GENETIC MUTATIONS CAUSE PRIMARY ALDOSTERONISM

By

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Genetic mutations cause primary aldosteronism

This thesis/dissertation is submitted by Namita Ganesh Hattangady and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Georgia Regents University.

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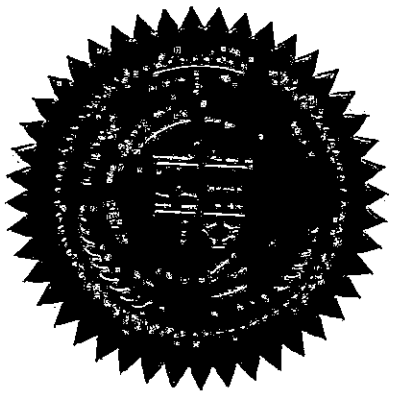

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NAMITA GANESH HATTANGADY

Genomic mutations cause primary aldosteronism

(Under the supervision of Dr. William E. Rainey, Ph.D.)

The human adrenal glands are complex endocrine organs that are physiologically located above the kidney. The cortex of the adrenal gland may be considered as a combination of three different steroidogenic tissue-types which form concentric zones within each adrenal. The three cortical zones include the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). Each zone, under independent regulation, produces unique steroid(s) which exhibit specific functions. The outermost ZG layer secretes the steroid, aldosterone, due to ZG-specific expression of aldosterone synthase (CYP11B2). Aldosterone regulates sodium reabsorption, and therefore, blood pressure. Aldosterone production is tightly regulated by the renin-angiotensin-aldosterone system. Thus, aldosterone levels are in direct proportion with renin levels. Other known physiological regulators of aldosterone production include serum K^+ levels and adrenocorticotrophic hormone.

A type of endocrine hypertension termed 'Primary Aldosteronism' (PA) is characterized by aldosterone secretion under conditions of suppressed renin levels. PA accounts for almost 10 % of hypertension. More recently, genetic mutations in an inward rectifying K^+ channel (KCNJ5) which occur as both somatic and germline cases, have been implicated in the pathology of PA. The goal of this dissertation is to define the role of KCNJ5 mutations in PA. In this dissertation, I will summarize my studies that describe the acute and chronic events involved in mutated KCNJ5-mediated aldosterone excess. In addition, I will define a novel mutation in KCNJ5 of germline nature identified at Georgia Regents University. Finally, I will also describe some interesting lessons we learned from the expression of mutated KCNJ5 in primary cultures of human adrenals.

The prevalence of a hereditary form of PA termed Familial Hyperaldosteronism type III (FH III) is very rare. Thus far, only a few mutations in the KCNJ5 gene, including T158A, G151R, G151E and I157S, are confirmed as causing FH III, following Mendelian genetics. Perhaps the most interesting feature of this disease is the varied phenotype between the different mutations. T158A-affected patients present with massive adrenal hyperplasia and require bilateral adrenalectomy. In contrast, patients affected by the G151E mutation have more severe hypertension, although their adrenals are near normal in appearance. In this study we identify a new germline mutation (Y152C). The index case was a 61 year old woman who underwent unilateral adrenalectomy. The patient with the Y152C mutation exhibited a milder hypertension phenotype (like the G151E-affected patient) with extensive hyperplasia (as seen in the T158A-affected patient). *In vitro* analyses of the Y152C mutation indicated a pathology similar to other known mutations in KCNJ5, including change in conductance to Na^+ ions and elevated calcium levels, and increased CYP11B2 mRNA and aldosterone production.

The inherent challenge presented by current studies utilizing constitutive expression of KCNJ5 mutations is the limitation in studying acute temporal events such as post translational modifications of steroidogenic enzymes and transcription factors. To address this issue, we generated a doxycycline-inducible cell model system for the T158A harboring KCNJ5 transgene. Herein, we demonstrate a useful system that is amenable to the study of acute and chronic events involved in mutated KCNJ5-mediated aldosterone excess. Our findings suggest that mutant KCNJ5 increases CYP11B2 expression through the stimulation of transcriptional activators of CYP11B2. Additionally, this is the first study to demonstrate that mutated KCNJ5 also activates steroidogenic acute regulatory protein (StAR) at the levels of translation and post translational phosphorylation. We also demonstrate the calcium channel blocker, verapamil, as an efficient blocker of mutated KCNJ5-mediated aldosterone production.

Finally, a key advantage of our study was the use of primary zonal cultures of human adrenal cells to confirm the effects of mKCNJ5. These studies have opened new avenues and questions regarding adrenal physiology and cell homeostasis. Overall, this study has improved our knowledge regarding the pathogenesis of PA caused by KCNJ5 mutations. Furthermore, it has identified calcium channel blockers as a potentially effective therapeutic strategy in the inhibition of aldosterone excess in PA caused by mutations in KCNJ5 .

KEY WORDS: Aldosterone, aldosterone synthase (CYP11B2), KCNJ5 mutations, primary aldosteronism (PA), familial hyperaldosteronism type III (FH-III), verapamil, primary human adrenal cells

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Key words: Aldosterone, CYP11B2, KCNJ5 mutations, primary aldosteronism (PA), familial hyperaldosteronism type III (FH-III)

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CHAPTER 1

Introduction

SECTION I. SPECIFIC AIMS

In normal physiology, aldosterone production is under the tight control of the renin angiotensin aldosterone system (RAAS). Dysregulation in the RAAS and excess aldosterone leads to hypertension. However, it has recently been shown that the prevalence of renin independent aldosterone excess, a condition termed primary aldosteronism (PA), is 8 - 14% amongst hypertension patients[1-3]. The two primary causes of PA are idiopathic primary hyperaldosteronism (IHA) and unilateral aldosterone producing adenoma (APA) [4]. After Choi et al. (2010) demonstrated that more than 30% of APA had point mutations in the selectivity filter of one of the inward rectifying potassium channels (KCNJ5), several studies across continents have revealed point mutations in familial and sporadic forms of PA [5-12]. **The overall goal of the proposed research is to define the mechanisms through which mutations in KCNJ5 lead to dysregulation of aldosterone production and expression of the enzyme aldosterone synthase (CYP11B2). To achieve these goals the following specific aims are proposed:**

Specific Aim 1: To elucidate the mechanisms through which mutations in KCNJ5 control CYP11B2 transcription. Using an adrenal cancer cell line, Oki K et al. (2012) demonstrated that the loss of selectivity of KCNJ5 channels leads to activation of Ca^{2+} signaling pathways including activation of voltage gated Ca^{2+} channels and calmodulin [13]. This aim seeks to define the downstream effects on CYP11B2 transcriptional events. For this purpose 2 sub-aims have been proposed:

a. To define the effect of KCNJ5 mutations on adrenal cell NURR1 mRNA and protein expression.

b. To define the effect of KCNJ5 mutations on ATF2 protein activation.

Hypothesis: Mutant KCNJ5 increases CYP11B2 transcription through stimulation of NURR1 expression and phosphorylation of ATF2 (Fig. 1.1).

Specific Aim 2: To characterize a novel KCNJ5 selectivity filter mutation identified through the Georgia Regents University (GRU) Adrenal Center. The aim is divided into the following sub-aims:

a. To define the effects of the KCNJ5 mutation on the selectivity of the KCNJ5 channel.

b. To define the effect of a novel germline KCNJ5 mutation on aldosterone production and CYP11B2 expression.

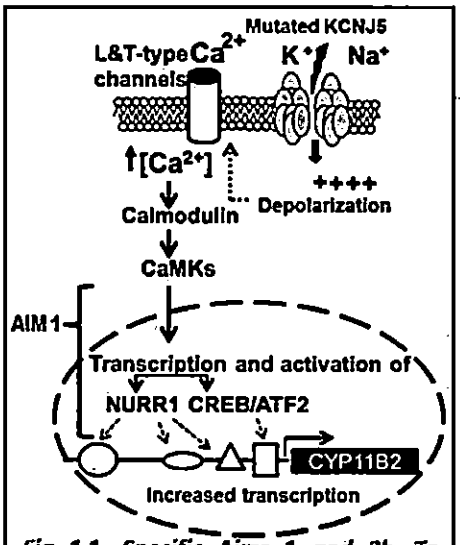


Fig 1.1. Specific Aims 1 and 2b. To define the effect of KCNJ5 mutation on CYP11B2 expression and aldosterone production.

Hypothesis: The newly described KCNJ5 mutation increases aldosterone production by increasing permeability to Na^+ ions, causing net depolarization of adenoma cells, an increase in Ca^{2+} signaling and culminating in the stimulation of CYP11B2 transcription (Fig. 1.1).

Specific Aim 3: To define and validate the effects of KCNJ5 mutations on aldosterone production in primary cultures of human adrenal glomerulosa cells (normal cells).

Hypothesis: Expression of KCNJ5 mutations in normal adrenal cells activates voltage gated calcium channels, calmodulin and calcium calmodulin kinases (CaMKs).

Significance: Although approximately 10% of hypertension patients suffer from PA, the causes of aldosterone production in these patients is not completely understood. In depth investigation of the mechanisms causing PA is therefore of clinical significance. The proposed aims are unique in the following aspects: 1) inclusion of a bank of human APA samples obtained through the GRU Adrenal Center and the Multidisciplinary Adrenal Cancer Clinic at the University of Michigan, 2) use of primary cultures of human adrenal glomerulosa cells, and 3) definition of a newly identified KCNJ5 mutation.

SECTION II. LITERATURE REVIEW AND RATIONALE

1.1. Overview of the human adrenal gland

The adrenal glands, otherwise known as the supra-renal glands, are a pair of triangular shaped multifunctional organs, each of which is located above the kidneys. In his article, 'Search the Scriptures', renowned endocrinologist from Georgia Regents University, Professor Robert Greenblatt states that the first account of the adrenal glands is to be found in the Bible [14, 15]. Herein, he speculates that Esau, one of monozygotic twins (Esau and Jacob), who is described as exceptionally hairy and strong, may have possibly suffered from the adrenal disease, congenital adrenal hyperplasia. In 1563, anatomist Bartolomeo Eustachi, described the anatomy of the adrenal gland in his detailed drawings of the kidney. In 1866, Arnold described the structural zonation of the adrenal gland [16].

The wet weight of the adrenal glands is ~6-8 g in adult humans, thus accounting for 0.01–0.02% of the total body weight [17, 18]. The adrenal gland comprises two different endocrine organs: the inner medulla, arising from the neuroectoderm and the outer cortex that originates from the mesoderm. The adrenal medulla is the innermost zone of the adrenal and is mainly comprised of chromaffin cells, which are similar to sympathetic ganglion in receiving pre-ganglionic synaptic innervation. However, instead of extending axons to target organs, the medullary cells secrete catecholamines directly into the circulation [17-19].

The adrenal cortex forms around 90% of the gland and lies sandwiched between the fibrous capsule (directly above) and the adrenal medulla

beneath. The cortex is both, histologically and functionally divided into three distinct zones: the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). The differential expression of steroidogenic enzymes in these zones leads to zone-specific steroid synthesis. The ZG, found directly beneath the capsule, is mainly composed of small round cells with scant lipid droplets, smooth endoplasmic reticulum-rich cytoplasm and a high nuclear-to-cytoplasmic ratio. This zone secretes mineralocorticoids, mainly aldosterone which regulates electrolyte homeostasis and blood pressure [17-20].

Histologically, the ZF consists of large polyhedral cells that extend centripetally towards the ZR. These ZF cells are characterized by large lipid droplets that give the cells a 'foamy' appearance [17-20]. These cells secrete glucocorticoids, mainly cortisol (corticosterone in rats and mice), which regulates glucose metabolism.

The innermost ZR replaces the organized radial pattern of the ZF by smaller-sized, irregular cords of cells that contain fewer lipid droplets and secrete dehydroepiandtrosterone (DHEA) and DHEA-sulfate (DHEA-S) which act as precursors that peripheral tissues use to produce sex steroids [17-20]. The exact functions of the DHEA and DHEA-S remain largely unknown. However, the downstream androgens-testosterone, androstenedione, and several other derivatives of these steroids have been shown to have reproductive functions in physiology and pathology.

The functional hypothesis behind the observed adrenocortical zonation is that stem cells derived from the capsule go through a series of differentiation steps, secreting different steroids as they migrate centripetally towards the

medulla, forming ZG cells, then ZF cells and finally ZR cells, after which they undergo senescence. The regulation of this process is still not completely understood, but is known to involve steroidogenic factor 1 (SF-1), DAX1 and members of the beta-catenin/WNT signaling pathway [21, 22]. The unique zonal histology of the adrenal is paralleled with a functional relevance, with each zone secreting a unique steroid (Section 1.2).

1.2. **Adrenal steroidogenesis**

1.2.1. Adrenal steroidogenic pathways

The process of the production of steroids or steroidogenesis, is a complicated process involving several enzymes and co-factors that convert the 27 carbon four-ring skeleton, to 21 carbon (C_{21}) steroids, namely progestagens, mineralocorticoids and glucocorticoids, 19 carbon (C_{19}) androgens and 18 carbon (C_{18}) estrogens.

C_{21} corticosteroids have a double bond at C4, a hydroxyl group at C21 and oxo-groups at the C3 and C20 positions. An α-hydroxyl group is present at the C17 position in cortisol, cortisone and 11-deoxycortisol, but not in deoxycorticosterone, corticosterone and aldosterone. A β-hydroxyl group may or may not be present at the C11 position. C_{19} adrenal androgens have hydroxyl or oxo group at the C17 position. Details of the steroidogenic enzymes can be found in Table 1. All adrenal steroidogenic enzymes are either cytochrome P450s (CYPs) or hydroxysteroid dehydrogenases (HSDs).

Steroidogenesis begins with the uptake of cholesterol and its delivery to the mitochondria. The human adrenal synthesizes cholesterol from several

different sources: 1) *de novo* from acetyl-CoA, by the action of 3-hydroxy-methylglutaryl co-enzyme A (HMG-CoA) reductase (the rate-limiting enzyme for cholesterol production), 2) plasma low density lipoproteins (LDLs) derived from dietary cholesterol [23, 24] and 3) high density lipoproteins (HDLs) [25] . Of these sources, LDL is the main source of cholesterol in man, while basal steroidogenesis is believed to be regulated by *de novo* synthesis of cholesterol [23, 26, 27]. Cholesterol is stored in the form of cholesterol ester, mainly in lipid droplets in adrenocortical cells. Following stimulation of steroidogenesis, cholesterol is made available for steroidogenesis by de-esterification by hormone sensitive lipase, after which is it transported to the mitochondria.

Table 1.1 Nomenclature of steroidogenic enzymes and peptides

Gene Symbol	***Enzyme Symbol***	***Enzyme***	***Subcellular localization***
StAR	StAR	Steroidogenesis acute regulator	Mitochondria
CYP11A1	CYP11A1	Cholesterol side-chain cleavage	Mitochondria
HSD3B2	HSD3B2	3β-hydroxysteroid dehydrogenase	Smooth ER
CYP21	CYP21	21-hydroxylase	Smooth ER
CYP11B2	CYP11B2	Aldosterone synthase	Mitochondria
CYP11B1	CYP11B1	11β-hydroxylase	Mitochondria
CYP17	CYP17	17α-hydroxylase/17,20-lyase	Smooth ER
SULT2A1	SULT2A1	Steroid sulfotransferase	Cytoplasm

Steroidogenic acute regulatory protein (StAR) transports cholesterol from the outer to the inner mitochondrial membrane, and is therefore considered the

rate limiting step in steroidogenesis. The adrenal steroidogenic pathway is illustrated in Fig 1.2.

It is important to note that glucocorticoids (corticosterone and cortisol) and the mineralocorticoid, aldosterone, are synthesized by the action of two isozymes: 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). In humans, 11β- and 18-hydroxylation, followed by 18-oxidation is mediated by a single enzyme, CYP11B2. This generates an aldehyde group at C18, thus producing aldosterone (aldehyde-sterone). The last step of cortisol production also involves the 11-hydroxylation of deoxycortisol to cortisol by 11β-hydroxylase. However, this enzyme only poorly catalyzes the 18-hydroxylation reaction and does not catalyze 18-oxidation.

1.2.2. Zonal nature of adrenal steroidogenesis

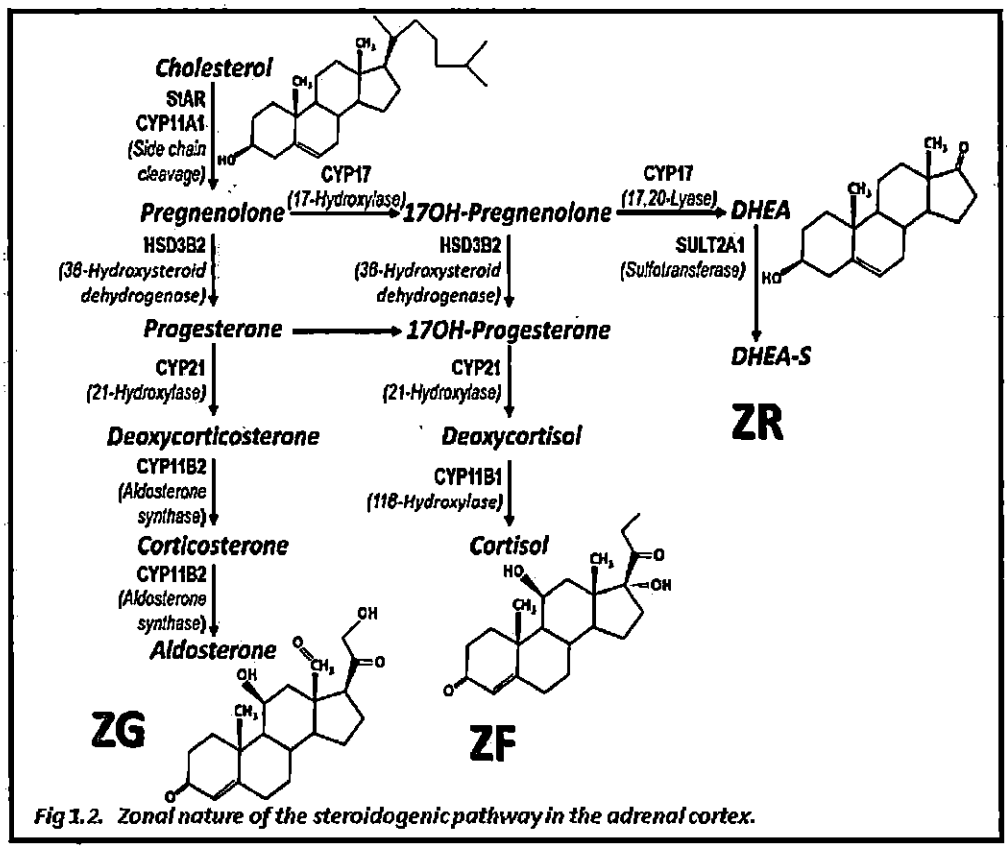


Fig 1.2. Zonal nature of the steroidogenic pathway in the adrenal cortex.

