening approach: first, we investigated the effects of chronic potassium substitution in mice. Microarray analysis of adrenal glands revealed a set of genes (set A) that were counter-regulated in a high potassium (HP) and low potassium substitution group, while others (set B) were highly upregulated in the HP intake group. In a second step, time dependency of expression changes of these pre-defined genes was studied following short-term potassium stimulation experiments *in vivo*. Thirdly, dose dependency of potassium-induced gene regulation was investigated *in vitro*. Finally, to provide indirect evidence for the potential relevance of the detected changes for autonomous aldosterone secretion, expression analysis of aldosterone-producing adenomas was compared with normal adrenal glands. While most investigated genes were similarly regulated following long- and short-term potassium stimulation *in vivo*, observed changes were reproducible in NCI h295R adrenocortical cells mostly for the set of genes identified in the HP group (set B). Similarly, in Conn's adenomas, mostly genes from set B displayed changes in expression pattern in comparison to normal adrenal glands, while genes from set A were mostly unchanged. Thus, while *in vivo* models can help in identifying genes potentially involved in potassium-dependent aldosterone secretion, these findings also underline the necessity to interpret potassium-induced gene regulation on the basis of the experimental setting.

Journal of Molecular Endocrinology (2010) **45**, 193–206

Introduction

Aldosterone through induction of sodium reabsorption and potassium secretion in the renal distal tubulus is a major regulator of electrolyte balance, intravascular volume, and blood pressure (Williams & Williams 2003). As evident in the context of primary aldosteronism, dysregulation of aldosterone secretion has deleterious effects with high incidence of cardiovascular events (Vasan *et al.* 2004, Milliez *et al.* 2005, Stowasser *et al.* 2005), metabolic alterations (Sindelka *et al.* 2000, Widimsky *et al.* 2000, Fallo *et al.* 2006), and renal damages (Rossi *et al.* 2006), which exceed those observed in patients with essential hypertension. Despite the high incidence of primary aldosteronism (Mulatero *et al.* 2004), the molecular mechanisms that contribute to autonomous aldosterone secretion only begin to emerge.

Aldosterone is released by the cells of the adrenal zona glomerulosa, but stored only in minimal quantities. Thus, *ad hoc* biosynthesis of aldosterone needs to be tightly regulated to maintain fluid and electrolyte homeostasis (Bassett *et al.* 2004b). Transcriptional activation and post-transcriptional modification of steroidogenic enzymes are the major regulatory mechanisms for acute and chronic modulation of adrenocortical steroid production and release (Christenson & Strauss 2001). There are two main rate-limiting steps for aldosterone synthesis: transport of cholesterol to the inner mitochondrial membrane is mediated by the steroidogenic acute regulatory protein (Lin *et al.* 1995, Cherradi *et al.* 1998) and the conversion of 11-deoxycorticosterone to aldosterone, a process which is catalyzed by aldosterone synthase (Curnow *et al.* 1991). Expression of aldosterone synthase, which is encoded by *CYP11B2*, is primarily

stimulated by extracellular potassium concentration and by angiotensin II. This transcriptional regulation had initially been described in the context of chronic (hours to days) *in vitro* stimulation experiments (Denner *et al.* 1996, Clyne *et al.* 1997). However, *in vivo*, adrenal *CYP11B2* levels increase even within minutes upon specific stimulation experiments (Spyroglou *et al.* 2009). Both potassium and angiotensin II can act independently and have equivalent effects on transcriptional regulation of *CYP11B2*. Glomerulosa cells are very sensitive to changes in extracellular potassium concentrations with aldosterone secretion *in vivo* (Boyd *et al.* 1971) and production *in vitro* (Boyd & Mulrow 1972, Varnai *et al.* 1998) stimulated by changes of potassium levels as low as 0.1 mM. An important mediator of potassium- and angiotensin II-induced aldosterone secretion is intracellular calcium content (Yagci & Muller 1996, Pezzi *et al.* 1997). Whereas elevation of extracellular potassium concentrations results in calcium influx through T- and L-type channels, angiotensin II also leads to calcium release from intracellular stores (Lotshaw 2001, Akizuki *et al.* 2008). The increase in intracellular calcium acts through the calcium-binding protein calmodulin, which activates calcium/calmodulin-dependent protein kinase I and IV (CaMK I/IV; Condon *et al.* 2002) which, in turn, activates various transcription factors including *NURR1*, *NGFIB*, *ATF-1*, and *CREB* (Bassett *et al.* 2004a,b). These transcription factors interact with defined sites in the 5'-flanking region of *CYP11B2* and enhance the transcription of aldosterone synthase (Clyne *et al.* 1997, Bassett *et al.* 2004b).

While these molecular mechanisms have been characterized in quite some detail over the last years, it is evident that potassium can induce a multitude of molecular changes that could also be involved in the regulation of aldosterone secretion. In an attempt to define novel pathways in the adrenal gland which are modulated in a potassium-dependent manner, we applied microarray techniques on adrenals from mice after specific stimulation paradigms. Upon long-term potassium substitution experiments, we identified a set of genes that were regulated in a potassium-dependent manner. These genes were further investigated in independent *in vivo* and *in vitro* experiments and finally explored for their potential involvement in autonomous aldosterone secretion.

Materials and methods

Animal experiments

All experiments were carried out following protocols examined and approved by the Regierung von Oberbayern and in accordance with the German guidelines for animal studies. Animals were maintained

under standard conditions of temperature (22 °C) with 12 h light:12 h darkness cycles and food and water *ad libitum*.