Adrenal biosynthesis of steroids is very closely associated with the observed histological zonation of the adrenal cortex due to differential expression of the steroidogenic enzymes across the zones. Aldosterone production is limited to the ZG due to several factors. Firstly, the selective expression of CYP11B2 in the glomerulosa creates a tightly controlled ZG-specific ability to synthesize aldosterone. While CYP11B2 is expressed in the entire ZG [28-30] in mice and rats, in human adrenals have relatively few subcapsular cell clusters expressing CYP11B2 [31]. A second cause of ZG specific aldosterone synthesis is the absence of CYP17 required for biosynthesis of cortisol and C_{19} androgens [32]. The absence of CYP17 from the ZG may be attributed to the expression of Ang II stimulated transcription factor cFos which inhibits the CYP17 transcriptional activator, steroidogenic factor 1 (SF-1) [33]. The centripetal flow of blood though the adrenal limits the precursors of aldosterone in the ZF from accessing the ZG-specific enzyme, CYP11B2. Finally, the ZG-specific expression of certain key transcription factors also enhances CYP11B2 promoter activation uniquely in the ZG.

HSD3B2 is expressed in both, the ZG and the ZF. CYP11B1 expression is highest in the ZF, then the ZR, with some limited expression in the ZG [28, 29], CYP17 expression is restricted to the ZF and ZR. The 17,20-lyase function of CYP17 lies exclusively in the ZR due to the ZR-specific expression of the cofactor CYB5. C_{19} androgens are thus synthesized solely in the ZR [24, 34-38]. Fig 1.2 represents the zonal nature of adrenal steroidogenesis.

1.3. Regulation of adrenal steroidogenesis

1.3.1. Renin–Angiotensin-Aldosterone System (RAAS)

The RAAS is a hormone-controlled that tightly regulates Na^+/water and K^+ homeostasis, aldosterone production and blood pressure. Renin, the first component of the RAAS, is secreted in the kidney. The macula densa acts as a sensor for lower Na^+ concentration in the luminal fluid and the juxtaglomerular (JG) cells secrete renin, which is stored in the secretory granules after cleavage of its precursor prorenin. In a series of steps, renin cleaves angiotensinogen secreted by the liver into angiotensin I, which in turn, is further cleaved by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). Ang II is the principal active agonist that stimulates aldosterone production in adrenal glomerulosa cells [19]. Ang II binds to the Ang II type 1 receptors (AT_1R) in the ZG, and initiates several signaling cascades (discussed in section 1.6) [39, 40]. The result is a net increase in the production and secretion of aldosterone. One of the actions of aldosterone is brought about by binding the mineralocorticoid receptors (MR) in the distal tubules and cortical connecting duct. This initiates an increase in re-absorption of Na^+ and water through the epithelial Na+ channels (ENaC), and leads to an increase in fluid/blood volume, and blood pressure. Na^+ absorption is also accompanied by the secretion of K^+ and H^+ ions [41]. Section 1.6 also discusses the non-renal effects of aldosterone which are mediated through genomic and non-genomic mechanisms.

1.3.2. Hypothalamic-Pituitary-Adrenal (HPA) axis

The HPA axis regulates several physiological processes [42] including glucose metabolism [43], emotions [44], response to stress (flight or fright response) [42] and

the immune system [45]. Under stress, the paraventricular hypothalamus secretes peptides corticotropin releasing hormone (CRH) and vasopressin, both of which can stimulate the anterior pituitary to synthesizes proopiomelanocortin (POMC). POMC is cleaved to form various active metabolites including ACTH, with a variety of essential functions. In the adrenal, the HPA axis mainly regulates glucocorticoid production (cortisol in humans and corticosterone in rats and mice) in the ZF. The resultant increase in glucocorticoid production has several physiological functions that include increasing immediate glucose availability to cope with "fight-or-flight" situations (gluconeogenesis, glycogenolysis, decreasing insulin), and up-regulating anti-inflammatory pathways [46]. Cortisol, in turn, directly feeds back on both, the hypothalamus and pituitary, to decrease secretion of CRH and ACTH, respectively [19, 47].

In addition to stimulating glucocorticoid production, ACTH is a potent agonist of C_{19} steroids, DHEA and DHEA-S in the adrenal ZR [19]. However, these steroids are unable to negatively feed-back on the HPA axis. Similarly, while aldosterone production may be stimulated by acute activation of HPA axis, it is not a part in the HPA axis. In fact, acute and chronic ACTH treatments have opposing effects on aldosterone production [41, 48]. These effects are discussed in detail in section 1.5 of this dissertation.

1.4. A historical perspective of aldosterone research

From the evolutionary perspective, it is believed that development of a Na^+ retaining mineralocorticoid, aldosterone, became essential as organisms from electrolyte-rich brackish waters became more terrestrial in nature.

History indicates that even without the scientific knowledge of aldosterone and electrolytes, our ancestors were aware of the importance of 'salt' in one's diet. This is amply reflected by the prominent role that proximity to naturally available salt played in determining the power of the world's great cities (due to its then limited availability). Since the mid-1900s, there has been an increase in research related to the role of aldosterone in electrolyte homeostasis and blood pressure regulation.

Pioneering work from the laboratory groups of Wintersteiner, Rechstein and Kendall and Mason first identified that the process of isolation of the then known adrenal steroids caused an amorphous fraction to be left over. This amorphous fraction after had substantial mineralocorticoid activity. Nearly 20 years later, in 1953, Simpson and Tait developed a technique to rapidly separate the uncharacterized compound and gave the name 'electrocortin'. Electrocortin was isolated in its pure crystalline form by Rechstein, in a large collaborative study with Tait, Simpson, and Winterstein, from 500 kg of beef adrenal extract using the then novel bioassay that was highly sensitive for mineralocorticoids [49-54]. Finally in 1954, the chemical structure of electrocortin was elucidated as '11β-21-dihydroxy-18-oxo-pregn-4-ene-3,20-dione'. The steroid was re-named 'aldosterone' by Rechstein, the key investigator in the identification studies. In 1955 Jerome Conn first described a patient with primary aldosteronism (PA) due to the presence of an adrenal aldosterone-producing adenoma [55]. Since then, thousands of independent studies have been conducted investigating the role of aldosterone in primary aldosteronism.

A broad range of studies have been carried out to identify key regulators of aldosterone: renin, angiotensin, serum potassium and to a lesser extent, adrenocorticotropin hormone (ACTH) [56-61]. The underlying molecular mechanisms involved in the regulation of aldosterone production are discussed in detail in Section 1.6 of this dissertation. The genes coding the isozymes CYP11B2 and CYP11B1 were cloned and characterized between1980-1990. More recent data have demonstrated several additional novel regulators of aldosterone production in physiology and disease.

The focus of aldosterone research, in the last couple of decades, has also shifted from the role of aldosterone in normal physiology, towards its contribution to cardiovascular disease. This might be attributed to the easy accessibility to salt, leading to the inclusion of ample Na^+ in one's diet, perhaps in excess of the daily human requirement. The Center for Disease Control and Prevention recommends a maximum daily Na^+ intake should be no more than 2300 mg in healthy individuals of ages 2-51 years. However, the current average Na+ intake is close to 3400mg per day. The effect of such an unhealthy diet combined with poor life style choices has adversely affected the general wellbeing of Americans. The American Heart Association currently estimates that every one in three American adults has high blood pressure. This dissertation also focuses on an aldosterone excess disorder termed Primary Aldosteronism (PA), with specific focus on the genetics and genomics underlying aldosterone excess.

1.5. Structure and regulation of human CYP11B2 (aldosterone synthase)

1.5.1. Overview of CYP11B isozymes

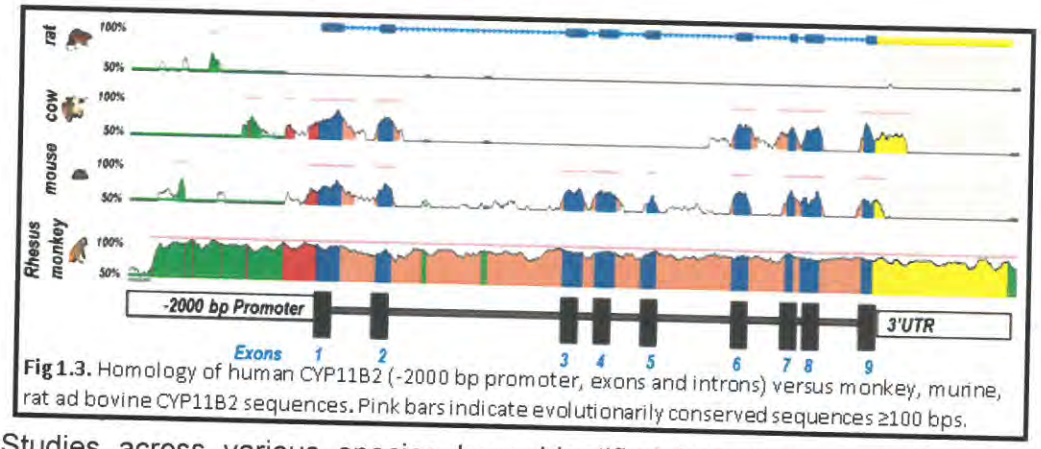


Fig 1.3. Homology of human CYP11B2 (-2000 bp promoter, exons and introns) versus monkey, murine, rat ad bovine CYP11B2 sequences. Pink bars indicate evolutionarily conserved sequences ≥100 bps.

Studies across various species have identified homology as well as key differences in the CYP11B isozymes (Fig 1.3). The bovine genome has a single CYP11B enzyme that is responsible for both cortisol and aldosterone production [62-65]. Humans [62, 66, 67], rats [68-70] and mice [71-74] have two CYP11B isozymes; CYP11B1 with 11β-hydroxylase activity for cortisol production and CYP11B2 with 11β-hydroxylase, 18-hydroxylase and 18-oxidase activities for aldosterone production. In addition, rats also express a third isozyme, CYP11B3 which is expressed at the neonatal stage [75, 76]. This isozyme has higher homology with the rat Cyp11b1 and lacks detectable 18-oxidase activity [77]. Rat isozyme CYP11B4 appears to be a pseudogene and is not expressed [70].

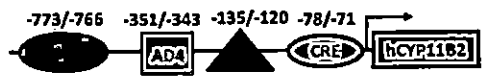
The isozymes CYP11B1 and CYP11B2 are believed to have developed through gene duplication during evolution. In humans, both isozymes are located on chromosome 8q22, ~ 40 kb apart, with CYP11B2 further towards

the 5' end. Each of the genes spans over 7000 bp and contains 9 coding exons. Their nucleotide sequences show a sequence similarity of over 94 % in the exons, 90 % in the introns, and 93% in the encoded proteins. Differences in key regulatory elements in the promoters of the genes, and zonal expression of transcription factors required for promoter activation lead to the CYP11B2 expression and aldosterone production in the ZG, and CYP11B1 expression and cortisol synthesis in the ZF [78].

1.5.2. Transcriptional regulation of the hCYP11B2 promoter

CYP11B2 expression is regulated by key regulatory elements in the promoter. These elements were first identified in the bovine CYP11B promoter, and were labeled 'Ad1-Ad6' [63-65, 79]. Several studies have identified homologous regulatory elements in the CYP11B2 promoter.

A. Structure of the human CYP11B2 promoter



B. Transcription factors that bind CYP11B2 regulatory elements

Cis-element	Transcription Factor	Binding sequence
-78/-71	CREB, ATF1, ATF2	TGACGTGA
-135/-120	COUP, SF1, NGFIB, NURR1	CTCCAGCCTTGACCT
-351/-343	SF1	CCAAGGCTC
-773/-766	NGFIB, NURR1	AAAGGCTA

Fig 1.4. Structure of hCYP11B2 (A) Regulatory elements in the CYP11B2 promoter and **(B)** Corresponding transcription factors that bind the consensus sequences.

Fig 1.4 describes the key *cis*-elements that regulate human CYP11B2 gene expression. These elements serve as binding sites for various transcription factors and nuclear receptors. The three key regulatory cis-elements in the human CYP11B2 promoter include: one cAMP response element (CRE)/Ad1 and two distal *cis*-elements (Ad5 and NBRE) that are able to bind members of the nerve growth factor-induced clone B family of transcription factors (NGFI-B or NR4A family) [40, 80-85]. These consensus sequences serve as binding sites for several specific transcription factors and nuclear receptors that are up-regulated and activated in response to agonist stimuli in the ZG. Mutational

analyses using luciferase reporter assay have indicated that the NBRE and Ad5 sites are crucial for promoter activation. Transcription factors that bind to these sites belong to the NR4A subfamily which includes three main members: NR4A1 (NGFIB), NR4A2 (NURR1) and NR4A3 (NOR). Electrophoretic mobility shift assays have suggested that NGFIB and NURR1 bind both, Ad5 and NBRE elements. However, immunostaining for protein expression in human adrenals suggest that while NURR1 is preferentially expressed in the ZG, NGFIB is expressed in both, the ZG and ZF [40, 86]. The over-expression of NURR1 has in fact been implicated in the development of aldosterone-producing tumors [87]. The Ad5 site consists of two half sites which bind several other transcription factors including AD4 binding protein/steroidogenic factor 1 (AD4BP/SF-1, encoded by NR5A1), COUP-TFI and COUP-TFII [88-90]. The activation of these transcription factors are also regulated by post translational modifications such as phosphorylation and sumoylation [88-92].

The Ad4 site is a nuclear receptor half-site and is particularly interesting. It serves as the binding site for SF-1. SF-1 is a monomeric nuclear receptor which is essential in development and function of the adrenal glands and the reproductive system. Studies have indicated that SF-1 activates almost all adrenal and gonadal steroidogenic genes. However, its role in the regulation of CYP11B2 is not yet clear. Deletion of the Ad4 site in the CYP11B2 promoter does not influence promoter activity. However, complete depletion of SF-1 in H295R cells and in mice decreases CYP11B2 expression [93, 94]. Thus, the effects of SF-1 seem to be dosage sensitive. A manuscript (in preparation) related to studies of the molecular mechanisms of the repressive effects of

SF-1 on CYP11B2 expression can be found in Chapter V (Appendix) of this dissertation. Although controversial, a T-344C polymorphism within the Ad4 site has also been associated with cardiovascular disease [95, 96].

The CRE site is closest to the transcriptional and translational start sites and serves as a bind site for all members of the cAMP response element binding protein family, including ATF1/2, CREB and CREM. Nogueira et al (2010) illustrate that transcription factors ATF/CREB and NGFI-B family members are rapid response genes which are upregulated in response to agonist stimulation of the ZG [40, 83]. Agonists elevate pathways involving kinases which cause activation of transcription factors. The activated transcription factors translocate to the nucleus, where they can bind the CYP11B2 promoter. Thus, they play a crucial role in the transcriptional regulation of CYP11B2 and the capacity of adrenal cells to produce aldosterone [83].

1.6. Physiological regulators of aldosterone production

1.6.1. Role of membrane potential in aldosterone production

As explained in earlier sections, the ZG cells are maintained at a hyperpolarized membrane potential of ~-70mV. This is achieved through the expression of K^+ channels which are constitutively open and acting as K^+ leak channels. Inhibition of these channels by the AT_1R or increased serum K^+ levels, depolarize glomerulosa cells and increases calcium influx to drive aldosterone secretion. Two recent studies using different mouse models, both with a deletion of genes encoding TWIK-related acid-sensitive K (TASK) channels, have provided interestingly different and complex primary aldosteronism phenotypes [97, 98]. In the studies by Heitzmann et al. (2008),

deletion of the TASK-1 channel resulted in a phenotype similar in pathology to a type of PA called glucocorticoid remediable aldosteronism (GRA), showing characteristics such as salt-insensitive hyperaldosteronism, hypokalemia and dexamethasone-suppressible aldosterone secretion[98]. The deletion of TASK-1 also seemed to change adrenal zonation and expression of CYP11B2, which was absent in the outermost zona glomerulosa but was expressed to a large extent in the zona fasciculata. This expression pattern seemed to be restricted only to females and to pre-pubertal males. On the other hand, Davies and colleagues [97] found that deletion of both TASK-1 and TASK-3 created a model with a phenotype resembling the pathology of IHA. Male mice showed increased aldosterone secretion that was not suppressed with a high-salt diet or the AT_1R blocker, candesartan [97]. Further studies will be required to understand the mechanism by which the different types of TASK channels interact and regulate adrenal function.

Besides TASK channels, the adrenal also harbors several other channels, exchangers, transporters and pumps which, assist in regulating membrane potential. Voltage gated L-type and T-type channels are key in responding to depolarization brought about by ZG agonists. These channels allow for elevation in intracellular calcium, thus activating a signaling cascade that culminates in an increase in CYP11B2 transcription and aldosterone production. Thus, the activity of membrane proteins, regulation of membrane potential and calcium signaling are all closely associated. Recently, Choi et al. have also identified that germ-line and somatic mutations in the selectivity filter of the inward rectifying potassium channel *KCNJ5* (Kir 3.4) result in PA [5]. This dissertation aims at determining the transcriptional mechanisms through

which these mutations cause elevated aldosterone production, expansion of aldosterone-producing cells and formation of adenomas.

1.6.2. Angiotensin II

As explained in section 1.3, Ang II is a potent regulator of CYP11B2 expression and aldosterone production, under the control of the RAAS. The regulation of aldosterone biosynthesis is divided into two key events in the steroidogenic pathway [99, 100]. Acutely (minutes after a stimulus), aldosterone production is controlled by rapid signaling pathways that increase the movement of cholesterol into the mitochondria where it is converted to pregnenolone. This "early regulatory step" is mediated by increased expression and phosphorylation of StAR protein [101-106].

Chronically (hours to days), aldosterone production is regulated at the level of expression of the enzymes involved in the synthesis of aldosterone (Fig 1.5) [40]. This has been called the "late regulatory step" and is particularly dependent on increased expression of CYP11B2. The chronic actions involve changes in the size of the zona glomerulosa as well as the glomerulosa cell's capacity to produce aldosterone. *In vitro* cell models have been particularly useful in defining the intracellular signaling mechanisms that lead to the chronic effects of Ang II.

The binding of Ang II to AT_1R not only inhibits activity of membrane proteins such as the outward leak K^+ channels and Na^+/K^+ ATPase [97, 98, 107, 108], but also activates secondary messenger pathways. The former results in depolarization of the cells, which causes the opening of voltage gated calcium channels and subsequent increase intracellular Ca^{2+} levels [40, 107]. Secondary

messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are activated when phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) [109-115]. IP3 is thought to elicit a transient increase in the cytosolic calcium concentration [39, 82, 116, 117]. Both these mechanisms lead to elevation in intracellular Ca^{2+} levels which leads to the activation of CaMKs.

Ang II-activated CaMKs increase the expression of transcription factors, as well as, activate them by phosphorylation. Activated transcription factors can translocate to the nucleus and bind the CYP11B2 promoter. This CaMK activity is clearly important, as inhibition of this enzyme decreases Ang II-induced aldosterone secretion [118-120]. However since complete inhibition is not observed [116], one may speculate that other signaling mechanisms are also involved.

DAG is produced not only PIP2, but also from PLD [121-124]. DAG activates protein kinase C (PKC), the activity of which has been controversial, as some groups demonstrate PKC-mediated stimulation in CYP11B2 [111, 115, 123, 125-127], while other groups have suggested an inhibitory role via activation of ERK-1/2 [128, 129]. DAG-activated PKC also plays a role in producing an aldosterone-secreting glomerulosa cell phenotype by inhibiting the expression of CYP17 [130, 131], possibly due to Fos-mediated repression of the CYP17 transcriptional activator, steroidogenic factor 1 (SF-1) [33]. Overall, Ang II-mediated signaling processes appear to increase CYP11B2 expression through the activation of its transcription. Using multiple glomerulosa cell models, microarray studies have defined additional transcription factors that are regulated by Ang II [132-134]. The role of these newly defined factors requires study.

Ang II has also been shown to upregulate the expression of LDL and HDL receptors [135] , enzymes involved in cholesterol synthesis [136], and increase aldosterone production by expansion of the zona glomerulosa via hypertrophy and hyperplasia, [137]. The fact that potassium can substitute for the effects of Ang II to increase adrenal expression of CYP11B2 and synthesis of aldosterone in mice with targeted deletion of the RAAS [138, 139] suggests that there might be an overlap in the mechanisms of potassium and Ang II stimulation of CYP11B2 expression.

1.6.3. Serum potassium

Elevated serum K^+ is a potent agonist of aldosterone production. High potassium diets in rats increase the expression of aldosterone synthase (CYP11B2) and aldosterone production [140, 141]. A recent study in mice subjected to 28 days of a high potassium diet reported a slight increase in the

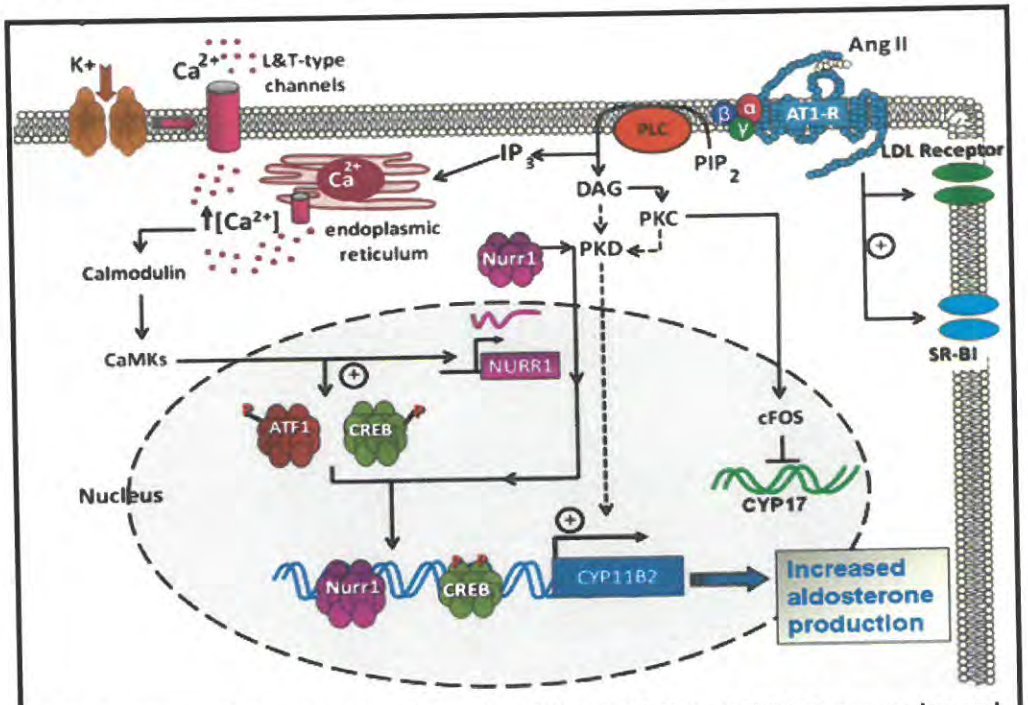


Fig 1.5. Pathways induced by chronic Ang II and K+ stimulation of CYP11B2 expression and aldosterone production.

thickness of the zona glomerulosa and suggested the role of several genes, including Mtus 1, Smoc 1 and Grp 48, in this process. However, *in vitro* experiments did not completely parallel these results [142]. A more significant increase in the thickness of the zona glomerulosa was found in rats on a high potassium diet for 2 – 7 weeks [143]. Attributing these observed changes solely to the chronic effects of potassium on aldosterone production is particularly challenging, since physiological potassium levels are tightly regulated by the renin-angiotensin system. Indeed, serum potassium levels following high potassium diets were not reported to be abnormally high.

Other *in vitro* studies using primary cultures of rat glomerulosa cells and the human adrenocortical H295R cell line have demonstrated increased CYP11B2 mRNA levels, promoter activation and aldosterone biosynthesis in response to elevated potassium levels in the growth media [144-147]. These results may help to explain the finding that in transgenic mice with targeted deletion in the renin-angiotensin system, potassium can induce CYP11B2 expression and synthesis of aldosterone in the adrenal [138, 139].

As with Ang II, a significant overlap exists between pathways activated during acute and chronic induction of CYP11B2 with elevated K^+. *In vitro and in vivo* studies indicate that the mechanism of potassium signaling in glomerulosa cells involves depolarization of cells to allow extracellular calcium influx through the T- and L-type calcium channels. This increase in calcium influx causes increased CYP11B2 expression. As in the case of Ang II, potassium-induced calcium activates CaMKs (types I and IV) [148], increase expression of, as well as phosphorylate and activate several transcription factors, such as of NURR1, ATF1, ATF2 and CREB [40, 80, 81, 83]. The CaMK antagonist KN93 and

the calmodulin inhibitor calmidazolium effectively inhibit potassium-induced CYP11B2 mRNA upregulation [40, 116] and promoter activation [40, 148], while treatment with calcium channel agonist BAYK8644 augments CYP11B2 mRNA expression in the H295R cell model [82, 116].

Interestingly, an extracellular K^+ concentration of less than 2 mM blocks Ang II-elicited calcium influx, suggesting a mechanism to prevent further hypokalemia by Ang II mediated aldosterone. In contrast, chronic high potassium increases long-term aldosterone production, sodium retention and ultimately blood pressure by increasing glomerulosa cell size and/or number as well as the cells' aldosterone synthetic capacity. However, a clearer understanding of chronic effects of potassium on the molecular pathways underlying secretion of aldosterone by adrenal glomerulosa cells is required.

1.6.4. ACTH

ACTH is considered the primary regulator of adrenal cortisol production, but only a secondary regulator of zona glomerulosa aldosterone production. It is clear that adrenal glomerulosa cells (both *in vivo* and *in vitro*) can acutely increase aldosterone production in response to ACTH. However, over time, ACTH treatment causes cultured glomerulosa cells to switch their phenotype to that of a cortisol-producing fasciculata cell [149, 150]. *In vivo* studies by Allen et al. demonstrated that ablation of the pituitary pre-proopiomelanocortin-secreting cells that produce ACTH, and the resultant low ACTH level, was accompanied by a steep decrease in the transcript levels of CYP11B1, but not of CYP11B2 [151]. In agreement with this observation, treatment with ACTH causes an initial increase in mRNA levels of CYP11B2

in the first 3 h; however, chronically, CYP11B2 expression decreases in response to ACTH *in vitro* in isolated rat adrenal cells [152]. Similarly, chronic low-dose infusion of ACTH in human subjects results in an initial increase in plasma aldosterone levels during the first 12 – 36 h, but a slow decline in these values over the next several days [153]. While the H295R adrenocortical cell lines express only low levels of ACTH receptors, treatment of these cells with cAMP analogs preferentially increases the expression of CYP11B1 over that of CYP11B2 [145]. The mechanism for chronic ACTH-mediated repression of aldosterone production and CYP11B2 expression is not fully understood. An interesting observation has been that cAMP signaling reduces the sensitivity of adrenocortical cells to Ang II by downregulating the expression of AT_1R [154, 155]. Another possible mechanism for the reduction in aldosterone production with chronic ACTH stimulation could be via the hormone's direct induction of CYP17, the activities of which direct the precursors of the steroidogenic pathway away from the production of aldosterone, and towards that of cortisol [130].

Since CYP11B2 has a cAMP-regulatory element (CRE) in its 5' promoter region [78], the mechanism preventing glomerulosa cells from responding to ACTH with increased CYP11B2 and excessive aldosterone production is not clear, two possible mechanisms have been suggested. First, at least in bovine glomerulosa cells, there is high expression of the inhibitory guanine nucleotide-binding protein G_i. Ang II signaling through the AT_1R, couples through G_i and inhibits ACTH-stimulated cAMP formation [156, 157]. Second, adrenal glomerulosa cells appear to express adenylyl cyclases 5 and 6 isoforms which are inhibited by a rise in intracellular calcium, a signaling

mechanism common to Ang II and potassium stimulation of aldosterone secretion [158]. The above provide evidence for a supportive, but not an obligatory, role of ACTH in aldosterone production.

1.6.5. Other aldosterone secretagogues

Besides Ang II, K^+ and ACTH, several other agents have been shown to increase aldosterone secretion. These agents include parathyroid hormone (PTH) [159, 160], bone morphogenetic protein 6 (BMP6) [161-165], endothelin-1 [166-170], orexins [171], microRNAs [172, 173], and regulator of G-protein (RGS) proteins [174, 175]. Reports suggesting elevated expression of several G-protein coupled receptors (GPCRs) in aldosterone producing adenomas (APA), such as the serotonin receptor [176-179], gonadotropin-releasing hormone receptor (GnRHR) [179, 180], and the luteinizing hormone receptor [181], has increased focus on their role in aldosterone production.

Chapter V (Appendix) of this dissertation includes a collaborative study with Nakamura and colleagues (Tohoku University, Sendai, Japan) wherein we demonstrate that the elevated expression and chronic activation of GnRHR in human adrenocortical H295R cells increases CYP11B2 expression and aldosterone production.

1.6.6. Functions of aldosterone

As mentioned earlier, aldosterone has both genomic and non-genomic effects in target tissues. The main sites of aldosterone action are the renal tubules, in response to elevation of the RAAS. Aldosterone binds the mineralocorticoid receptors (MR) in the cortical connecting duct. The genomic

effects include the transportation of this complex to the nucleus where it activates the transcription of several genes including ENaCs, serum and glucocorticoid sensitive kinases (SGKs), Na/K ATPase, Na/H exchanger and neural precursor cell-expressed developmentally downregulated gene 4 (Nedd4). The non-genomic actions of aldosterone include the activation of PKC, through DAG signaling pathways. Together these proteins help absorption of Na^+ ions along with water, while secreting K+ ions.

Aldosterone has also been reported to elicit effects in extra-renal tissues such as the vascular, cardiac and neural tissues [182-184]. These effects are more pronounced in conditions of aldosterone excess. They may be brought about through the action of aldosterone on its own, or synergistically with Ang II [185-187]. Aldosterone can activate reactive oxygen species by increasing the expression of NADPH oxidase subunits, decreasing the activity of glucose-6-phosphate dehydrogenase, which reduces the conversion of $NADP^+$ to NADPH [183, 188]. Aldosterone also activates enzyme protein phosphatase 2A (PP2A) which prevents phosphorylation of a Serine residue in nitric oxide synthase, resulting in the formation of reactive oxygen species (ROS). ROS are known to cause tissue inflammation. Aldosterone, along with Ang II, also activates endothelin-1, TGFβ and plasminogen activator inhibitor-1 (PAI1) mRNA expression through MR-dependent pathways [182, 183, 189, 190]. These pathways prevent the formation of plasmin, thus decreasing plasmin-activated matrix metalloproteinases (MMPs), causing accumulation of the extracellular matrix, which finally result in renal failure. Several MR blocker clinical trials including the RALES trial using spironolactone and EPHSUS trial using eplerenone demonstrated a significant reduction in morbidity and mortality in

in patients with ventricular dysfucntion [183, 184, 191-193]. This indicates that MR plays an important role in mediating the deleterious role of aldosterone. Similarly, MR antagonism has also been suggested to alleviate kidney disease by reducing urinary albumin excretion in hypertension [194, 195].

Aldosterone also binds MR in the brain, to increase Na^+ appetite, vasopressin release and sympathetic activity [196]. Gomez-Sanchez EP [197] demonstrated that low doses of aldosterone, when administered directly into the brain, induces hypertension in rats. This hypertension was remediable with mineralocorticoid antagonist RU28318 [198]. The cerebellum and hippocampus, also express Cyp11b2 transcript and protein, albeit at levels more than 1000 fold less than that found in the adrenal [199-202]. As seen in the adrenal, Cyp11b2 expression in these tissues also seem to be augmented in response to elevated peripheral renin and Na^+ restriction [199]. Together, these results indicate both autocrine and paracrine effects of aldosterone in rat neural tissue.

1.7. Dysregulation of adrenal aldosterone

1.7.1. Hypertension

As explained above, aldosterone secretion is under the tight regulation of its agonists - the RAAS and serum K^+. Therefore, an inappropriate increase in aldosterone would cause retention of fluid volume and hypertension. Hypertension is categorized based on the cause of the disease. The World Health Organization (WHO) defines primary hypertension (essential hypertension or idiopathic hypertension) as 'systemic hypertension of unknown cause that results from dysregulation of normal homeostatic control

mechanisms of blood pressure in the absence of detectable known secondary causes'. Secondary hypertension, as defined by WHO, is 'systemic hypertension due to an underlying disorder'. Secondary hypertension accounts for 10% of cases of hypertension [203]. The underlying disease may be renal or endocrine in nature. Primary aldosteronism is a type of endocrine hypertension where the underlying disease is found in the adrenal gland.

1.7.2. Primary Aldosteronism

PA is characterized by renin independent aldosterone excess. Around 8 - 10% cases of hypertension are a result of PA. [4, 204, 205]. Inappropriately high aldosterone levels are known to causes excessive sodium and water retention, and eventually, hypertension. In exchange for sodium absorption, potassium and hydrogen ions are seccreted in the kidney cortical connecting ducts. If this occurs over a prolonged period of time, and is severe enough, hypokalemia and metabolic alkalosis may be possible outcomes.

1.7.2.1. Types of Primary Aldosteronism

The various types of PA and their causes can be found in Table 1.2 (adapted from the US Endocrine Society guidelines [206] and Chao et al, 2013 [207]. PA caused by APA, or Conn's Syndrome, was first identified by Dr. Jerome W. Conn in 1955 after an intensive and elaborate study that lasted over 240 days, on a female patient with seemingly resistant hypertension, and exhibiting all the classical symptoms of what we now call PA. The cause of PA was identified by surgical removal of an adrenal tumor that cured the patients hypertension and hypokalemia. This dissertation focuses specifically on

Conn's Syndrome, with emphasis on the molecular mechanisms by which genetic mutations in APA lead to aldosterone excess.

Table 1.2. Classification and causes of PA (adapted from [208])

PA subtype	*Prevalence*	*Causes*	*Major symptoms*
SPORADIC PA			
a) Idiopathic hyperaldosteronism(IHA) /Bilateral adrenal hyperplasia (BAH)	60-65 %	Unknown	Hyperplasia of both adrenals, causing lack of lateralization in adrenal vein sampling (Table 1.3)
b) Unilateral aldosterone producing adenoma (APA)	30-35 %	Unknown. However mutations have been found in several channels in APA expressing higher CYP11B2 mRNA levels	• Hyperplasia of single adrenal • APA harboring mutations observed in younger population, sometimes with gender bias
c) Adrenocortical carcinoma (ACC)	<1 %	Unknown	• Metastatic, aggressive tumors. • No mutations reported thus far.
FAMILIAL PA			
a) Familial Hyperaldosteronism Type I (FH I)/ Glucocorticoid Remediable Aldsoteronism (GRA)	7 %s	Unusual crossover of genes causing CYP11B1 promoter to drive the expression of CYP11B2. Sensitive to ACTH levels.	• Childhood or adult onset • Bilateral adrenal hyperplasia • Possible nodular formation • Cortisol suppressible with inhibitors HPA axis
b) Familial Hyperaldosteronism Type II (FH II)	<1 %	Unknown, but has been shown to be associated with chromosome 7p12	• Adult onset • IHA or APA
c) Familial Hyperaldosteronism Type III (FH III)	<1 %	Germline mutations in KCNJ5 (G151R, G151E, T158A)	• Childhood onset • Massive bilateral hyperplasia • Nodular formation some cases

1.7.2.2. Diagnosis and current therapies for PA

As described above, the classical features of PA is renin-independent aldosterone production. Therefore, the initial test for PA involves defining the aldosterone to renin ratio. Most laboratories use a radioimmunoassay to measure aldosterone levels. Renin is quantified as either plasma renin activity (PRA) or direct renin concentration (DRC). Due to lack of uniformity in diagnostic protocols, the ARR cut-off used across referral centers is different. However, most centers consider ARR elevated in the range of 20 -40 (aldosterone ng/dL / renin ng/mL/h) when the test is performed in the morning on a seated ambulatory patient.

However, besides elevated ARR, the plasma aldosterone concentration should also be elevated (≥15 ng/dl). Fulfilment of both these criteria is important considering the fact that elderly patients and populations of certain ethnic races often have extremely low PRA. This results in the false positive diagnosis of an effectively high ARR. Thus, a combination of two factors serves as the initial indicators of PA: 1) PAC ≥15 ng/dl and 2) an elevated ARR.

If elevated ARR leads to suspicion of PA, salt loading tests are used to confirm this diagnosis (Table 1.3). Once confirmed, the subtype of PA is determined through a systematic flow in diagnostic methods. The flow chart in Fig 1.6 details these methods. The plan of therapy to be used for a patient depends on the specific PA subtype that he is diagnosed with. Adrenal vein sampling (AVS) has been very useful and successful as tool to diagnose PA subtype due to its ability to test the lateralization index and therefore identify the affected adrenal. Furthermore, current medical research is also testing the

ability to measure hybrid steroids including 18-oxocortisol (serum), 18-hydroxycorticosterone (urinary and serum) and 18-hydroxycortisol (urinary and serum) as a method of diagnostic workup for determining the subtype of PA [208-210].

Table 1.3 Confirmatory tests for PA (adapted from [208])

Test	*Protocol*	*Threshold value for PA*
Fludrocortisone/ Hydrocortisone suppression test	0.1 mg fludrocortisone/6 hourly and sodium supplementation (slow release natrium 30 mmol/day) over 4 days	Upright plasma aldosterone at day 5 at 10am >6 ng/dl
Saline infusion test	2000 ml 0.9% saline i.v. over 4 hours (while recumbent); beginning between 8am and 10am	Plasma aldosterone after saline infusion>5 ng/dl
Oral sodium loading	3 days of oral sodium loading (urinary sodium >200 mEq/day), collection of 24 hours urine	Urinary aldosterone after sodium loading >12 µg/day
Captopril test	25 mg Captopril p.o., patients in seated position	Plasma aldosterone after 2 hours >15 ng/dl
Urinary aldosterone metabolites	Tetrahydroaldosterone in a 24 hour urine sample	Tetrahydroaldosterone >66 mg/day

As explained in Fig 1.6, PA caused due to unilateral APA is treated with adrenalectomy. Adrenalectomy cures hypertension in ~50% of the patients; the remainder 50 % of the patients also require treatment with antihypertensive drugs after adrenalectomy [211-213].