Long-term stimulation experiments

To explore long-term effects of potassium stimulation on aldosterone secretion and concurrent transcriptional regulation, 8-week-old male CD-1 mice (Charles River Laboratories, Sulzfeld, Germany) were separated in three groups of 30 animals each and maintained under different diets for 28 days. While the low potassium (LP) group received chow with 7171 mg/kg potassium and distilled water, the high potassium (HP) group was fed with chow containing 11 380 mg/kg potassium and drinking water that was substituted with 2% KCl. Control (Co) animals were kept with regular chow containing 9100 mg/kg potassium and distilled drinking water.

On day 28, mice were euthanized, and trunk blood was collected for hormonal measurements within 1 min after initial handling. All adrenals were immediately cleaned from adjacent tissue using a stereomicroscope, snap frozen in liquid nitrogen, and stored at −80 °C until further processing. From each animal, one adrenal was used for RNA extraction, while the other was utilized for morphological examination.

Short-term potassium stimulation

For short-term potassium stimulation, 12-week-old female C3H mice (Jackson Laboratories, Bar Harbor, ME, USA) received drinking water *ad libitum* that was supplemented with 2% KCl. After 1, 4, and 7 days respectively, five mice per time point were euthanized for collection of trunk blood and adrenal samples. For baseline controls, another group of mice was euthanized without specific prior treatment. Blood and tissue sampling was performed as described above.

Surgical tissue samples

Diagnosis of aldosterone-producing adenomas was made according to established criteria including documentation of primary aldosteronism, aldosterone production by the tumor, histological diagnosis, and relief of symptoms upon surgical intervention. Adenoma samples were collected during the German and Austrian Adrenal Network Multicenter Trial (Saeger *et al.* 2003), whereas normal adrenal glands were obtained from patients undergoing nephrectomy and adrenalectomy because of kidney cancer. After removing adjacent fat tissue, the adrenal samples were snap frozen in liquid nitrogen and immediately stored at −80 °C until analyzed. The study protocol was approved by the local ethics committees of the

Universities of Würzburg and Freiburg. A total of 12 patients with aldosterone-producing adenomas and 6 normal adrenal samples were included in the expression studies.

Morphological examinations

For morphological evaluation of murine adrenal glands, tissue samples were fixed in paraformaldehyde, embedded in paraffin, sectioned, and azan stained following standard procedures. Stained sections were examined with a standard light microscope using 50× magnification, and areas representing the zona glomerulosa were quantified as square pixels using the SPOT software (Version 3.5.5; Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Aldosterone measurement

Aldosterone was determined with an in-house time-resolved fluorescence immunoassay as recently described in detail (Manolopoulou *et al.* 2008). Analytical sensitivity of the assay was 8 pg/ml; intra-assay coefficients of variation at concentrations of 18, 34, and 139 pg/ml were 7.3, 6.3, and 4.4% respectively. Inter-assay coefficients were 15.2, 15.1, and 8.0% respectively.

Microarray analysis

Twenty-two thousand murine cDNA microarrays were produced and processed essentially according to the Stanford protocol described by Eisen & Brown (1999). The library of 22 000 murine cDNAs was obtained as bacterial stocks from the National Institute of Aging (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>, http://lgsun.grc.nia.nih.gov/cDNA/NIA_7_4k.html), kindly distributed by the MRC, Cambridge, UK (Tanaka *et al.* 2000, VanBuren *et al.* 2002). Plasmids were purified using the Qiagen 96-well Turbo Kit (Qiagen). Inserts were purified by PCR using vector primers flanking the individual inserts (5'-CTG CAA GGC GAT TAA GTT GGG TAA C-3' and 5'-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3'). The PCR products were purified by ethanol precipitation, and resuspended in H₂O. The aliquots were dried and resuspended in 3× SSC to a final concentration of ~40 ng/μl for printing. Microarray printing was performed on aminosilane-coated slides (CMT-GAP II Slides; Corning Inc., Wiesbaden, Germany), using an arrayer that was assembled according to specifications by the Stanford group. The arrayer was operated using software kindly provided by Joe de Risi (<http://cmgm.stanford.edu/pbrown>).

Twenty-four micrograms of either tissue or reference RNA were transcribed into cDNA in the presence of Cy3- or Cy5-labeled dUTP, using Superscript-II reverse

transcriptase (Invitrogen). Hybridizations were performed in the presence of an equal amount of reference RNA (Stratagene, La Jolla, CA, USA) as described by Boldrick *et al.* (2002). All other steps, including hybridization, were performed according to the protocol provided by Brown *et al.* (<http://cmgm.stanford.edu/pbrown>).

Spot signal intensities were measured with an Axon 4000A scanner using commercially available software (GenePix 4.1, Axon Instruments, Union City, CA, USA). Printing artifacts were screened for and excluded manually if not detected by the software. Image and data files, array layout, and all relevant information requested by the minimum information about a microarray experiment (MIAME) guidelines (Brazma *et al.* 2001) were transferred to the GeneTrafficDuo database (Iobion Informatics, La Jolla, CA, USA). In order to exclude artifacts near background range, all cDNA spots were eliminated when sample intensity or reference intensity was <50 or less than the local background. Local background was subtracted from spot intensities. A locally weighted scatter plot smoother subgrid normalization method was used for normalization. Following an approach proposed by Dudoit *et al.* (2002), the computed expression ratios depend on the intensity of the spots. Thus, a smooth nonlinear least-squares fit was computed to correct for an intensity-dependent bias. Initially, the log ratio of measured Cy3 and Cy5 values obtained from the image analysis software was computed. The complete set of expression data has been made available at <http://www.endocrine-research.mki.klinik.uni-muenchen.de/en/download/>.