Treatment with mineralocorticoid receptor (MR) blockers such as spironolactone or eplerenone is recommended for patients with other forms of PA. Spironolactone, although effective, has side effects such as gynecomastia, and erectile dysfunction in males, and abnormal menstrual

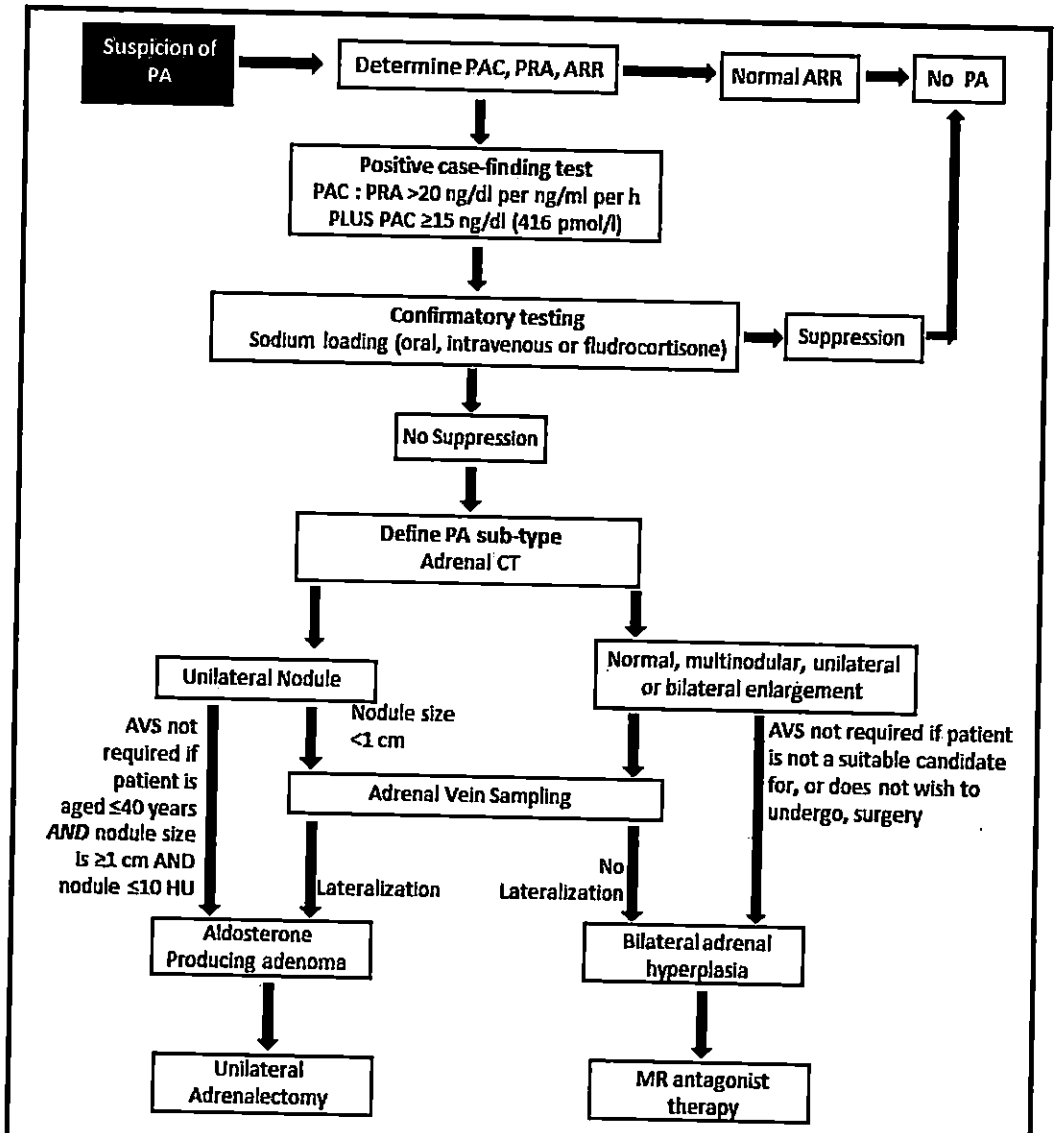


Fig 1.6. Flow chart for diagnosis of the subtype of PA (Adapted from [220, 223, 224])

cycles in females, due to its affinity for the progesterone and androgen receptors [214, 215]. These side-effects may be resolved with MR-specific eplerenone treatment [216]. Interestingly, spironolactone treatment has also shown to cause some toxicity in the adrenal by the formation of 'spironolactone bodies' [217]. A caveat of MR blockade is the possibility of chronic activation of the renin angiotensin system. Therefore, whether long term MR treatment should be accompanied by Ang II receptor blockers is still a topic of discussion in the medical community [212]. Finally, in light of genetic

mutations in APA that involve Ca^{2+} dysregulation in PA (Section 1.7), the use of calcium channel blockers for a targeted population has been suggested [218].

1.7.3. Hyperaldosteronism induced end organ damage

As explained in section 1.6, aldosterone has also been shown to cause cardiovascular disease through genomic and non-genomic actions. Elevated aldosterone in PA also have psychological effects such as anxiety and depression thus impacting the quality of life [184].

1.8. Genetic and genomic mechanisms of primary aldosteronism

1.8.1. Mutations in KCNJ5

Recent studies have implicated APA as the cause for 35% of PA cases. A study by Choi et al in 2011, identified mutations in the selectivity filter of an inward rectifying, G-protein coupled potassium channel (GIRK4 or KCNJ5) [5]. Over the last 3 years, several more mutations have been found in KCNJ5, most of them being in the selectivity filter. This dissertation (Specific Aims 1 and 3) focus specifically on APA harboring these mutations and seeks to identify the transcriptional mechanisms involved in mutant KCNJ5 mediated elevation in CYP11B2 expression and aldosterone production. Specific Aim 2 identifies and characterizes a new germline mutation in KCNJ5.

1.8.1.1. Structure and role of KCNJ5 in normal physiology

KCNJ5 or GIRK4 encodes a G-protein coupled inward rectifying potassium channel Kir 3.4. The gene lies on chromosome 11 and consists of 2 coding exons. Functional Kir3.4 channels are a result of homo or hetero-tetrameric assemblies of the proteins Kir 3.1-4 [219]. Kir 3.4 and Kir 3.1 have been shown to heteromerize in a stoichiometric ratio of 1:1 [220].

The structure of the Kir 3.4 channel (Fig 1.7) consists primarily of two membrane spanning domains (M1 and M2) linked by the pore (H5 or P region) that contains the selectivity filter (a conserved G-Y-G motif), and cytoplasmic amino (-NH2) and carboxyl (COOH)- terminals. Overall, the four groups of associated β-sheet-rich NH2 and COOH termini form a cylindrical structure around the selectivity pore. Kir 3.4 lacks the conserved region present in voltage dependent channels and is therefore insensitive to membrane voltage. Thus, in the absence of Kir3.4 activators, the channel is active at all membrane potentials. The inward rectifying function is a result of the block of outward K^+ flux by intracellular substances such as Mg^{2+} and polyamines. Transmembrane units also play a critical role in the gating of Kir3.4. Mutations in this region can interfere with channel gating.

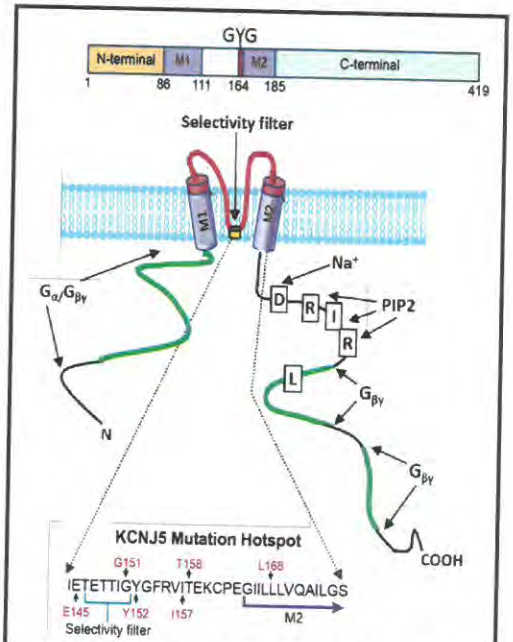


Fig 1.7. Structure of the Kir 3.4 channel with emphasis on hot spot for mutations. Adapted from Mark M.D. et al 2000

Kir 3.4 channels are activated by the binding of the $G_{\beta\gamma}$ subunit released from an activated GPCR. In fact, when the cytoplasmic domains of Kir 3.4 and Kir 3.1 associate in a heteromer, $G_{\beta\gamma}$ binding is synergistically elevated. A large number of residues in the cytoplasmic region of Kir 3.4 have been identified as potential binding sites for the $G_{\beta\gamma}$ subunit. A single point mutation in the C terminus from leucine (L) to glutamate (E) has been shown to decrease binding of the $G_{\beta\gamma}$ subunit. The C terminus also harbors an aspartate (D)

residue which serves as a binding site for Na^+, as well as positively charged R-I-R residues that serve as binding sites for phosphoinositol-4,5-biphosphate (PIP2). PIP2 is essential for the sustenance of normal function of the many Kir channels. Finally, an increase in extracellular K^+ also augments Kir 3.4 conductance, in agreement with the multi-ion pore model. Although the exact function of Kir 3.4 in normal adrenal ZG physiology is not clearly understood, recent studies indicate that Ang II and K^+ actions involve the inhibition of KCNJ5 activity. Therefore, Ang II may inhibit KCNJ5 not only via the GPCR (explained ahead), but also by depleting PIP2 reserves.

Deactivation of the Kir3.4 channel is regulated by RGS proteins as well as by pathways parallel to the release of $G_{\beta\gamma}$ during GPCR activation. The synergistic activation of $G\alpha_{i/o}$ and $G\alpha_q$ pathways accelerates pathways that cause deactivation of GIRKs faster than the $G\alpha_{i/o}$ alone. Activation of $G\alpha_q$ pathway includes the activation of PLC which has dual methods of deactivation of Kir 3.4: 1) by the depletion of PIP2 and 2) by the activation of PKC which directly phosphorylates the C-teminus of the channel, reducing its affinity for PIP2. The Gα mediated depletion of PIP2 is probably one of the mechanisms by which the Ang II mediated signaling pathway prevents hyperpolarization by Kir 3.4. RGS proteins, on the other hand, accelerate the GTPase activity to increase hydrolysis of GTP to GDP on the $G\alpha_{i/o/q}$ subunits [220-223].

1.8.1.2. Mutations in KCNJ5 in APA

Mutations in KCNJ5 have been identified in cardiac disease [224-226]. Choi et al (2011) used whole exome sequencing APA (with a mean diameter ~2.8 cm) obtained from 22 patients presenting with hypertension, variable

hypokalemia and high ARR [5]. Eight of 22 APA were found to harbor G151R and L168R mutations in the KCNJ5 selectivity filter. A germline mutation (T158A) in an American family was also identified. Thus mutations in KCNJ5 have been found to be the cause of FH III. Electrophysiological studies suggested that the mutations result in a change in conductance of the channel, such that Kir3.4 loses its inward rectifying ability and instead, acts as a conductor for Na^+ ions (Fig 1.1). Following this study, there have been several other studies that have identified additional mutations in the KCNJ5 gene, mostly in the selectivity filter [7, 11, 12, 227-229]. The pore/selectivity of Kir 3.4 thus appears to be a 'hotspot' for mutations that cause PA.

So far, all reported mutations in KCNJ5, both sporadic and familial, have been heterozygous. *In vitro* studies aimed at investigating the mechanism by which mutated-KCNJ5 increases CYP11B2 expression and aldosterone production have identified a large role of calcium channels [13, 230]. The hypothesis is that Na^+ influx that occurs with mutations in KCNJ5, depolarize the APA cells, which open voltage gated calcium channels which increase CYP11B2 expression. Supporting this, treatment of adrenal cells expressing mutated Kir3.4 with calcium channel blocker nifedipine decreased CYP11B2 expression [13, 227].

APA with mutated Kir 3.4 have also shown to have disrupted adrenocortical zonation [231]. Unpublished data from our lab (Nishimoto et al, in preparation), has shown an increasing gradient in Kir 3.4 expression from the ZR to the ZG. The expression of wild type and mutant Kir 3.4 in primary cultures of human

ZG (capsular) cells and ZF/ZR cells, will give insight into any effects of KCNJ5 expression on adrenocortical zonation.

Access to adrenal banks at the University of Michigan and Georgia Regents University allowed for expansion of the APA cohort to update the prevalence of KCNJ5 mutations. This cohort is a mixed ethnic bag, consisting of APA obtained from Japan, Italy and various regions of the United States. Our unpublished data (Table 1.4) indicate a 50 % prevalence of KCNJ5 mutations. The prevalence is higher in Japan, and lower in a Caucasian population (Table 1.4). During this process, we identified a novel mutation in the selectivity filter of an APA (*Specific Aim 2).*

Despite the sudden spurt in research on KCNJ5 mutations in PA caused by APA, there are several aspects of PA caused by APA are far from being clearly understood. For example, there have been contradicting reports on KCNJ5 mutations harboring APA being larger in size and having lower CYP17 and higher KCNJ5 expression than APA without KCNJ5 mutations [6, 9]. Another unanswered question is whether the source of APA formation is the ZG or ZF [232].

Table 1.4. Current prevalence of APA harboring KCNJ5 mutations (unpublished data)

	Sex	Padova (Italy)	USA	Japan	Total
Total no. of samples		58	50	70	178
No of KCNJ5 mutant APA	F	22	14	16	52 (58%)
	M	5	4	28	37 (42%)
	T	27 (47%)	18 (36%)	44 (63%)	89 (50%)
No of KCNJ5 WT APA	F	11	14	8	34 (37 %)
	M	20	18	18	56 (63 %)
	T	31 (53%)	32 (64%)	26 (37%)	89 (50%)

1.8.2. Other mutations in PA

Following the identification of mutations in the KCNJ5 gene, in a similar study, APA of patients characterized with hypokalemia were subjected to exome sequencing to identify mutations in novel genes in APA: the ATP1A1 gene which codes for Na^+/K^+ ATPase, the ATP2B3 gene which codes for a Ca^{2+} ATPase found in the cell membrane, [233] the CACNA1D gene which encodes the alpha sub-unit of the $Ca_v1.3$ channel [218, 234]. The mutations perturb ion transport in the cell [233, 234]. The prevalence of mutations in the ATP1A1 and ATP2B3 genes was reported to be ~ 5 % and 1.7 %, repectively in APA, with a male bias[233, 235]. Our findings on prevalence of mutations in ATPases in a mixed ethnic APA cohort indicated a similar prevalence. None of the mutations in the ATP1A1, ATP2B3 and CACNA1D genes reported so far are concurrent with KCNJ5 mutations. Furthermore, mutations in ATP1A1 and ATP2B3 described thus far have been somatic in nature. While germline mutations in CACNA1D have been reported, these mutations were not identified in the parents. As a result, thus far, mutations in KCNJ5 are the only known causes for FH III. This does, however, emphasize the importance of calcium signaling in PA. This may also suggest the use of calcium channel blockers as a target therapy for PA caused by gene mutations that elevate calcium signaling in adenoma cells.

1.9. Significance of Proposed study

Although the role of calcium signaling in aldosterone production has been identified in APA with mutant Kir 3.4, the transcriptional regulation of CYP11B2 has not yet been studied. This dissertation is therefore designed to

define the role of transcriptional regulation of CYP11B2 through NURR1, ATF1/2 and CREM rapid response proteins, and validate the results in primary cultures of human ZG and ZF/ZR cells. This study also has the potential to indicate any effects of KCNJ5 expression on adrenocortical zonation. Finally, this study expands the prevalence study of APA harboring KCNJ5 mutations and also identified a new germline mutation in the selectivity filter of the KCNJ5 channel.

CHAPTER 2

Material and Methods

2.1. Cell models

The current studies have used a variety of human adrenal cell models including primary cultures of human adrenal cells as well as the adrenocortical carcinoma cell lines, HAC15 and H295R. These cell lines have been further modified to develop inducible gene expression systems to best suit the purpose of the respective study.

The human adrenocortical carcinoma cell line, H295R, was developed by Dr. William E. Rainey as a sub-strain of the original NCI-H295 cell line [236]. This cell line represents cells of all three adrenocortical zones (ZG, ZF and ZR) and has been a beneficial tool in the investigation of adrenal steroids biosynthesis, as well as in the study of several adrenal signaling pathways initiated by agonists of steroid production, including Ang II and K^+[82, 108, 116, 117, 144, 145, 148, 236-239].

HAC15 (clone 15) is a second adrenocortical cell line, vastly used in *in vitro* studies related to adrenal physiology. The HAC15 cell line was generated in the laboratory of Dr. William E. Rainey by Dr. Jeniel Parmar as a clone of the H295R cells, expressing higher basal aldosterone production[236]. Specifically, clone 15 of the HAC15 cells have retained the ability to respond to agonist Ang II and K^+, and additionally, exhibit modest response to ACTH. The HAC15 cells are therefore, the current preferred cell line for studies on aldosterone production. For this dissertation, the H295R and HAC15 cell lines have been used extensively to generate a variety of cell lines.

The lentivector pLenti-CMV-TRE3G-KCNJ5^{T158A} was a kind gift from our collaborator, Dr. Celso Gomez Sanchez. These cells constitutively express the rtTA3 protein, a reverse trans-activating protein. When treated with doxycycline, rtTA3 can bind to the Tet operator and conditionally induce the expression of KCNJ5-T158A. These cells have been used to study the transcriptional events underlying elevated CYP11B2 expression mediated by mutant KCNJ5 (Aim 1).

The Human Donor Adrenal programs at Georgia Regents University as well as the University of Michigan made it possible to procure adrenals from cadaveric and kidney transplant donors. The adrenals were logged into the Rainey bank for Donor Adrenal Normal (DAN), and tested for morphology by hematoxylin-and-eosin staining, CYP11B2 expression as a functional test for the identification of ZG-rich adrenals by immunostaining and finally used for zonal adrenal cell isolation (capsular/ZG cells and ZF/ZR cells). Capsular/ZG cells obtained from adrenals exhibiting CYP11B2-rich ZG, minimal APCC expression and CYP11B2 response to Ang II, were utilized to demonstrate the effect of KCNJ5-T158A expression in these cells. Similar studies were conducted in the ZF/ZR adrenal zonal cultures. The use of these tissues has been approved by the Institutional Review Boards at Georgia Regents University (Augusta, GA) and at the University of Michigan (Ann Arbor, MI).

2.1.1. Cell Culture

All cell models were routinely grown in Dulbecco's Modified Eagles/Ham F12 (DME/F12) medium supplemented with sera as described below:

Name of cell line	Serum	Other supplements common to all cell lines	Selection
HAC15	10% cosmic calf serum (Hyclone, Logon, UT)	• Dulbecco's Modified Eagles/Ham F12 (DME/F12) • 1% ITS Plus Premix (containing insulin, human transferrin and selenous acid) (Corning, NY) • 1% Penicillin/Streptomycin (Gibco) 0.01% Gentamycin (Sigma Aldrich, St Louis, MO)	None
HAC15-CMV-rtTA3	5% cosmic calf serum (Hyclone, Logon, UT)		Hygromycin
HAC15-TRE-mKCNJ5	5% cosmic calf serum (Hyclone, Logon, UT)		10 µg/mL Puromycin (Sigma Aldrich)
HAC15- T158A	10 % cosmic calf serum (Hyclone, Logon, UT)		5 mg/ml blasticidin (Life technologies)

All cell types were incubated at 37°C and 5% CO_2, and the medium was changed every alternate day. Cells were grown in T75 flasks up to 75-80% confluence and sub-cultured thereafter (~ 5-6 days after initial inoculation of cells). Sub-culturing involved aspirating the growth medium and washing the cells briefly in 10 mL of serum-free DME/F12 medium (Gibco). Cells were then incubated with 6 mL of 0.05% Trypsin-EDTA (Gibco) for 5 minutes. The flask was tapped on the sides and from the bottom to loosen the adherent cells. After pipetting the cell solution a few times, the trypsin was neutralized by transferring the solution into a 50 ml tube containing 12 ml growth medium.

Cells were pelleted by centrifuging at 1200 rpm for 4 minutes. After aspirating the supernatant, the cell pellet was broken by gentle tapping of the tube. Cells were resuspended in 10 ml growth medium. Clumping of cells was reduced by passing the cell suspension through a 70 micron filter prior to plating. For expansion of cell lines, cells were plated at a density of 5 million cells/T-75 flask.

Primary cultures of human adrenal Capsular/ZG and ZF/ZR cells were isolated by sectioning the adrenal, and separately digesting the Capsular/ZG and ZF/ZR cells in DME/F12 medium containing 1 mg/mL collagenase-dispase and 0.25 mg/ml DNase-I (F. Hoffman-La Roche Ltd, Switzerland). Repeated digestion and mechanical dispersion were carried out (4-8 times, as required) for 1h each at 37°C. Cells from each digestion were collated. Cells were plated in 4 wells, at a density of 80,000 cell/well, in a 24-well dish, for testing response to agonists Ang II, K^+ and ACTH. The remaining cells were frozen in freezing medium (50% growth medium, 40% Cosmic Calf Serum and 10% DMSO) in cryovials, and stored at -150 °C, until ready for use.

2.1.2. Cell Treatment

Cells were grown to 75-80% confluence, plated at densities described below, and grown 48 hours in regular growth medium.

Dish-type	Density	Purpose
6-well dish	1 million cells/well	Nuclear protein isolation for westerns
12-well dish	400,000 cells/well	Transient transfections (luciferase assays), western analyses, steroid assays

24-well dish	200,000 cells/well	RNA isolation (qPCR), westerns analyses
48-well dish	25,000 cells/well	RNA isolation (qPCR), westerns analyses, steroid assays

After 48 h, the medium was replaced with 0.1% low serum medium, and incubated overnight. The next day, cells were treated with agonists Ang II (10 nM), K^+ (13.6 mM) or Forskolin (10 μM) and incubated, as required.

2.1.3. Viral transductions

2.1.3.1. Development of the HAC15-TRE-mKCNJ5 cell line

The pLenti-CMV-rtTA3-Hygro vector[240] was used to develop a lentivirus to generate the HAC15-CMV-rtTA3-hygro cell line expressing the reverse tetracycline-controlled transactivator 3 (rtTA3) [240]. These cells were further transduced with a lentivirus expressing KCNJ5 harboring the T158A mutation (mKCNJ5) under the CMV promoter containing a Tet Operator element (TO), to generate the HAC15-TRE-mKCNJ5 cells. Incubation with doxy prevents rtTA3 from physically binding to the TO, thus permitting expression of mKCNJ5. The HAC15-CMV-rtTA3-hygro and HAC15-TRE-mKCNJ5 cells were grown in Dulbecco's Modified Eagles/Ham's F:12 (DME) medium containing 5% Cosmic calf serum (source), 1% ITS (source) and antibiotics (pencillin-streptomycin and gentamicin). HAC15-TRE-mKCNJ5 cells were further selected with 10 μg/mL puromycin

2.1.3.2. Transduction of Primary cells

Primary adrenal cells, were plated towards the end of the day, at a density of 25,000 cells per well (cell lines) or 25,000 cells/well (primary cells) in a 48

well dish, and grown in the respective growth medium, for 24 h. For transduction, the growth medium was aspirated out and replaced with 200 µl of antibiotic free-growth medium containing 10 µg/ml polybrene (Millipore) as well as 50 µl of 'viral solution'. The viral solution consisted of the required volume of the stock viral solution and antibiotic-free, polybrene-free growth medium to make a 50 µl 'viral solution'. For non-transduced controls, cells were incubated in 250 µl of antibiotic-free and polybrene-free growth media. An example of the calculations for transductions can be found below:

	MOI 1	***MOI 3***	***MOI 5***	***MOI 10***
Cells/well	25,000	25,000	25,000	25,000
Conc of the viral stock solution (TU/µL)	5,000	5,000	5,000	5,000
Required viral particles/well (TU)	25,000	75,000	125,000	250,000
Volume of viral stock solution/well (µL)	2.5	7.5	12.5	25
Volume of growth media/well (µL)	47.5	42.5	37.5	25
Total volume of viral solution/well (µL)	50	50	50	50

Plates were wrapped in clingwrap to avoid spillage, and spinnoculated at 1200 rpm for 2 h. After overnight incubation, cells were recovered the following morning by the addition of 500 µl (2X of original transduction volume) of antibiotic-free and polybrene-free growth media. Recovery was carried out for 48 h, after which, the medium was replaced with 0.1% low serum medium (overnight) to arrest cells in the G_0 phase. The next morning, the medium was replaced with fresh 0.1% low serum medium overnight with/without agonists, as required. NT cells treated with Ang II (10 nM, for ZG cells and HAC15 cells) or ACTH (10 nM, for ZF/ZR cells) for 24 h, served as a

positive controls. At the end of the experiment, medium was collected and stored at -20 °C for steroid assays and the plates were store at -80°C for RNA and/or isolation. Generation of the HAC15-TR3G-T158A cell line was carried out by similarly transducing cells in a T-75 flasks and subsequently selecting for transduced cells with 2 µg/mL puromycin.

2.2. RNA extraction and Reverse Transcription (RT) and real-time quantitative RT-PCR (qPCR)

2.2.1. RNA extraction

Total RNA (collation of cytoplasmic and nuclear RNA) was isolated from cells using the RNeasy Mini Plus Kit (Qiagen), following manufacturer's recommendations. Briefly, cells were lysed in RLT buffer, homogenized by repeated pipetting, transferred to the gDNA eliminator column and centrifuged at 10,000 rpm for 30 s. The flow-through was collected, and 1x volume of 70 % Ethanol (prepared from 200-proof absolute ethanol diluted in nuclease free water) was added. After repeated pipetting, the entire volume was transferred to the RNeasy RNA Spin Column, and centrifuged at 10,000 rpm for 30 s. This process traps the RNA in the filter of the Spin Column. Therefore, the RNA column was washed with RW1 (once) and RPE (twice) to purify and precipitate the RNA. Each time, the flow through in the collection tube was discarded. Finally, after a dry spin in a new collection tube, the filters were transferred to sterile microfuge tubes. Nuclease free water (30 µl) was added to the filter, incubated for 2 min, and RNA solution was eluted by centrifuging the tube at 10,000 rpm for 1.5 min.

RNA isolation for transduced cells included an additional step of DNase treatment to ensure minimal detection of the transgene inserted in the cellular genome. For this purpose, the RNA spin column containing the RNA was washed with 350 µL RW1, incubated for 15 min in 80 µL DNase I diluted in RDD buffer (Qiagen, following manufacturer's recommendations), and further washed with RW1 and RPE. This was followed by elution in nuclear free water.

The concentration and purity of the RNA was determined using the ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ratio of absorbance at 260nm and 280nm was used to determine RNA purity. Generally, for RNA, a 260/280 ratio of ~2.0 is generally accepted as 'pure', although a ratio 2.0-2.2 is commonly considered acceptable. Low 260/280 ratios indicate possible contamination by EDTA, ethanol and other organic solvents. Similarly, the ratio of absorbance at 260 nm and 230 nm is a secondary control. RNA was stored at -80C until further use or reverse transcribed immediately.

2.2.2. Reverse Transcription (RT)

Reverse transcription of RNA to generate cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 100ng – 2 µg of RNA, as determined by the nanodrop, was collected in 0.2 ml PCR tube. The volume was brought up to 25 µl with nuclease free water, and an equal volume of '2X-RT master mix' was added to each tube. The 2X-RT master mix was prepared by addition of manufacturer provided 10X random primers, 10X RT buffer, 25X dNTPs, 20X

Multiscribe reverse transcriptase and nuclease-free water. This total RNA solution of 50 µl was subjected to the RT reaction in an Eppendorf Mastercycler Gradient System (Eppendorf) using the program below:

	Step 1	Step 2	Step 3	Step 4
	25°C	37°C	85°C	4°C
Temperature Time	10 min	120 min	5 sec	Hold

2.2.3. Real time quantitative PCR (qPCR)

Real-time qPCR was performed using the Step One Plus PCR System (Life Technologies). In-house, gene sequence-specific primers and a fluorescence-labeled Taqman® probe have been previously designed in the Rainey laboratory and are custom ordered from Integrated DNA Technology Co. (Coralville, IA). The probe is designed to recognize the region between the primer spanning sequences consists of a 5'FAM dye and 3'Black Hole Quencher 3 (BHQ3). Stock solutions of the primers and probes were prepared. A 20X mix of the primer-probes were prepared by adding 180 µL each of the forward and reverse primer stock solutions, 50 µL of the probe, and 590 µL of NFW. The detailed sequences for in-house primer-probes are given below:

Gene	***Enzyme***	***Sequence (5' → 3')***
CYP11B2	Aldosterone synthase	Forward: GGCAGAGGCAGAGATGCTG Reverse: CTTGAGTTAGTGTCTCCACCAGGA Probe: CTGCACCACGTGCTGAAGCACT

Gene	*Enzyme*	*Sequence (5' → 3')*
CYP11B1	11β-hydroxylase	Forward: GGCAGAGGCAGAGATGCTG Reverse: TCTTGGGTTAGTGTCTCCACCTG Probe: TGCTGCACCATGTGCTGAAACACCT

Primer-probe mixes for the endogenous control cyclophillin A (PPIA), KCNJ5, SF-1 (NR5A1) and NR4A2 were purchased from Life technologies. All primer-probes used were designed to cross from inter-exon boundaries, to minimize detection of genomic DNA. A qPCR reaction consisted of 5 µL of cDNA, 10 µL TaqMan Fast Universal PCR Master Mix (2X; Applied Biosystems) or Kapa Probe fast qPCR kit master mix ABI Prism (Kapa Biosystems, Boston MA), 1 µL of the 20X primer probe mix (a total of 900nM of each primer, and 400nM probe, per reaction) in a total volume of 20 µL in a MicroAmp® Fast 96-well plate. Negative controls consisted of nuclease-free water in place of cDNA. The PCR program was set up as follows, and the last two stages were performed for 40 cycles:

Stage Name	**Temperature**	**Time**
Activation	95°	0.2 s
Melt	95 °	3 s
Anneal/Extend	60 °	30 s

Normalization of gene expression within each sample was performed by using the respective expression levels of endogenous PPIA (cyclophilin A) to calculate the ΔCt. Change in gene expression (ΔΔCt) was calculated with

respect to the respective basal ΔCt for that experiment. The relative fold change in gene expression was obtained using the equation $2^{-\Delta\Delta Ct}$.

2.3. Protein isolation and estimation

2.3.1. Total protein isolation

Total protein from each well was isolated by lysing cells in 50- 100 µL of MPER® Mammalian Lysis buffer (Thermo Pierce) by repeated pipetting. The protein lysate was further homogenized using a sonicator (Eppendorf) for 10 s. The shaft of the sonicator was rinsed in 70 % Ethanol, three times after each sample. Protein content was estimated using the Micro BCA Kit (Thermo Pierce). Protein samples were stored in the -80°C freezer until further use.

2.3.2. Nuclear protein isolation

HAC15-TRE-mKCNJ5 cells were plated at a density of $1x10^6$cells/well in a 6-well dish and incubated for 48 h. After serum starvation in 0.1 % cosmic calf serum, cells were treated with fresh low serum medium with/without 1 µg/ml doxycycline for indicated times.

After termination of the treatments, cells were immediately washed twice with PBS containing protease and phosphatase inhibitors (Thermo Scientific). Nuclear protein was isolated using the NE-PER™ Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific). Briefly, cells were gently scraped in 500 µl of chilled PBS containing 0.1M phosphate, 0.15M chloride (pH 7.2) and 1X Halt protease and phosphatase inhibitors (Thermo Scientific), and collected in a 1.7 ml microfuge tube. Cells were pelleted by centrifuging at 500 rpm for 5 min. After two such washes, the supernatant was discarded. The

cell pellet was re-suspended in 200 µl of ice cold CER I solution, vortexed vigorously for 15 s at the highest speed setting and incubated on ice for 10 min. To this solution, an additional 11 µl of ice-cold CER II was added. The tube was vortexed for 5 s at the highest speed setting, and centrifuged for 5 min at 16000 rpm. The supernatant containing the cytoplasmic extract was stored in a microfuge tube, while the pellet was processed for nuclear protein. The insoluble pellet fraction was suspended in 100 µL of chilled NER solution and vigorously vortexed at the high speed for 15 s. The tube was then transferred on ice, and vortexed for 15 s, every 10 min, for a total of 40 min. Nuclear protein was finally isolated by collecting the supernatant after centrifuging the NER fraction for 10 min at 16,000 rpm. Protein was estimated in both cytoplasmic and nuclear protein fractions using the Micro BCA Kit (Thermo Pierce). 20 µg aliquots of the protein were stored in the -80°C freezer until used for western analyses of transcription factors.

2.4. Western analyses for protein

Protein (20 µg/sample) was aliquoted in a microfuge tube and mixed with NuPage LDS sample buffer, NuPage Sample reducing agent, and MPER® Mammalian Lysis buffer (Thermo Pierce). Samples were boiled at 95°C, chilled on ice and separated on a 10% BisTris gel by gel electrophoresis (200 V, 50 min) using the XCell SureLock system (Life Technologies). Protein bands were then transferred to an activated PVDF membrane (1.5 h, 40 V). After transfer, the membrane was blocked for 1h at room temperature with either 5 % BSA/non-fat dry milk in 1X Tris Borate Saline-0.1% Tween (TBST). The membrane was then incubated with indicated primary and HRP-conjugated or fluorescent secondary antibodies. Membranes incubated with

fluorescent secondary antibody were washed and bands were visualized on the Licor fluorescent imager. For membranes incubated with HRP-conjugated secondary antibodies, the ECL Kit (Thermo Pierce) was used for signal development. Endogenous β-actin (Sigma Aldrich), GAPDH (Thermo Pierce) or Lamin (Santa Cruz) was used for normalization, as indicated.

CHAPTER 3

Manuscripts

SECTION I.

A NOVEL Y152C KCNJ5 MUTATION RESPONSIBLE FOR FAMILIAL HYPERALDOSTERONISM TYPE III

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ABSTRACT

Context: Primary aldosteronism is a heterogeneous group of disorders comprising both sporadic and familial forms. Mutations in the *KCNJ5* gene, which encodes the inward rectifier K+ channel 4 (GIRK4, Kir3.4), cause familial hyperaldosteronism type III (FH-III) and are involved in the pathogenesis of sporadic aldosterone-producing adenomas (APA).

Objective: To characterize the effects of a newly described *KCNJ5* mutation *in vitro*.

Patients and Methods: The index case is a 62 year old female affected by primary aldosteronism, who underwent left adrenalectomy after workup for adrenal adenoma. Exon 1 of *KCNJ5* was PCR amplified from adrenal tissue and peripheral blood and sequenced. Electrophysiological and gene expression studies were performed to establish the functional effects of the new mutation on the membrane potential and adrenal cell *CYP11B2* expression.

Results: *KCNJ5* sequencing in the index case revealed a new p.Y152C germline mutation; interestingly, the phenotype of the patient was milder than most of the previously described FH-III families. The tyrosine to cysteine substitution resulted in pathological Na^+ permeability, cell membrane depolarization and disturbed intracellular Ca^{2+} homeostasis, effects similar, albeit smaller, to the ones demonstrated for other *KCNJ5* mutations. Gene expression studies revealed an increased expression of *CYP11B2* and its transcriptional regulator *NR4A2* in HAC15 adrenal cells overexpressing KCNJ5-Y152C compared to the wildtype channel. The effect was

clearly calcium-dependent, since it was abolished by the calcium channel blocker nifedipine.

Conclusions: Herein we describe a new germline mutation in *KCNJ5* responsible for FH-III.

Key words: KCNJ5 mutations, primary aldosteronism (PA), familial hyperaldosteronism (FH-III)

INTRODUCTION

Primary aldosteronism (PA) is a heterogeneous group of disorders comprising both sporadic [bilateral adrenal hyperplasia (BAH) and aldosterone-producing adenoma (APA)] and familial forms. Three forms of familial hyperaldosteronism have been reported thus far, named familial hyperaldsteronism types I, II and III (FH-I to FH-III) [241]. The first family affected by FH-III was described as an early-onset and particularly severe form of PA, with profound hypokalaemia, resistant hypertension and marked bilateral adrenal hyperplasia [242]. Subsequently, the molecular basis of FH-III was idientified as a germline mutation (p.T158A) in the *KCNJ5* gene encoding the G-protein-activated inward rectifier K· channel 4 (GIRK4). Adrenal zona glomerulosa cells display high resting K^+ conductance, responsible for cell membrane hyperpolarization. Electrophysiological studies demonstrated that the p.T158A KCNJ5 mutation, located near the selectivity filter of the channel, results in loss of ion selectivity and sodium entry, thus leading to membrane depolarization [5]. Following these original findings, an additional six families and three germline *KCNJ5* mutations (p.G151R, p.G151E, p.I157S) were reported [8, 11, 243]. Further in vitro studies established that the over-expression of *KCNJ5* mutations in HAC15 adrenocortical cells resulted in Na_+ and Ca_{2+} influx, increased transcription of *CYP11B2* and of the associated regulatory factors, *NR4A2* and *NR4A3*, thus increasing aldosterone production [13, 230].

Interestingly, mutations in the *KCNJ5* gene have also been implicated in the pathogenesis of around 40% of sporadic APA [241]. Herein we describe a patient with a mild form of primary aldosteronism, caused by a newly described germline point

mutation (p.Y152C) in the *KCNJ5* gene and report the functional characterization of the mutation *in vitro*.

MATERIALS AND METHODS

Detailed materials and methods are provided in the Supplementary File.

RESULTS

Phenotype of the index case

The index case is a 62 year old African-American female. She was first diagnosed with hypertension at the age of 48, when she presented for nausea and headache at the Georgia Regents University Emergency Department. She displayed resistant hypertension (180/110 mmHg) on four antihypertensive medications and severe hypokalemia (2.1 mEq/L) despite potassium supplementation. In 2008, she again presented to the Emergency Department for nausea, vomiting and headache and elevated blood pressure (250/130 mmHg). Abdominal CT scanning showed nodularity in the left adrenal gland (several subcentimeter nodules, the largest being 8 mm). Hormonal testing under treatment with ACE-I revealed plasma aldosterone 24 ng/dL and suppressed PRA (<0.6 ng/mL/h). Catecholamine levels and dexamethasone suppression test were normal. The patient underwent AVS, but the right adrenal vein was not cannulated successfully; left adrenalectomy was performed on the basis of CT findings and difficult to control blood pressure. Post-operatively, plasma aldosterone levels had decreased, but PRA remained suppressed. Fourteen months after surgery (May 2010), plasma aldosterone increased and PRA remained suppressed. She was not treated with mineralocorticoid receptor antagonists because of reduced renal

function that progressed to end-stage renal disease. All ten siblings and five sons were hypertensive.