In vitro experiments

The NCI h295R tumor cell line obtained from the ATCC collection (ATCC-LGC, Wesel, Germany) was cultured as described earlier (Betz *et al.* 2005) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% insulin–transferrin–selenium supplement, 1% penicillin/streptomycin, and 1×10⁻⁸ M hydrocortisone. For stimulation experiments, cells were split onto 6-well culture dishes in a density of 850 000 cells/well and allowed to grow for 48 h. Cells were then treated with 0, 5, or 20 mM potassium chloride added to the culture medium for a period of 48 h. From each individual well, supernatant was collected for aldosterone measurement, and cells were harvested for RNA extraction respectively.

Luciferase experiments

NCI h295R cells were split onto 96-well culture dishes in a density of 20 000 cells/well and allowed to grow for 48 h. Cells were co-transfected with the phRL-CMV plasmid and the pGL4.10[luc2]-hCYP11B2 plasmid

(kind gift from Dr C Gomez-Sanchez, University of Mississippi) using TransFast Transfection Reagent (Promega). Cells were then treated with 0 to 20 mM potassium chloride added to the cell culture medium for a period of 48 h. Supernatant from each individual well was collected for aldosterone measurement. Luciferase activity was determined with a luminometer (Mithras Reader, Berthold Technologies, Bad Wildbach, Germany) using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's protocol. Firefly luciferase activity from pGL4.10[luc2]-hyCYP11B2 plasmid was normalized to the renilla luciferase activity from phRL-CMV plasmid. Results were expressed in percentage in comparison to the untreated control.

Real-time PCR

RNA from murine adrenals, surgical samples, and *in vitro* experiments was extracted using the SV Total RNA Extraction system (Promega) followed by reverse transcription (RevertAid H Minus First Strand cDNA Synthesis Kit, Fermentas, St Leon-Rot, Germany) in accordance with the manufacturer's protocol. For real-time PCR analyses, the FastStart DNA MasterPLUS SYBR Green I reaction mix was utilized in the Light-Cycler 1.5 (Roche). The cycling program for real-time PCR started with a pre-incubation step at 95 °C for 10 min, followed by an amplification step that consisted of 40 cycles at 95 °C for 15 s, annealing for 30 s (annealing temperature primer specific mentioned below), and an extension step at 72 °C for which the time depending on product length in bps divided by 25 (Roche). Primer sequences as well as product length and annealing temperatures are summarized in Table 1.

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 house-keeping gene β -actin for murine samples and *HPRT* for human samples. To facilitate overall comparison of individual real-time PCR experiments, expression levels of the particular genes were set as 100% for the control. For each time point, expression levels were expressed as percentage increase or decrease from baseline levels respectively.

Statistical analysis

All results are expressed as mean $\pm$ S.E.M. Statistical significance was determined by an unpaired *t*-test using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was defined as $P < 0.05$ and is

denoted by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in the figures if not stated otherwise.

For statistical analysis of differentially expressed genes from the microarray experiments, a two-sample *t*-test was used. The computed *P* values were adjusted using a false discovery rate-controlling algorithm (Benjamini & Hochberg 1995). Genes with an adjusted *P* value of < 0.05 were selected, and agglomerative hierarchical clustering as introduced by Kaufman & Rousseeuw (1990) was performed using the R statistical software package (www.r-project.org).

Results

Global analysis of potassium-induced gene regulation

In the first instance, we investigated long-term effects of potassium supplementation on murine adrenal morphology and transcriptional regulation of aldosterone synthase *in vivo*. Despite the fact that no significant differences in serum potassium levels were observable in the applied *in vivo* models, upon chronic potassium substitution over 4 weeks, treated animals displayed a 3.5-fold increase in plasma aldosterone (1151 ± 98 vs 315 ± 46 pg/ml; $P < 0.001$, Fig. 1A) and a 12.5-fold increase in adrenal *Cyp11b2* expression (1279.2 ± 102.3 vs $100 \pm 5.4\%$; $P = 0.0003$, Fig. 1B) compared to mice under regular diet. On a morphological scale, only minor changes were evident with a slight increase of zona glomerulosa thickness in HP-treated mice in comparison to the control group (122.2 ± 9.5 vs $100 \pm 3.8\%$; $P = 0.04$, Fig. 1C). In contrast to animals from the HP group, no significant changes in these endpoints were detectable between animals kept under LP diet and Co (plasma aldosterone, 266 ± 47 vs 315 ± 46 pg/ml; $P = \text{NS}$; *Cyp11b2* expression, 186.5 ± 16.4 vs $100 \pm 5.4\%$; $P = \text{NS}$; area of zona glomerulosa, 117.4 ± 5.9 vs $100 \pm 3.8\%$; $P = \text{NS}$).