Identification of a new KCNJ5 mutation

A 610bp of the exon 1 of the *KCNJ5* gene was PCR amplified and sequenced. In both adrenal nodule and adjacent tissue and, peripheral blood the heterozygous point mutation c.455A>G was identified, corresponding to a tyrosine (Y) to cysteine (C) substitution at amino acid position 152 (Figure 3.1.1A). We also searched for the p.Y152C mutation by SNP assays on genomic DNA extracted from patients (n=100) affected by BAH; we could not identify the 455A>G substitution in any of the analyzed samples.

Immunohistochemistry and microarray analyses of adrenal tissue

Immunohistochemical staining of the adrenal tissue showed nodular structures with diffused KCNJ5 expression and variable CYP11B2 expression. The adjacent cortex showed a variable degree of glomerulosa hyperplasia (Fig 3.1.S1).

Microaarray analysis for steroidogenic enzymes in the adrenal tissue harboring the Y152C mutation showed 1.8-fold higher *CYP11B2* levels than normal adrenals. Along with *CYP11B2*, transcription factors *NR4A2* and *NR4A3* were also up-regulated (Fig 3.1.S2).

Gene expression studies on HAC15 cells

To investigate the effects of the KCNJ5-Y152C mutation in adrenal cell function HAC15 cells were transfected with pcDNA3.1/KCNJ5-Y152C. pcDNA3.1/KCNJ5^{WT} and pcDNA3.1 empty vector and gene expression was analyzed by real-time PCR.

We observed a significant up-regulation of *CYP11B2* (4-fold) and its transcription factor *NR4A2* (5.4-fold) in cells overexpressing KCNJ5-Y152C compared *to* $KCNJ5^{WT}$ and cells transfected with empty vector (Figure 3.1.1B). No statistically significant difference was observed between HAC15 cells overexpressing $KCNJ5^{WT}$ and pcDNA3.1 mock-transfected cells for either of the two selected genes. In cells transfected with the mutant KCNJ5, treatment with nifedipine (10 µmol/L) decreased *CYP11B2* and *NR4A2* over-expression by 90% and 57%, respectively (Fig 3.1.B).

Biophysical properties of KCNJ5-Y152C

HEK293 cells were transiently co-transfected with mutant or wildtype KCNJ5 and KCNJ3. At high extracellular K^+ (50 mM), wildtype transfected cells displayed a large inward (negative) current that was absent after replacement of 45 mM extracellular K^+ with Na^+ (con; 5 mM K+) (Fig. 3.1.2A). Replacement of extracellular Na^+ by the larger cation NMDG (Na+-free) had no further effect. Cells expressing KCNJ5-Y152C /KCNJ3 also displayed a reduced inward current after replacement of extracellular K^+ by Na^+ (Fig 3.1.2B). However, replacement of extracellular Na^+ by NMDG (Na^+-free) reduced the inward current further, consistent with a pathological Na^+ permeability of the mutated channel (Fig 3.1.2B and D). Mock-transfected cells showed a significant albeit smaller Na+ conductance (Fig 3.1.2E). The membrane voltage of cells expressing KCNJ5-Y152C /KCNJ3 was depolarized due to the pathological Na^+ influx through the mutated channel (-37 mV in KCNJ5-Y152C /KCNJ3 vs. -74 mV in $KCNJ5^{WT}$/KCNJ3 cells). Accordingly, cells expressing the mutated channel were hyperpolarized when bath Na+ was replaced by NMDG (Fig. 3.1.2F and G).

The current blocked by the K+ channel blocker Ba^{2+} (Ba^{2+} sensitive current) displayed the expected rectification of the inward current in wildtype KCNJ5/KCNJ3 expressing cells. In KCNJ5-Y152C /KCNJ3 cells, the Ba^{2+}-sensitive current was very small and similar to that of mock-transfected cells (Fig 3.1.S3A). To augment the inwardly rectifying current, the extracellular K^+ was increased from 5 to 50 mM. Under these conditions, KCNJ5-Y152C /KCNJ3-expressing cells showed a slightly increased Ba^{2+}-sensitive current compared to mock-transfected cells suggesting that the KCNJ5-Y152C mutant is at least partially Ba2+-sensitive (3.1.S3B) in contrast to KCNJ5-G151R and KCNJ5-G151E that are Ba^{2+} insensitive [8].

Disturbed intracellular Ca^{2+} homeostasis

The effect of the KCNJ5-Y152C mutant on the Fura-2 ratio, a measure of intracellular Ca^{2+} concentration, was studied in transfected HEK293 cells. Under control conditions (extracellular Ca^{2+} 1.8 mM), the Fura-2 ratio was higher in KCNJ5-Y152C /KCNJ3 expressing cells compared with wildtype KCNJ5/KCNJ3 cells but lower than cells expressing the mutant $KCNJ5^{G151E}$/KCNJ3 [11]. The highest increases in cytosolic Ca2+ were observed at 5 mM extracellular Ca^{2+} where clear differences were evident between cells expressing the mutated KCNJ5 and KCNJ5WT (Fig. 3.1.2H). This is illustrated by the initial rate of change of the Fura-2 ratio at 5 mM extracellular Ca^{2+} which shows a strong increase in cytosolic Ca2+ induced by 5 mM extracellular Ca^{2+} in cells expressing both KCNJ5 mutants compared to the $KCNJ5^{WT}$ (Fig. 3.1.2I). To further investigate the nature of the increased intracellular Ca^{2+} levels, Ca^{2+} was completely removed from the bath solution and replaced by EGTA (Fig 3.1.S4). Under these conditions, intracellular Ca^{2+} levels of cells expressing KCNJ3/KCNJ5-Y152C channels

strongly decreased. In cells expressing KCNJ5WT and in non-transfected cells, removal of bath Ca^{2+} had no such effect. Next, the purinergic agonist ATP (100 µmol/l) was added to the bath under Ca^{2+}-free conditions to stimulate Ca^{2+} release from IP3-sensitive intracellular stores. This store-release of Ca^{2+} was not modified by the expression of the mutated channels. These data indicate that the increased intracellular Ca^{2+} of cells expressing KCNJ3/KCNJ5-Y152C channels is not caused by increased release of Ca^{2+} from IP3-sensitive stores but reflects pathological transport of Ca^{2+} across the plasma membrane.

DISCUSSION

Mutations in the *KCNJ5* gene have been implicated in the pathogenesis of both sporadic APA and FH-III. Seven FH-III families with four different *KCNJ5* mutations have been reported so far: with the exception of those carrying the p.G151E mutation, affected members displayed severe hyperaldosteronism and uncontrolled hypertension from childhood, thus requiring bilateral adrenalectomy. By contrast, G151E affected patients have a milder phenotype, with normal appearing adrenals, treatable with medical therapy [5, 11].

In this study, we describe a patient affected by PA due to a novel germline mutation in *KCNJ5*. The index case presented with resistant hypertension and hypokalemia. She underwent unilateral adrenalectomy on the basis of adrenal CT findings. *KCNJ5* sequencing revealed a point c.455A>G mutation, leading to the Tyr152Cys (Y152C) substitution. Tyrosine 152 belongs to the GlyTyrGly motif of the K+ selectivity filter of the channel, and is highly conserved among orthologs and paralogs.

As for p.G151E [11], the p.Y152C mutation was associated with a milder phenotype in terms of both clinical and biochemical parameters compared to other FH-III families. In particular, the patient displayed minimal changes at adrenal CT scanning, complete cortisol suppression after dexamethasone administration and aldosterone was produced at a relatively lower rate. However, electrophysiological studies showed that KCNJ5G151E channels were associated with a much larger Na^+ conductance compared to other mutated channels and consequent Na^+-dependent cell lethality. Therefore, both the G151E mutation with a severe impact on the channel functions and the Y152C mutation with a little effect on sodium Na^+ conductance, are associated with a mild clinical phenotype.

The index case was diagnosed with PA at the age of 48, whilst most FH-III patients were diagnosed before 7 years of age. However, on the basis of the severe target organ damage, with left ventricular hypertrophy and renal function impairment, we can speculate that the patient had been hypertensive for a considerable time before diagnosis.

The electrophysiological consequences of the p.Y152C mutation were similar to those described for other *KCNJ5* mutations. The overexpression of the mutant channel resulted in cell membrane depolarization, due to a pathological Na+ permeability (albeit smaller than the one observed in other *KCNJ5* mutations) [8, 11]. Under physiological conditions, adrenal zona glomerulosa cell membrane depolarization results in voltage-gated Ca^{2+} channel opening which in turn leads to *CYP11B2* transcription and aldosterone production.

In agreement with these data, we observed an impairment of cytosolic Ca^{2+} handling of KCNJ5-Y152C expressing cells (increased intracellular Ca^{2+} activity and a disturbed capacity to lower intracellular Ca^{2+}) and at least three mechanisms could underlie this phenotype: i) activation of voltage-gated Ca^{2+} channels via the depolarization induced by the Na+ influx through KCNJ5-Y152C ; ii) impairment of Ca^{2+} extrusion via Na^{+}/Ca^{2+} exchange mechanisms because intracellular Na+ is increased and the membrane is depolarized and iii) direct influx of Ca^{2+} through mutant channels (however this has not yet been shown).

In addition, we showed that overexpressing KCNJ5-Y152C in adrenocortical cells resulted in a significant up-regulation of *CYP11B2* and its transcription factor *NR4A2,* thus driving aldosterone production.

In conclusion, we report a new germline *KCNJ5* mutation responsible for a milder and late onset form of FH-III. This further broadens the spectrum of the phenotypic presentation of FH-III patients and highlights the importance of considering this disease in apparently late onset familial PA.

Acknowledgements

We are grateful to Prof. Celso Gomez-Sanchez (University of Jackson, Mississippi) for providing the antibodies for CYP11B2.

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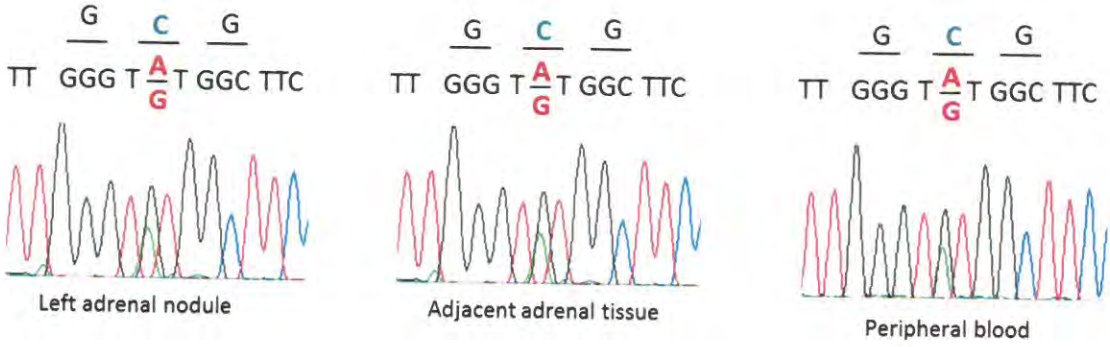
FIGURES

Figure 3.1.1 (A) Sequences of tumor cDNA, adjacent adrenal tissue and peripheral blood genomic DNA of *KCNJ5* codons 151-153 showing the c.455A>G substitution resulting in the p.Y152C mutation. **(B)** Real-time PCR analysis of *CYP11B2* and *NR4A2* in HAC15 cells overexpressing KCNJ5-Y152C compared with cells overexpressing $KCNJ5^{WT}$ and pcDNA3.1 empty vector. Each bar represents the mean ± SE of relative fold change of gene expression in three independent experiments. Each assay was performed in triplicate, and GAPDH was used as endogenous control. *, P <0.05 compared with WT.

(A)



(B)

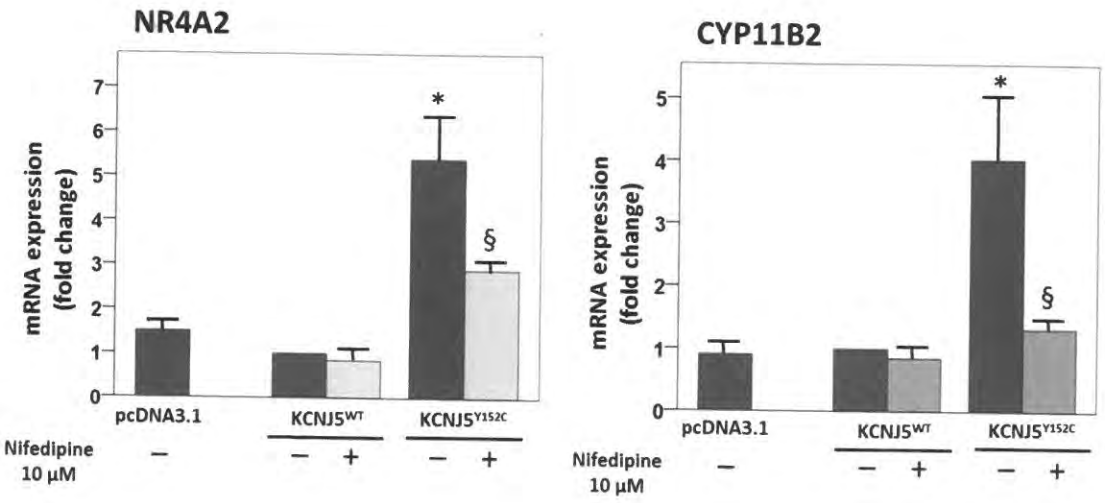
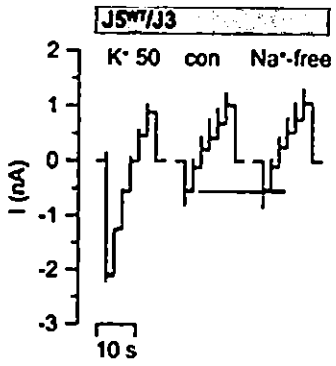
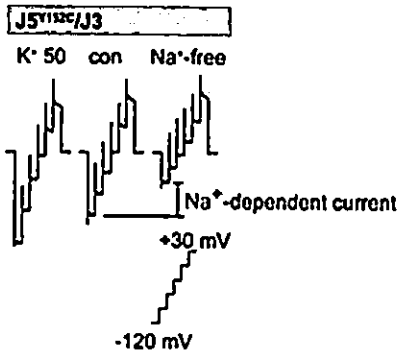


Figure 3.1.2. Basic characteristics of the KCNJ5-Y152C mutant channel. **(A)** and **(B)**: Representative current traces of a wildtype KCNJ5/KCNJ3 (J5WT/J3; left panel) and of a mutant KCNJ5-Y152C /KCNJ3 (J5Y152C/J3; right panel) expressing HEK 293 cell are shown. Current traces were recorded at 50 mM extracellular K^+ (K+ 50), 5 mM extracellular K^+ (con), and after replacement of extracellular Na+ by NMDG (Na+-free). **(C-E):** I/V curves of similar whole cell experiments as shown in (A) and (B). **(F)** Effect of Na+ replacement on the membrane voltage. Asterisks indicate significant differences compared to control conditions. **(G)** Na+-dependent conductance (calculated from the conductance between -120 and -90 mV from the data shown in (C-E)) was highest in KCNJ5-Y152C /KCNJ3 cells. **(H)** Effects of extracellular Ca^{2+} concentration on cytosolic Ca^{2+} concentration. (Fura-2 fluorescence). Mean values of the Fura-2 ratio (340nm/380nm) ± SEM in cells expressing KCNJ5WT/KCNJ3 (WT), KCNJ5G151E/KCNJ3 (G151E), and KCNJ5-Y152C /KCNJ3 (Y152C) at various extracellular Ca^{2+} concentrations. Numbers of experiments are shown in parenthesis. **(I)** Initial rate of Fura-2 ratio changes induced by 5 mM extracellular Ca^{2+}. The period labeled with red dots in (H) was used for the linear fitting and values were normalized to that of the wildtype. Values are mean values ± SEM. Asterisks indicate significant differences compared to wildtype channels expressing cells.