Using this *in vivo* model, we applied microarray expression analysis to identify genes in the adrenal gland that were differently regulated under chronic potassium supplementation. In accordance with the initial phenotypic characterization, differences in gene regulation were much more prominent in the HP group (set B, 2189 genes regulated > 1.5 -fold over Co group with $P < 0.05$; Table 2) than in animals following LP treatment (72 regulated genes). For further real-time PCR analysis, we choose those genes (set A) that displayed opposite regulation in the HP and LP groups in comparison to the Co animals (Fig. 2A). Based on these experiments, expression of *Mtus1* (HP, 354.5 ± 41.3 versus Co, $100 \pm 7.3\%$, LP, $85.3 \pm 16.7\%$; $P < 0.01$), *Smoc1* (HP, $531.9 \pm 25.9\%$ versus Co, $100 \pm 15.4\%$, LP, $44.9 \pm 6.1\%$; $P < 0.001$), and *Gpr48*

Table 1 Primer sequences used for quantitative PCR analysis

	Species	Primer sequence	Annealing temperature (°C)	Amplification product (bp)
Gene (accession number)				
<i>Cyp11b2</i> (NM_009991.2)	Mouse	5'-CAGGGCCAAGAAAACCTACA-3' 5'-ACGAGCATTTTGAAGCACCT-3'	63	368
<i>Mtus1</i> (NM_001005863)	Mouse	5'-GCATTGGTTGACAAAGCTGAA-3' 5'-TTCCCATGAGAGGGTCAGTC-3'	55	193
<i>Tcf4</i> (NM_009333.2)	Mouse	5'-TCCGCACCCTCCAGATTAC-3' 5'-GAGTGAGCCGACGTCAGTC-3'	55	295
<i>Gpr48</i> (NM_172671.2)	Mouse	5'-GACTTCGCATTACCAACCT-3' 5'-GCCTGAGGAAATTCATCCAA-3'	54	148
<i>Wwox</i> (NM_019573.2)	Mouse	5'-CGAGTCCCACAGATTACAG-3' 5'-GCAGTTCATTGGAGAAGAGG-3'	142	62
<i>Smoc1</i> (NM_022316.1)	Mouse	5'-CACCACAGACATGGTTCAGG-3' 5'-TGTTTCAGGTCGCAGTAGTCG-3'	55	241
<i>Sos1</i> (NM_009231.2)	Mouse	5'-GGAACAGTAAGAGGTAAGCA-3' 5'-TTCTATTGGGTGTAAGGTGAG-3'	62	194
β -actin (NM_007393)	Mouse	5'-TCATGAAGTGTACGTGGACATCC-3' 5'-CCTAGAAGCATTTGCGGTGGACGATG-3'	56	282
<i>MTUS1</i> (NM_001001925.2)	Human	5'-GGACAACAACACAGCATTGG-3' 5'-TCCACAGAAGCTCCTCGTTT-3'	54	193
<i>TCF4</i> (NM_030756.3)	Human	5'-GGAAGTTTGGAAAGACGG-3' 5'-ATTTTCATCTGGAGATAGGTTTCGG-3'	60	157
<i>GPR48</i> (NM_018490.2)	Human	5'-GCCTGAATGGGCTAAATCAA-3' 5'-TGCTCCTGGAGGCTGTTATC-3'	55	180
<i>WWOX</i> (NM_016373.1)	Human	5'-TGGAGCTAATTCAGGAATAGGG-3' 5'-ATGAAGAGGCACATTCTTG-3'	60	225
<i>SOS1</i> (NM_005633.3)	Human	5'-AAACGAAGACTGAGTGAATCTG-3' 5'-CTCCCAACCATCAATATTCTTCTG-3'	61	110
<i>HPRT</i> (NM_000194.2)	Human	5'-TGCTGACCTGCTGGATTACA-3' 5'-CCTGACCAAGGAAAGCAAAG-3'	57	229
<i>NKTR</i> (NM_00385)	Human	5'-CAGTTCAGAAGAGCCAAGGA-3' 5'-CAGGAATCTTCGGTTCAGGT-3'	62	211
<i>LPHN3</i> (NM_015236.4)	Human	5'-AAACATATCAAGCAGTCAGAGG-3' 5'-CAAATCAGGAGACAACAAGG-3'	60	271
<i>SPP1</i> (NM_001040058.1)	Human	5'-GTGCCATACCAGTTAAACAG-3' 5'-TCATCTACATCATCAGAGTCGT-3'	60	294
<i>MAGI1</i> (NM_004742.2)	Human	5'-AATACCACCAACCAAGCAG-3' 5'-CATCACCAATCCTCATCTTTCC-3'	60	213
<i>HMOX2</i> (NM_001127204.1)	Human	5'-AACCAAATGAGAATGGCTGAC-3' 5'-GGCTGAGTATGTGAAGTAAAGTG-3'	60	164
<i>RNF144B</i> (NM_182757)	Human	5'-TGAAAGAGAAGTTCATCTGGAC-3' 5'-TCATTGCGTTTCGATATAAACCC-3'	59	287

(HP, $297 \pm 31.9\%$ versus Co, $100 \pm 6\%$, LP, $87.9 \pm 14.4\%$; $P < 0.01$) was significantly upregulated in HP-treated mice compared with controls and mice after LP treatment. Furthermore, we detected a significant increase in expression levels for *Tcf4* (LP, $143 \pm 4.8\%$ versus Co, $100 \pm 9\%$; $P = 0.006$), *Sos1* (LP, $148.1 \pm 8.5\%$ versus Co, $100 \pm 6.9\%$; $P = 0.005$), and *Wwox* (LP, $202.8 \pm 13.3\%$ versus Co, $100 \pm 4.9\%$; $P = 0.0003$) in the LP group in comparison to the Co group. For *Tcf4* (LP, $143 \pm 4.8\%$ versus HP, $84 \pm 8\%$; $P = 0.0007$) and *Sos1* (LP, $148.1 \pm 8.5\%$ versus HP, $90.5 \pm 7.1\%$; $P = 0.002$), a significant increase in gene expression was also detectable after LP treatment in comparison to the HP stimulation.