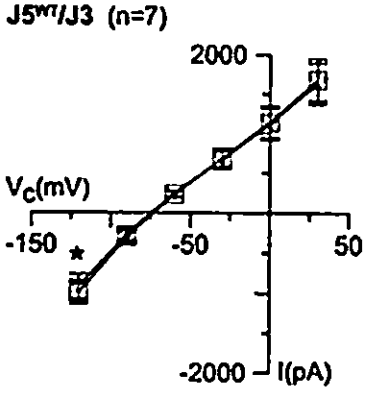
(A)


J5WT/J3
K+ 50
con
Na+-free
2
1
0
-1
-2
-3
I (nA)
10 s

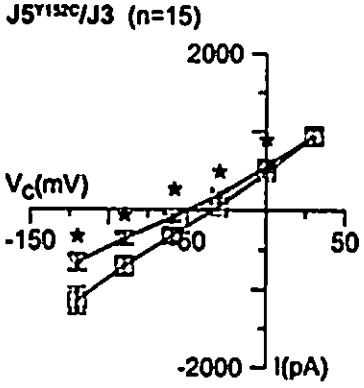
(B)


J5Y152C/J3
K+ 50
con
Na+-free
Na+-dependent current
+30 mV
-120 mV

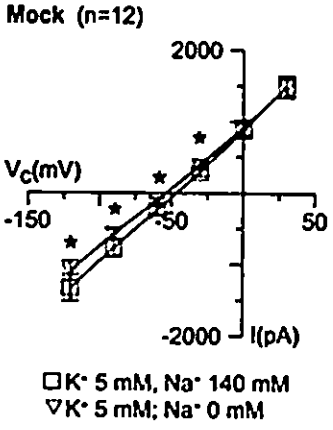
(C)


J5WT/J3 (n=7)
2000
Vc(mV)
-150
-50
50
-2000
I(pA)

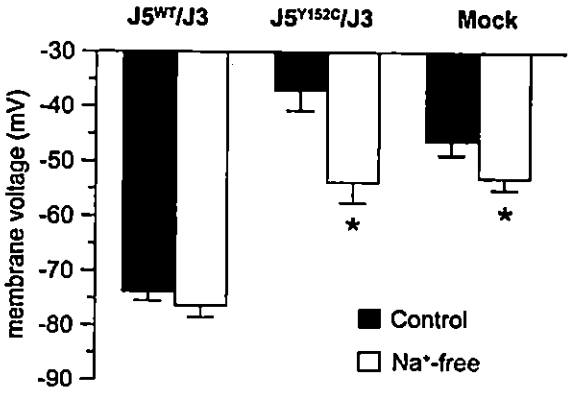
(D)


J5Y152C/J3 (n=15)
2000
Vc(mV)
-150
-50
50
-2000
I(pA)

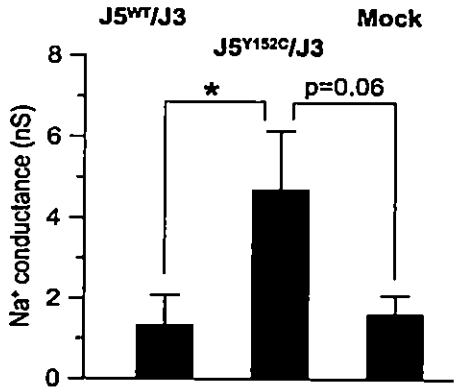
(E)


Mock (n=12)
2000
Vc(mV)
-150
-50
50
-2000
I(pA)
K+ 5 mM, Na+ 140 mM
K+ 5 mM; Na+ 0 mM

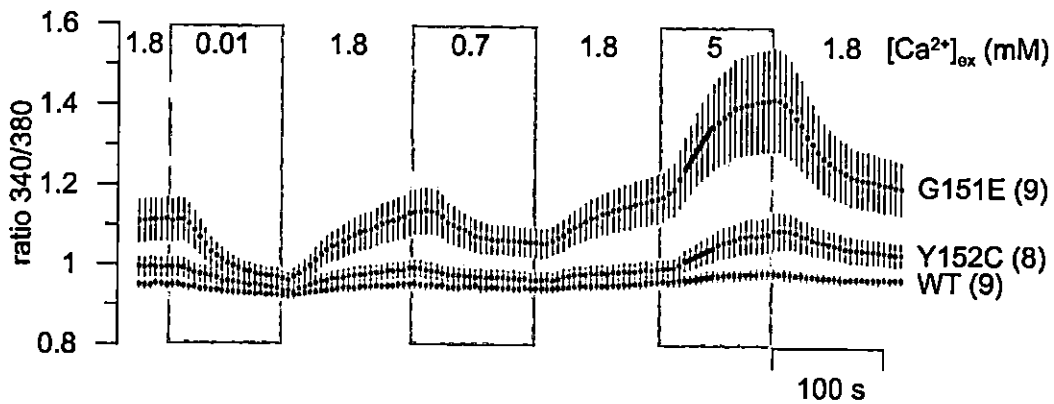
(F)


J5WT/J3
J5Y152C/J3
Mock
-30
-40
-50
-60
-70
-80
-90
membrane voltage (mV)
Control
Na+-free

(G)


J5WT/J3
Mock
J5Y152C/J3
*
p=0.06
Na+ conductance (nS)
8
6
4
2
0

(H)


1.6
1.4
1.2
1
0.8
ratio 340/380
1.8
0.01
1.8
0.7
1.8
5
1.8
[Ca2+]ex (mM)
G151E (9)
Y152C (8)
WT (9)
100 s

(I)

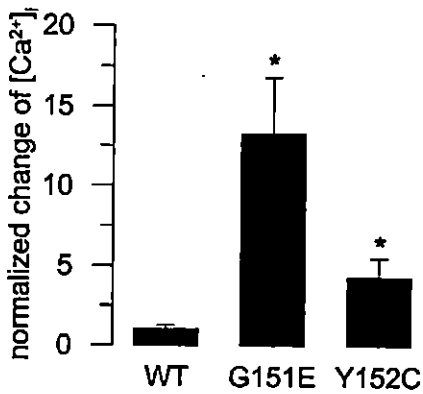

normalized change of [Ca2+]i
20
15
10
5
0
*
*
WT
G151E
Y152C

Figure 3.1.S1 Sections of adrenal tissue harboring the Y152C mutation stained with hematoxylin and eosin (H&E) (A,D) and immunostained using antibodies against human aldosterone synthase (CYP11B2) (B,E) and human Kir 3.4 (KCNJ5) (C,F).

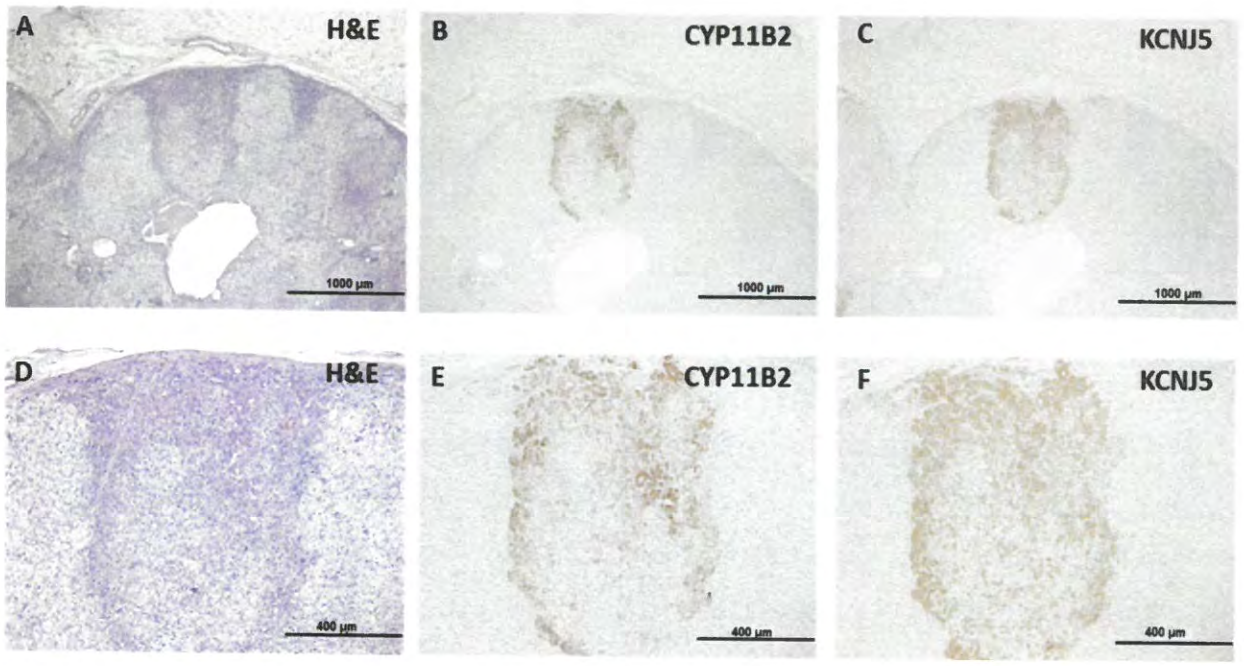


Figure 3.1.S2. Heatmap comparing steroidogenic enzymes in adrenal tissue harboring the Y152C mutation in the KCNJ5 gene versus normal adrenal tissues obtained from patients of renal cancer or from cadaver donors. Heatmap colors are based on log_2signal intensity. Absolute fold change (FC) is also provided.

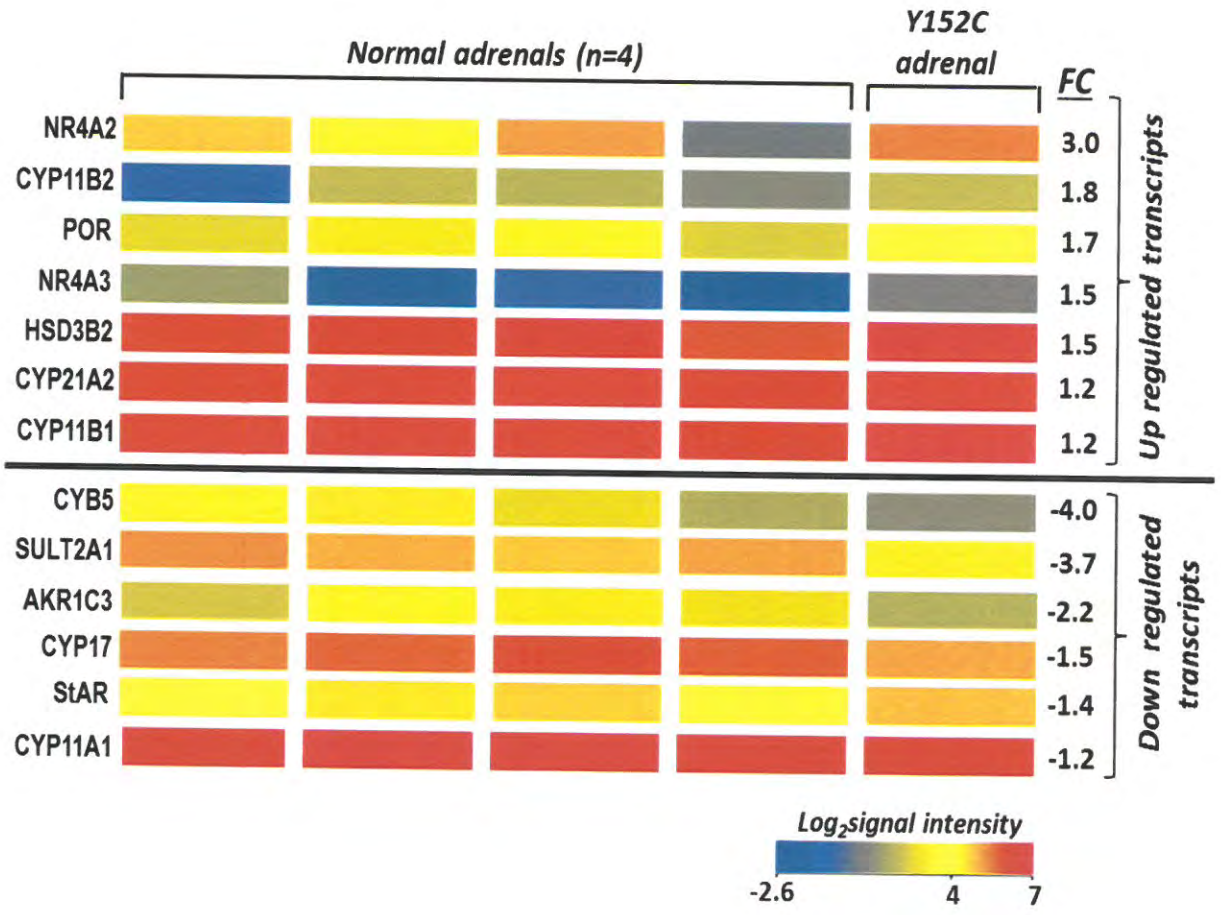


Figure 3.1.S3. Ba^{2+}-sensitive currents as a measure of the KCNJ5/KCNJ3-induced current in HEK cells. (A) Ba^{2+}-sensitive currents at low extracellular K^+. (B) Ba^{2+}-sensitive currents at high extracellular K^+. Values are mean values ± SEM.

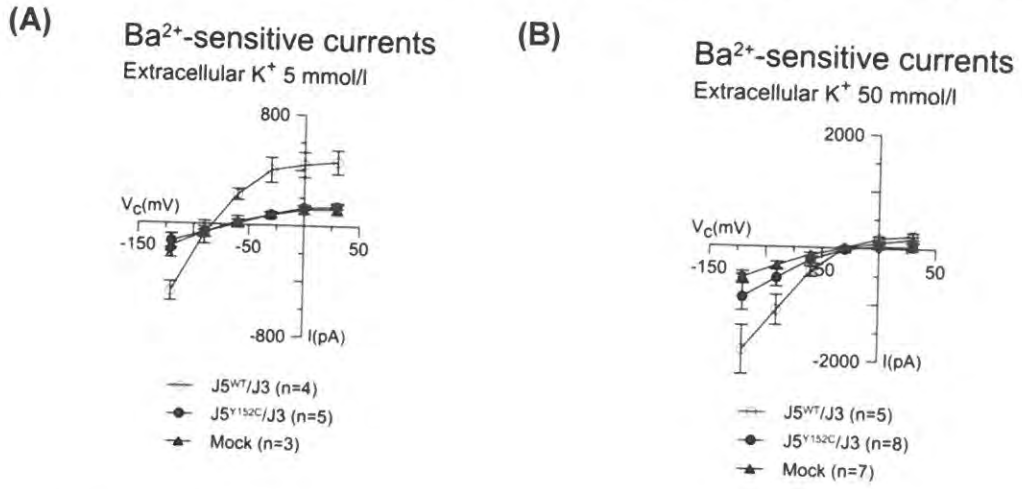
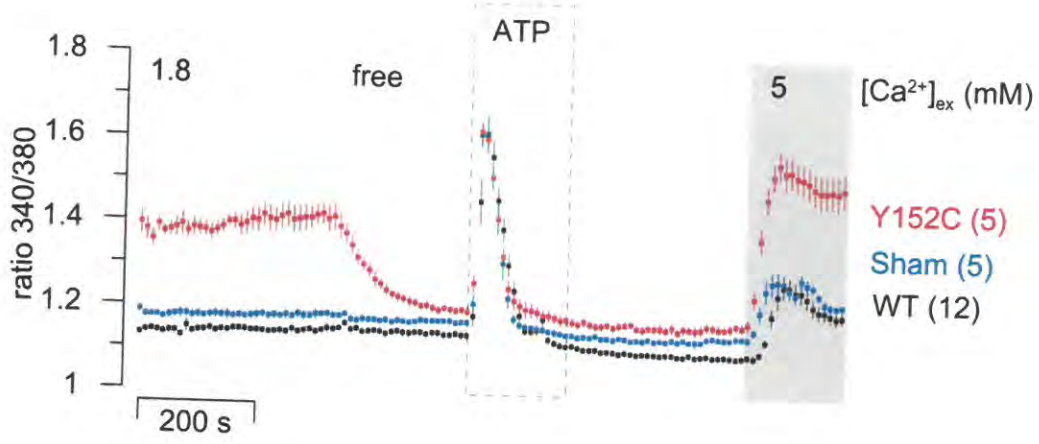


Figure 3.1.S4. ATP-induced release of Ca^{2+} from intracellular stores under Ca^{2+}-free conditions. HEK293 cell were co-transfected with KCNJ3/KCNJ5-Y152C (Y152C) or KCNJ3/KCNJ5WT (WT) or non-transfected cells (sham). After removal of bath Ca^{2+} (free), ATP (100 µmol/l) was added to stimulate purinergic receptors and induce release of Ca^{2+} from IP_3-sensitive intracellular stores. The amounts of released Ca^{2+} were similar in all three types of cells. Values are mean values ± SEM, number of cells in brackets.



SUPPLEMENTAL FILE

Materials and methods

Patient selection and tissues

Patients with BAH were recruited at the Division of Internal Medicine and Hypertension, University of Torino, Italy, as previously described[244]. Briefly, patients were screened using the serum aldosterone:plasma renin activity (PRA) ratio (ARR) and PA was confirmed using the intravenous saline loading test. All patients underwent adrenal CT scanning and AVS. The diagnosis of BAH was performed when the aldosterone:cortisol ratio from one adrenal was < 4 times that of the other adrenal gland [245].

In the index case carrying the p.Y152C KCNJ5 mutation, the diagnosis of APA was performed on the basis of an adrenal CT finding of a left adrenal nodule. AVS was performed, but right adrenal vein cannulation was not successful. First degree relatives of the index case were not available for genotyping.

Normal human adult adrenals were obtained from patients undergoing laparoscopic nephrectomy for localized renal carcinomas (in all cases histological examination excluded the involvement of the adrenal in the tumor lesion) and from human cadaver donors through the Cooperative Human Tissue Network (Philadelphia, PA) and Clontech (Palo Alto, CA).

KCNJ5 sequencing

KCNJ5 cDNA from APA and adjacent adrenal tissue was PCR amplified using intron spanning primers as described elsewhere [230].

SNP genotyping.

SNP genotyping was performed on genomic DNA extracted from 100 BAH patients according to the manufacturer's instructions (Applied Biosystem, Foster City, CA). Briefly, each reaction was performed in a total volume of 25 µL containing 12.5 µL of TaqMan Universal PCR Master Mix, 0.625 µL of SNP Genotyping Assay Mix, 5µL of gDNA (20-25 ng) and 6.875 µL of nuclease-free water.

Cell culture and transfection.

HAC15 human adrenocortical carcinoma cells were cultured in Dulbecco's Modified Eagle/F12 medium (Invitrogen, Carlsband, CA) supplemented with 10% Cosmic Calf Serum (HyClone, Logan, UT), 1% insulin/transferrin/selenium Premix (BD Biosciences, Sparks, MD) and antibiotics.

HAC15 cells were electroporated using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany) in 100 µL of nucleofector solution R. After electroporation, cells were plated in 6-well plates with 5 mL of growth medium/well and allowed to recover for 24 h, then starved overnight in 0.1% low serum media (0.1% Cosmic calf serum and antibiotics).

For incubations in the presence of nifedipine, nucleofected cells were plated directly in 5-mL complete medium supplemented with nifedipine (10µmol/L; Sigma-Aldrich) added from 50 mg/mL of stock solutions in dimethyl sulfoxide. This resulted in a final concentration of 0.007% dimethyl sulfoxide that was maintained for all of the cell incubations.

Immunohistochemistry

Sections of 10 µm thickness from formalin-fixed, paraffin-embedded adrenal tissue from the patient harboring the Y152C germline KCNJ5 mutation was quenched for endogenous peroxidase using H_2O_2 and then incubated with antibodies against human KCNJ5 (Sigma HPA071353, diluted 1:500) or human CYP11B2 (a generous gift from Dr. Celso Gomez Sanchez, diluted 1:100) for 1 h. After a short rinse, the EnVision reagent (Dako, Carpinteria, CA) coupled with peroxidase-labeled polymer was incubated as secondary antibody for 30 min. The proteins were visualized using 3,3'-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, and mounted.

RNA extraction and gene expression assay

Adrenal tissue was homogenized in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) with 700 µL of RLT buffer (Qiagen, Hilden, Germany) with 10% β-mercaptoethanol. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. For cDNA generation, 2 µg total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. qPCR was performed in triplicate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for KCNJ5, nuclear receptor subfamily 4, group A, member 2 (NR4A2) and human aldosterone synthase (CYP11B2). Gene expression

levels were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method, using GAPDH as endogenous control.

Microarray analysis

RNA samples used for microarray experiments were assayed for purity and quality using an Experion Automated Electrophoresis System (Bio Rad, Life Sciences Group, Hercules, CA). RNA samples were hybridized to an Illumina bead chip containing more than 48,000 probes representing over 25,000 human genes (Illumina, San Diego, CA). The arrays were scanned at high resolution on the iScan system (Illumina) at the GRU core facility. Results were analyzed using GeneSpring GX (version 11.5) software (Silicon Genetics, Redwood City, CA). Fold changes for genes differentially regulated in the KCNJ5 mutation-bearing adrenal tissue versus the 4 control adrenal samples were calculated, and used to generate a heat map based on $\log_2$ signal intensity.