Regulation of candidate genes following short-term potassium stimulation *in vivo*

To further explore the observed transcriptional changes in a time-dependent manner in an independent experiment, groups of animals of different gender and genetic background were kept under HP supplementation between 1 and 7 days followed by measurement of plasma aldosterone and *Cyp11b2* expression levels in the adrenals. As expected, potassium treatment significantly increased plasma aldosterone levels in comparison to baseline values over the observation period with a peak after 1 day (day 1, 298 ± 59 pg/ml, $P < 0.01$; day 4, 226 ± 11 pg/ml, $P < 0.001$; day 7,

Figure 1 Serum aldosterone concentrations (A), aldosterone synthase (*Cyp11b2*) expression level (B), and ratio of zona glomerulosa area (C) in murine adrenal glands after different potassium substitution (LP, low potassium; Co, control; HP, high potassium). Asterisks denote significant differences in comparison to untreated controls.

170 ± 24 pg/ml, $P < 0.05$ versus baseline, 88 ± 11 pg/ml; Fig. 3A). Similarly, for adrenal *Cyp11b2* expression levels, a twofold elevation over baseline was detectable upon potassium substitution in comparison to untreated controls (day 1, 191.2 ± 26.7%, $P < 0.03$; day 4, 192.6 ± 59.9%, $P = \text{NS}$; day 7, 215 ± 48.4%, $P = \text{NS}$ versus baseline, 100 ± 20%; Fig. 3B).

In a second step, we analyzed the expression levels of those candidate genes that we had identified to be differently regulated under long-term potassium stimulation (set A). A significant increase in expression over baseline values was detectable after 1, 4, and 7 days of potassium substitution for *Mtus1* (day 1, 153 ± 20.4%, $P = 0.041$; day 4, 162 ± 28.4%, $P = 0.036$; day 7, 156.7 ± 30%, $P = \text{NS}$ versus baseline, 100 ± 12.2%;

Fig. 3C) and also for *Smoc1* (day 1, 106.9 ± 18%, $P = \text{NS}$; day 4, 149.5 ± 25.3%, $P = \text{NS}$; day 7, 192.9 ± 17.4%, $P = 0.02$ versus baseline, 100 ± 25.3%; Fig. 3G). Conversely, a significant decrease in expression over baseline was detectable for *Gpr48* (day 1, 49.7 ± 5.5%, $P = 0.03$; day 4, 37.8 ± 7.3%, $P = 0.013$; day 7, 30.4 ± 4.4%, $P = 0.006$ versus baseline, 100 ± 17.3%; Fig. 3E), *Tcf4* (day 1, 42 ± 4.5%, $P = 0.0097$; day 4, 35 ± 4.2%, $P = 0.0052$; day 7, 45.2 ± 9.1%, $P = 0.02$ versus baseline, 100 ± 15.6%; Fig. 3D), *Sos1* (day 1, 51.3 ± 7.5%, $P = 0.0045$; day 4, 30.8 ± 7.5%, $P = 0.0005$; day 7, 24 ± 5.9%, $P = 0.0002$ versus baseline, 100 ± 10%; Fig. 3H), and *Wwox* (day 1, 72.4 ± 20.2%, $P = \text{NS}$; day 4, 41.1 ± 11.8%, $P = 0.0022$; day 7, 50.9 ± 11.4%, $P = 0.0058$ versus baseline, 100 ± 8.2%; Fig. 3F). Thus, with the exception of *Gpr48*, the investigated candidate genes displayed comparable changes in expression levels upon chronic and short-term potassium supplementation even as for those experiments (long versus short supplementation), animals of different age, gender, and genetic background had been chosen.

Effects of *in vitro* potassium stimulation on the expression levels of candidate genes

To further substantiate our results in a well-defined *in vitro* system, the human adrenocortical tumor cell line NCI h295R was incubated with various amounts of potassium chloride (ranging from 5.8 to 25.8 mM final concentration) added to the medium. After incubation for 48 h, aldosterone synthase promoter activity and aldosterone secretion were measured. As expected and in accordance with the literature (Clyne *et al.* 1997), potassium supplementation resulted in a dose-dependent upregulation of *CYP11B2* promoter activity and aldosterone production in the cell culture supernatant (Fig. 4A and B). Accordingly, potassium-dependent induction of endogenous *CYP11B2* expression could be demonstrated in a dose-dependent manner (5.8 mM K^+ , 100 ± 10.8%; 10.8 mM K^+ , 367.0 ± 85.3%, $P = 0.036$; 25.8 mM K^+ , 1985 ± 212.3%, $P = 0.0009$; Fig. 4C). Despite these expected and clear responses in well-defined experimental endpoints, changes in expression levels in the pre-defined candidate genes from set A (Fig. 4D) were either not significantly different from baseline levels (for *TCF4*, *GPR48*, and *SOS1*, Fig. 4E, F, and H) or displayed differences that were even opposed to those observed in the *in vivo* experiments (WWOX: 10.8 mM K^+ , 149 ± 15.3%; $P = 0.0102$; 25.8 mM K^+ , 262 ± 21.5%; $P < 0.0001$ vs 5.8 mM K^+ , 100 ± 2%). Only *MTUS* was upregulated by potassium similar to what had been detected in long- and short-term potassium stimulation *in vivo* (10.8 mM K^+ , 105 ± 7.5%; $P = \text{NS}$; 25.8 mM K^+ , 130 ± 9%; $P < 0.05$ vs 5.8 mM K^+ , 100 ± 6.8%).

Table 2 Genes upregulated in adrenals from mice under chronic high potassium supplementation in comparison to control animals (set B)

Gene name	Fold change	P value	
Gene ID			
BG068446	RIKEN cDNA A830008O07 gene	6.725	5.75×10^{-5}
BQ553208	IBR domain containing 2	3.476	0.006746
BG063118	Inner centromere protein	3.423	2.00×10^{-5}
BG064470	Membrane-associated guanylate kinase, WW and PDZ domain containing 1	3.375	0.0002006
BG070062	Secreted phosphoprotein 1	3.276	0.0008055
BQ551455	Latrophilin 3	3.274	0.002254
AA408927	Natural killer tumor recognition sequence	3.254	2.79×10^{-5}
BG075413	RIKEN cDNA C230081A13 gene	3.204	0.005806
BG081591	Heme oxygenase (decycling) 2	3.145	0.003419
BG069986	Cyclin A2	3.141	2.90×10^{-5}
BQ555253	Chromogranin B	3.079	8.12×10^{-5}
BG077012	Cell division cycle 6 homolog (<i>S. cerevisiae</i>)	3.063	0.0007093
BQ561647	DNA segment, Chr 12, ERATO Doi 551, expressed	3.043	0.004591
BG075763	F11 receptor	2.989	0.0006479
BG064926	Antigen identified by monoclonal antibody Ki-67	2.978	0.001053
BG062945	Synaptojanin 2 binding protein	2.975	1.29×10^{-5}
BG076175	DNA segment, Chr 18, ERATO Doi 653, expressed	2.965	0.002324
AA408929	Zinc finger CCCH type containing 5	2.962	0.0002755
BG075611	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	2.952	0.003591
BG071965	Cyclin B1	2.944	0.002913
BG065028	Ubiquitin-like, containing PHD and RING finger domains, 1	2.928	0.001997
BQ560671	Cysteine-rich protein 61	2.926	3.88×10^{-5}
BQ552612	Chromodomain helicase DNA binding protein 6	2.901	0.000353
BG063496	Centrosomal protein 55	2.889	0.0008679
BQ562474	CD44 antigen	2.876	0.0009502
BQ555316	Chromogranin A	2.873	0.00198
BG063063	UDP-N-acetyl- α -D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7	2.866	0.000162
BG076012	ATPase type 13A3	2.851	0.0001381
BG068695	Transcribed locus	2.845	0.0005665
AW538243	Integrin α 1	2.825	0.0002094

In contrast, genes from set B displayed more consistent transcriptional changes in accordance with the long-term *in vivo* substitution experiment: from the investigated genes, only *NKTR* showed potassium-induced downregulation (10.8 mM K⁺, $111.7 \pm 13.5\%$, $P = \text{NS}$; 25.8 mM K⁺, $57.6 \pm 8.0\%$; $P < 0.05$ vs 5.8 mM K⁺, $100 \pm 11.7\%$), while *LPHN3* (10.8 mM K⁺, $126.5 \pm 25.9\%$, $P = \text{NS}$; 25.8 mM K⁺, $199.5 \pm 24.1\%$; $P < 0.01$ vs 5.8 mM K⁺, $100 \pm 19.9\%$), *SPPI* (10.8 mM K⁺, $105.2 \pm 6.0\%$, $P = \text{NS}$; 25.8 mM K⁺, $145.6 \pm 16.4\%$; $P < 0.05$ vs 5.8 mM K⁺, $100 \pm 4.8\%$), *MAGII* (10.8 mM K⁺, $180.6 \pm 18.6\%$, $P < 0.001$; 25.8 mM K⁺, $315.6 \pm 39.7\%$; $P < 0.001$ vs 5.8 mM K⁺, $100 \pm 3.4\%$), *HMOX2* (10.8 mM K⁺, $105.1 \pm 11.1\%$, $P = \text{NS}$; 25.8 mM K⁺, $143.6 \pm 15.7\%$; $P < 0.05$ vs 5.8 mM K⁺, $100 \pm 4.3\%$), and *RNF144B* (10.8 mM K⁺, $123.0 \pm 24.9\%$, $P = \text{NS}$; 25.8 mM K⁺, $191.0 \pm 23.8\%$; $P < 0.05$ vs 5.8 mM K⁺, $100 \pm 19.6\%$) followed potassium-induced upregulation (Fig. 4E).

Analysis of expression levels of candidate genes in aldosterone-producing adenomas

To further explore the potential relevance of potassium-dependent gene regulation for autonomous aldosterone production causing primary hyperaldosteronism, we analyzed samples of human aldosterone-producing adenomas for alterations in the expression levels of the previously identified genes in comparison to normal adrenal tissues. While for *MTUS1* expression, a significantly lower expression was detectable in aldosterone-producing adenomas in comparison to normal adrenal tissues (54.7 ± 7.1 vs $100 \pm 17.4\%$; $P = 0.01$), for other genes from set A such as *SMOCL*, *GPR48*, *TCF4*, *SOS1*, and *WVOX*, no significant differences between the two groups were evident (Fig. 5A). More significant alterations in gene expression were detectable for genes from set B with *NKTR* (202.1 ± 12.2 vs $100 \pm 26.2\%$; $P < 0.05$),

Figure 2 (A) Schematic overview of genes identified by microarray analysis to be significantly regulated in adrenal glands from mice upon long-term low (LP) and high potassium (HP) supplementation in comparison to control animals (Co). (B) Real-time PCR analyses displaying expression levels of selected candidate genes (*Mtus1*, *Tcf4*, *Gpr48*, *Wwox*, *Smoc1*, and *Sos1*) from the same experiment. Asterisks denote significant differences in comparison to untreated controls. Grey stars denote significant differences between the low potassium and the high potassium groups.

LPHN3 (720.6 ± 75.0 vs $100 \pm 17.4\%$; $P = 0.0002$), *MAGI1* (267.1 ± 38.3 vs $100 \pm 23.5\%$; $P < 0.01$), and *RNF144B* (241.7 ± 45.1 vs $100 \pm 20.6\%$; $P < 0.05$) being significantly upregulated, while *SPPI* (24.4 ± 11.7 vs $100 \pm 26.2\%$; $P = 0.01$) being lower expressed in Conn's adenomas in comparison to normal adrenal glands (Fig. 5B).

Discussion

Although potassium is a well-known secretagogue for aldosterone secretion from the zona glomerulosa, molecular changes in the adrenal gland that are induced by potassium supplementation *in vivo* have not been defined in detail. As a screening approach,

Figure 3 Serum aldosterone levels (A) and adrenal aldosteronase expression (B) in wild-type mice after 1, 4, and 7 days of potassium stimulation. (C–H) Effects of short-term potassium stimulation on expression levels of *Mtus1* (C), *Tcf4* (D), *Gpr48* (E), *Wwox* (F), *Smoc1* (G), and *Sos1* (H) in murine adrenal glands. Asterisks denote significant differences in comparison to baseline controls.

we applied an experimental paradigm of HP and LP intake in mice that was maintained over 4 weeks and identified genes that displayed opposite changes in expression levels in the LP and HP groups as compared to a Co group of animals.

Diet-induced changes in serum potassium are difficult to induce in the context of normal kidney function. In fact, we were not able to demonstrate significant changes in serum potassium levels between

the experimental groups, which are most likely due to compensatory mechanisms including those affecting the renin–angiotensin–aldosterone system. However, as we clearly demonstrate that adrenal expression levels of *Cyp11b2* and aldosterone levels in animals can be induced by means of HP intake in a highly significant manner, the applied experimental setting with potassium supplementation was able to provoke expected endocrine changes. Interestingly, only slight

Figure 4 Potassium-dependent increase in *CYP11B2* promoter activity (A), secretion of aldosterone (B), and endogenous *CYP11B2* expression (C) in NCI h295R. Effect of potassium stimulation on expression levels of genes from set A (D) and from set B (E) in NCI h295R. Cells were treated with medium supplemented with 5 mM (final concentration 10.8 mM) or 20 mM (final concentration 25.8 mM) KCl for 48 h. Asterisks denote significant upregulation compared with untreated controls (5.8 mM KCl).

enlargement of zona glomerulosa thickness was observable with a concomitant more than tenfold increase in *Cyp11b2* expression levels. Thus, in this experimental setting, transcriptional regulation or changes in mRNA stability seem to play a more important role in comparison to morphological changes such as zonation.

Following the chronic potassium stimulation, a set of genes (referred to as set A) including *Mtus1*, *Gpr48*, and *Smoc1* were found to be upregulated in the HP group, while other genes such as *Tcf4*, *Wwox*, and *Sos1* expression were elevated in those animals kept on a LP diet. Five out of six investigated genes displayed the same changes during an independent time course experiment with an observation period between 1 and 7 days. In contrast, expression of *Gpr48*, which was clearly upregulated after 4 weeks of potassium stimulation was decreasing in a time-dependent fashion within the first week of potassium supplementation. *Gpr48*, a G-protein-coupled receptor, which is currently

classified as an orphan receptor, has been implicated in playing a role in tumor cell invasiveness and metastasis (Gao *et al.* 2006) and bone remodeling (Luo *et al.* 2009) among others. Owing to its functional interdependency with cyclin-dependent kinase inhibitor p27^{Kip1} (Gao *et al.* 2006), we can only speculate that the observed bimodal regulatory pattern of *Gpr48* could be involved in the regulation of potassium-induced cell cycle control, which differs between the earlier and later observation time points. Furthermore, as animals of different age, gender, and genetic background had been used for the long-term experiments versus short-term experiments, we cannot exclude the possibility that these factors might have affected *Gpr48* expression levels. Conversely, the overall similarity of expression patterns despite the differences in the experimental outline provides indirect evidence for a functional relevance of these candidate genes.

Similar to *Gpr48*, the majority of genes identified by this screening approach have not been described in the

Figure 5 Expression levels of genes from set A (A) and from set B (B) in aldosterone-producing adenomas (APA) in comparison to normal adrenal glands (Co). Asterisks denote significant differences between the two groups.

context of adrenal physiology or aldosterone autonomy (Bassett *et al.* 2005, Lenzini *et al.* 2007). In contrast, *WWOX*, which has been identified as a tumor suppressor gene in a variety of human malignancies (Aqeilan & Croce 2007), has also been shown to be required for steroidogenesis (Aqeilan *et al.* 2009). Although the endocrine phenotype in *Wwox* knockout animals was mainly related to the ovaries and testes, gonadal genes also required for adrenal steroidogenesis such as *Cyp11a1* were described to be downregulated in the context of *Wwox* deficiency. Thus, although it remains to be shown, it is tempting to speculate that *WWOX* might play a role also in the regulation of potassium-induced aldosterone secretion.

Independent of its assumed function, the observed changes in expression levels *in vivo* could be interpreted as a direct potassium-induced effect on the adrenal gland or as an indirect effect mediated by other factors, which itself had been modulated by potassium supplementation. In an attempt to differentiate between these two possibilities, we proceeded with *in vitro* experiments by treating NCI h295R cells with different potassium concentrations. Following these experimental conditions, both an increase in aldosterone release and promoter activation of *CYP11B2* were readily detectable in potassium-treated cells. Similarly, the majority of genes identified in the HP group (set B) displayed a potassium-dependent upregulation in NCI h295R cells. In contrast, with exception of *MTUS*, the expression levels of the predefined genes from set A did

not change or – as for *WWOX* – did display even opposite changes to those observed *in vivo*. Thus, it is possible that potassium-induced modulation of regulatory networks such as the renin–angiotensin–aldosterone system or the hypothalamus–pituitary–adrenal axis could overcome direct potassium-induced effects on the adrenocortical cell.

However, other potential explanations could also account for the observed discrepancies: although an *in vitro* approach has the advantage of well-characterized experimental conditions, artifacts induced by the absence of zonal integrity and paracrine networks that also could modulate transcriptional regulation in the glomerulosa cell have to be taken into account. Furthermore, it is possible that species-dependent differences between rodents and humans could apply for potassium-dependent regulation of steroidogenesis. Finally, disruption of regulatory pathways in a tumor cell line might have affected the resulting expression levels that differ from those observed in a normal adrenocortical cell. In fact, genes required for normal aldosterone regulation could provide good candidates for genes that are involved in dysregulation of aldosterone production in primary aldosteronism. Indeed, recent examples from knockout mouse models have highlighted this concept. Specifically, deletion of potassium channels that are required for sensing potassium concentration by the glomerulosa cell such as the large-conductance, voltage- and Ca^{2+} -activated K^+ (BK) channel (Sausbier *et al.* 2005) or members of

the 2P domain family potassium channels TASK1 (Davies *et al.* 2008, Heitzmann *et al.* 2008) have been demonstrated to result in a phenotype resembling many aspects of primary aldosteronism.

To collect further indirect evidence for a potential role of the defined candidate genes for dysregulation of aldosterone production in primary aldosteronism, we compared expression levels in aldosterone-producing adenomas with normal adrenal glands. Autonomous aldosterone secretion in Conn's adenomas is accompanied by low renin levels and (a tendency to) hypokalemia. Thus, expression analysis of the pre-defined candidate genes could provide indirect evidence whether regulation of the gene in a potassium-dependent manner is still intact (downregulated) or possibly part of the autonomous dysregulation (upregulated). Our expression analyses demonstrated a significant downregulation for *MTUS1* gene expression in aldosterone-producing adenomas in comparison to normal adrenal tissues, while the remaining genes did not differ significantly between the two groups.

As *Mtus1* expression was consistently upregulated in a potassium-dependent manner *in vivo* and *in vitro*, we would assume that the observed downregulation in Conn's adenomas should be interpreted as the consequence of lower potassium levels in these patients. Furthermore, the lack of clear-cut changes of the remaining candidate genes from set A makes transcriptional dysregulation as the cause of autonomous aldosterone secretion unlikely. Of those genes with significant differences in expression in Conn's adenomas from set B, *LPHN3* has been described also to be more abundantly expressed in fetal than in adult adrenal glands (Xing *et al.* 2009). Notably, another of the candidate genes from set B, *SPPI*, has very recently been identified in meta-analysis of genome-wide gene expression analysis as one of 36 genes for which the expression differed between the pre-hypertensive phase and established hypertension (Marques *et al.* 2010). As *SPPI* coding for secreted phosphoprotein 1 was decreased during the development of hypertension and also lower expressed in Conn's adenomas, these data provide indirect arguments for a potential functional relevance of *SPPI* in the pathophysiology of hypertension.

However, a number of limitations have to be taken into account for the interpretation of our findings: first, species differences (mouse versus human) in the regulation of aldosterone secretion could apply. Secondly, an *in vitro* system is unlikely to reflect all aspects present in an organ and whole organism respectively. Finally, given the fact that aldosterone-producing adenomas might have a phenotype that resembles that of a glomerulosa cell, comparison of expression levels of a Conn's adenoma

with whole adrenal glands could overestimate expression of a given gene.

In summary, we have identified a number of genes regulated *in vivo* in a potassium-dependent manner. Robust differences in expression levels within a specific experimental setting could be observed. However, discrepancies between different *in vivo* and *in vitro* paradigms highlight the complexity of potassium-dependent gene regulation that is likely to be modulated within a cellular and endocrine network. Although experimental conditions have to be taken into account for the proper interpretation of these results, the defined candidate genes can serve as the starting point for further functional studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by grants from the Landesstiftung Baden-Württemberg (P-LS-ASN/5) and a grant from the Deutsche Forschungsgemeinschaft (BE2177/8-1) to FB.

Acknowledgements

The authors thank Lilia Spady and Inga Johnsen for excellent technical assistance and to Dr Martin Bidlingmaier for help with the hormone measurements. The authors are further indebted to Dr Ceslo Gomez-Sanchez (University of Mississippi, Jackson, MS) for providing us with a *CYP11B2*-promoter construct. This work is part of the doctoral thesis of A D and S S.

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Received in final form 10 July 2010

Accepted 20 July 2010

Made available online as an Accepted Preprint 20 July 2010