Electrophysiological characterization of the KCNJ5-Y152C mutant

Human KCNJ3 and KCNJ5 cDNAs were purchased from Invitrogen/Geneart. The mutation c.455A>G of KCNJ5 (resulting in the mutant protein KCNJ5-Y152C) was generated by site-directed mutagenesis. For electrophysiological measurements, KCNJ3 cDNA was subcloned into pIRES2-AcGFP (Clontech), whereas KCNJ5 and KCNJ5-Y152C were subcloned into pIRES2-DsRed (Clontech) expression vectors. For functional studies, 50% confluent HEK 293 cells were co-transfected with wildtype KCNJ3 and wildtype or mutated KCNJ5 using Lipofectamine (0.5 µg of each plasmid per 35 mm dish). For patch-clamp experiments, only cells with both green (KCNJ3 expressing) and red (wildtype KCNJ5 or KCNJ5-Y152C expressing) fluorescence were used. Patch-clamp recordings were performed using an EPC-10 amplifier without leak

subtraction (HEKA, Germany). The following solutions were used (all concentrations in mM): control solution: pH 7.4; 10 HEPES; 140 NaCl; 5 KCl; 1.8 $MgCl_2$; 1.8 $CaCl_2$. High K^+ solution: pH 7.4; 10 HEPES; 95 NaCl; 50 KCl; 1.8 $MgCl_2$; 1.8 $CaCl_2$. Na+-free solution: pH 7.4; 10 HEPES; 5 KCl; 1.8 $MgCl_2$; 1.8 CaCl2; 140 N-methyl-D-glucamine chloride ($NMDG^+$). Pipette solution: pH 7.4; 5 HEPES; 140 KCl; 4 $MgCl_2$; 1 $CaCl_2$; 1 EGTA. Ba^{2+}-sensitive currents were calculated by subtracting the currents in the absence and presence of Ba^{2+} (5 mM).

Ca^{2+} measurements

For cytoplasmic Ca^{2+} measurements KCNJ3, KCNJ5, KCNJ5-Y152C and $KCNJ5^{G151E}$ cDNAs were subcloned into the bicistronic expression vector pIRES-CD8 [246]. Cytoplasmic Ca^{2+} measurements were performed by loading the cells for 1 h with 5 µM Fura-2 AM in the presence of Power Load permeabilizing reagent (Molecular Probes, Darmstadt, Germany). To measure the effect of extracellular Ca^{2+} on intracellular Ca^{2+} levels, cells were superfused with solutions containing 0.1, 0.7, 1.8 or 5 mM Ca^{2+}. The Fura-2 ratio of emissions after excitations with 340 nm and 380 nm was used as a measure of intracellular Ca^{2+} concentrations. For comparison, the known mutant $KCNJ5^{G151E}$ was used [8]. All the experiments were performed at room temperature.

Statistical analyses

IBM SPSS Statistics 19 (SPSS INC, Chicago, IL) was used for statistical analyses. Data are expressed as mean ± S.D. or S.E. and individual experiments were repeated at least three times. Differences between variables were evaluated using unpaired t test or Mann-Whitney test. A probability of less than 0.05 was considered statistically significant.

SECTION II.

EFFECTS OF MUTANT KCNJ5 ON ACUTE AND CHRONIC REGULATION OF ALDOSTERONE SYNTHASE (CYP11B2) EXPRESSION AND ALDOSTERONE PRODUCTION.

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ABSTRACT

Background: Primary aldosteronism (PA) is the most common cause of secondary hypertension. Recently, somatic and germline mutations in the inward rectifying K+ channel (KCNJ5) have been identified in 40-70% aldosterone producing adenomas, and in adrenal nodular hyperplasia. A role for calcium signaling has been determined in mutated KCNJ5 (mKCNJ5) mediated elevated CYP11B2 gene expression and in aldosterone production. However, few studies have demonstrated the effects of mutated KCNJ5 on acute events and chronic transcriptional mechanisms involved in aldosterone production.

Hypothesis: Mutant KCNJ5 increases CYP11B2 expression and aldosterone production through the acute activation of steroidogenic acute regulatory protein (StAR), as well as transcription factors NURR1 and ATF2 that regulate the expression of CYP11B2.

Methods: An adrenal cell line with doxycycline (doxy) inducible mKCNJ5, harboring the T158A mutation (HAC15-TR-mKCNJ5), was developed and utilized for this study. Primary cultures of human adrenal fasciculata-reticularis (ZF/ZR) cells were used to confirm the effects of mKCNJ5. Real-time quantitative PCR was used to analyze changes in gene expression, and western analyses were used to demonstrate changes in protein expression or in the activation of protein by post translational modification.

Results: Doxy incubation caused a dose- and time-dependent increase in mKCNJ5 and CYP11B2 mRNA and protein levels. Transcript levels peaked at 36 h for KCNJ5, and at 60 h for CYP11B2. A corresponding increase in aldosterone production was seen at 60

h of doxy treatment. mKCNJ5 also increased the expression of NURR1 mRNA (in a time-dependent manner) and nuclear protein (at 48 h doxy incubation). Doxy incubation also increased the phosphorylation and activation of ATF2 (another transcriptional activator of CYP11B2) after 18 h doxy treatment. All of these stimulatory effects of mKCNJ5 were inhibited by the L-type Ca^{2+} channel blocker, verapamil. mKCNJ5 stimulated acute events regulating aldosterone production, including increase in 30 kDa total StAR and phosphorylated StAR. Finally, constitutive expression of mKCNJ5 primary adrenal cells caused a dose dependent increase in transcript levels of KCNJ5, but not in CYP11B2 or in aldosterone production. Treatment with calcium ionophores (data not shown) indicated that primary cells may have a 'muted' calcium pathway.

Conclusion: Overall, mKCNJ5 increases CYP11B2 expression and aldosterone production by upregulating both acute and chronic regulatory events in the adrenocortical carcinoma cell line. While these results could not be reproduced in primary human ZF/ZR cells, it suggests mechanisms in the ZF/ZR that repress CYP11B2 expression, and also causes speculation about hypotheses regarding ZF cells being the origin for APA harboring KCNJ5 mutations. Finally this study bolsters the use of verapamil as an efficient therapy in abrogating mKCNJ5 mediated aldosterone excess in PA.

Key words: KCNJ5 mutations, primary aldosteronism (PA), aldosterone, CYP11B2, steroidogenic acute regulatory protein (StAR), verapamil

INTRODUCTION

In normal physiology, aldosterone production is tightly regulated by the renin-angiotensin-aldosterone system. Primary aldosteronism (PA) is characterized by renin independent aldosterone excess and forms the most common cause of secondary hypertension. The most common and main causes of PA are the formation of aldosterone producing adenomas (APA) or bilateral adrenal hyperplasia. In 2011, Choi and colleagues reported mutations in the selectivity filter of the inward rectifying K^+ channels (KCNJ5, coding for the Kir 3.4 protein) in over 35 % of APA, with a higher prevalence in females [5]. In the past three years, numerous groups including ours, have also described several other mutations localized in and around the KCNJ5 selectivity filter, causing a change in conductance from K^+ ions to influx of Na^+ ions [5, 11, 12, 227-230]. As indicated by electrophysiological studies, the Na^+ influx causes the activation of voltage gated Ca^{2+} ions, activating the calcium signaling pathway, and leading to elevated expression of aldosterone synthase (CYP11B2) and aldosterone production [13, 227, 228, 230]. The use of L-type Ca^{2+} channel blockers, such as nifidipine, have been elucidated as efficient therapies for the blockade of aldosterone excess, mediated by mutated KCNJ5[13, 227, 230, 247]. In an elegant study by Tauber and colleagues, comparison of inhibitors of KCNJ5 channels, Ca^{2+} channels and the Na^+/Ca^{2+} and H^+/Cl^-exchangers indicated that verapamil could most potently inhibit mutant KCNJ5 mediated dysregulation of calcium [247]. However, the effect on target gene CYP11B2 and aldosterone production was not studied.

Most *in vitro* studies of mutant KCNJ5 have used strategies with constitutive transgene expression in adrenocortical cells. While useful findings have been provided by these

studies, these strategies make it difficult to define the acute actions of mutant KCNJ5 on adrenal cell function. Herein, we developed a doxycycline (doxy) inducible system for KCNJ5 harboring the T158A mutation (mKCNJ5) in order to define the effect of mKCNJ5 on acute and chronic events in aldosterone production. This cell line permitted the definition of mKCNJ5 effects on the acute regulation of aldosterone production through activation of steroidogenic acute regulatory (StAR) protein and key transcription factors needed for CYP11B2 transcription.

MATERIALS AND METHODS

Cell Culture

Human adrenocortical carcinoma (HAC15) cells were cultured as described earlier [227, 230, 236, 248]. The pLenti-CMV-rtTA3-Hygro vector[240] was used to generate a lentivirus to generate the HAC15-CMV-rtTA3-hygro cell line expressing the reverse tetracycline-controlled transactivator 3 (rtTA3) [240]. These cells were further transduced with a lentivirus expressing KCNJ5 harboring the T158A mutation (mKCNJ5) under the CMV promoter containing a Tet Operator element (TO), to generate the HAC15-TRE-mKCNJ5 cells. Incubation with doxy prevents rtTA3 from physically binding to the TO, thus permitting expression of mKCNJ5. The HAC15-CMV-rtTA3-hygro and HAC15-TRE-mKCNJ5 cells were grown in Dulbecco's Modified Eagles/Ham's F:12 (DME) medium containing 5% Cosmic calf serum (source), 1% ITS (source) and antibiotics (pencillin-streptomycin and gentamicin). HAC15-TRE-mKCNJ5 cells were further selected with 10 µg/mL puromycin. For experiments, cells were plated at a density of 50,000 cells/well in a 48-well dish (for studies involving gene expression analyses) or at

1 million cells/well in a 6 well dish (for nuclear protein isolation) for 48 h. After low serum incubation for 24 h, mKCNJ5 induction was initiated with 1 µg/mL doxy for the indicated times. Inhibitor studies involved pre-incubation for 30 min in 10 µM verapamil.

Viral transduction

Cells were plated for 24 h, treated with appropriate amount of virus in growth medium devoid of any antibiotics and containing 8 µg/mL polybrene. Cells were spinnoculated at 1200 rpm for 2 h, followed by overnight incubation. Recovery was performed with the addition of 2X growth medium devoid of any antibiotics/polybrene. After additional 48 h incubation, cells were sub-cultured and maintained in normal growth medium with 10 µg/mL puromycin.

RNA isolation and real time quantitative PCR (RT-qPCR)

Gene expression analyses were performed using Taqman primer-probes (to make a total of 900nM of each primer, and 400nM probe, per reaction; Life Technologies, Carlsbad, CA) and Kapa Probe fast qPCR kit master mix ABI Prism (Kapa Biosystems, Boston MA). Negative controls consisted of nuclease-free water in place of cDNA. The PCR program, consisting of 40 cycles of amplifications, was performed as per company recommendations. Normalization of gene expression within each sample was performed by using the respective expression levels of *PPIA* (cyclophilin A) to calculate the ΔCt. Relative increase in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein isolation and western analyses

Total protein from each well was isolated by lysing cells in 5 MPER Mammalian Lysis buffer (Thermo Pierce) by repeated pipetting for homogenization. For nuclear protein isolation, after termination of the treatments, cells were immediately washed twice with PBS containing 1X Halt™ Protease Inhibitor Cocktail (Thermo Scientific) and 1X PhosSTOP EASYppack (phosphatase inhibitor cocktail, Roche). Nuclear protein was isolated using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific). Protein was estimated using the Micro BCA Kit (Thermo Pierce). 20 µg aliquots of cell protein were stored at -80°C until use for western analyses. Protein samples were reduced by boiling at 95°C for 5 min and separated on a 10% BisTris gel by electrophoresis (200 V, 50 min) using the XCell SureLock system (Life Technologies). Protein bands were then transferred to an activated PVDF membrane (1.5 h, 40 V). After transfer, the membrane was blocked for 1 h at room temperature with either 5 % BSA/ non-fat dry milk in Tris Borate Saline-0.1% Tween (TBST). Primary and secondary antibodies were diluted using the blocking solution as the diluent. The regular ECL Kit or the ECL SuperSignal West Pico Kit (Thermo Pierce) was used for signal development. Immunodetection of β-actin (Sigma Aldrich), GAPDH (Thermo Pierce) or Lamin A/C (Santa Cruz) was used for normalization, as indicated.

RESULTS

Doxy causes time dependent induction of mKCNJ5

The HAC15-TRE-mKCNJ5 was generated with Doxy regulated mKCNJ5 expression using lentiviral transduction. HAC15-TRE-mKCNJ5 cells were treated with 1 µg/mL doxy

for the indicated times to induce mKCNJ5 expression. mKCNJ5 exhibited a time dependent increase in mRNA (*Figure 3.2.1, Panel A*) and protein (*Figure 3.2.1, Panel B*). Transcript expression increased after 6 h of doxy incubation, peaked at 36 h (increasing by ~ 40-fold) and plateaued thereafter. Western analyses indicated corresponding increase in Kir 3.4 protein levels. Semi quantitative densitometric analysis indicated that Kir 3.4 increased approximately 5 fold to 10 fold between 24 h to 60 h.

Mutated KCNJ5 increased CYP11B2 expression and aldosterone production, which is reversible by blockade of calcium channels

Temporal effects of the incubation of HAC15-TRE-mKCNJ5 cells with 1 µg/mL of doxy on CYP11B2 and aldosterone production were investigated. Data suggested that, alongside of mKCNJ5, CYP11B2 mRNA expression also increased in a time-dependent manner (*Figure 3.2.2., Panel A*). Transcript expression peaked at 36 h and plateaued thereafter. A similar temporal increase in CYP11B2 protein was also observed with doxy incubation (*Figure 3.2.2., Panel B*). After 24 h doxy incubation, protein levels doubled every 12 h, attaining a 5 fold increase over basal at 48 h and almost 10 fold over basal at 60 h. Treatment with agonist Ang II served as a positive control and it increased CYP11B2 mRNA (by ~60-fold over basal) and protein (5 fold over basal) (*Figures 3.2.2, Panels C and D*). Investigation of aldosterone levels after 60 h treatment with doxy or Ang II demonstrated that both treatments elevated aldosterone production (normalized to protein) by over 3-fold and 4 fold (compared to basal), respectively (*Figure 3.2.2, Panel E*). Finally, treatment with L-type Ca^{2+} channel blocker verapamil (10 µM) blocked the stimulatory effects of mKCNJ5 on CYP11B2 mRNA and protein (by

approximately 85%) and Ang II (by approximately 70%) (*Figures 3.2.2, Panels C and D*). Stimulatory effects of mKCNJ5 and Ang II on aldosterone production were also inhibited by approximately 50 % and 30 % respectively (*Figure 3.2.2, Panel E*).

Effect of mKCNJ5 on transcriptional events that regulate CYP11B2

Transcription factors NURR1 and ATF2 form the main regulators of CYP11B2 promoter activation, and are regulated by intracellular calcium levels [39, 86, 239, 249]. We, therefore, investigated the effect of mKCNJ5 on the expression of NURR1 and the activation of ATF2 by phosphorylation. Analyses for transcript levels for mRNA indicate a time-dependent increase with doxy incubation, with NURR1 mRNA peaking at 8 fold over basal at 36 h and plateauing thereafter (*Figure 3.2.3, Panel A*). A corresponding increase in nuclear localized NURR1 was also observed at 48 h of mKCNJ5 activation by doxy treatment (*Figure 3.2.3, Panel B*). Stimulation of NURR1 mRNA and protein was inhibited by pre-treatment of cells with verapamil (10 μM) by approximately 70% (*Figure 3.2.3, Panel B).* Treatment with Ang II as a positive control similarly elevated NURR1 transcript and protein by 5 fold at 60 h incubation. Verapamil pre-treatment inhibited the Ang II mediated effects by approximately 50 %. To define the effect of mKCNJ5 on the activation of ATF2, a transcription factor of the cAMP response element binding protein (CREB) family, nuclear protein from cells incubated with doxy or Ang II in the presence/absence of verapamil was assessed for phosphorylation. Trending similar to Ang II, phosphorylation of ATF2 (normalized to total ATF2 levels) increased with doxy (by ~ 3 fold) and Ang II (~2.5 fold) treatment (*Figure 3.2.3, Panel C*). These effects were reversible by calcium channel blockade by verapamil which inhibited doxy and Ang II effects by 50 % and 30%, respectively. Overall, Figure 3.2.3 indicates

mKCNJ5 mediated calcium dysregulation leads to the activation of chronic transcriptional activation of CYP11B2.

Effect of mKCNJ5 on acute events involved in aldosterone production

To further characterize the role of acute events in mKCNJ5 mediated events, we investigated the effects in steroidogenic acute regulatory protein (StAR), an established acute regulator of CYP11B2. The regulation of StAR at the levels of transcription as well as post translational modification was determined. *Figure 3.2.4, Panel A* is a representative of three western analyses for phosphorylated StAR, total StAR and GAPDH (as a loading control). After 24 h of mKCNJ5 expression by doxy incubation, phospho-StAR was elevated by 1.2 fold ($p<0.05$) after normalization to both, total StAR and GAPDH (*Figure 3.2.4, Panel B)*. An increase in total StAR by 1.2 fold by mKCNJ5 was also observed (*Figure 3.2.4, Panel C)*. Although incubation with Ang II for 24 h did not increase total or phosphorylated StAR, the peaks for increase in total StAR was confirmed at 12 h. Phosphorylated StAR levels increased as early as 30 min post Ang II treatment, decreasing after 3 h incubation (data not shown). A dramatic increase in phosphorylated StAR was also confirmed by stimulating the HAC15-TRE-mKCNJ5 cells with dbcAMP (data not shown).

mKCNJ5 does not increase aldosterone production in primary cultures of human adrenal cells

The expression of mKCNJ5 in primary human adrenal cells was adopted to compare the results obtained from the adrenocortical carcinoma cell line. In three independent sets, human adrenal fasciculata-reticularis (ZF/ZR) cells which form the majority of the

human adrenal cortex were transduced with lentiviruses constitutively expressing mKCNJ5, in a dose dependent manner in primary cells. Constitutively expressed turboGFP (in the same backbone) was used as a control to calculate fold change in transcript expression. GFP visualization indicated successful transduction of the primary cells *(Figure 3.2.5, Panel A)*. Real time qPCR indicated a dose dependent increase in KCNJ5 mRNA in all the three independent sets, although mRNA levels were significant only at the highest dose of virus (over 100 fold increase at a multiplicity of infection of 20) due to variability between sets (*Figure 3.2.5, Panel B)*. However, mKCNJ5 was unable to increase CYP11B2 mRNA and aldosterone production (data not shown).

DISCUSSION

Primary aldosteronism (PA) is the most common cause of secondary hypertension and adrenal disease, characterized by renin independent aldosterone excess. The major causes of PA are idiopathic hyperaldosteronism (IHA), characterized by bilateral adrenal hyperplasia and by the formation of aldosterone producing adrenomas (APA). Approximately 50 % of APA are known to harbor mutations in KCNJ5, however, the molecular events mediated by mutant KCNJ5 in the transcriptional regulation of CYP11B2 are not known in entirety. Herein, our studies defined acute and chronic events involved in the signaling.

For this purpose, we developed a doxycycline inducible cell line for the conditional expression of KCNJ5 harboring the T158A mutation (mKCNJ5), a mutation which occurs as both, somatic and germline, in APA[11, 13]. Findings from transient or stable constitutive expression of mutated KCNJ5 demonstrate increases in aldosterone

production and expression of CYP11B2, by inducing cell depolarization and resultant activation of calcium dependent kinases [13, 227, 228, 230]. Specifically, a sharp advantage of the conditional expression of mKCNJ5 over these models of constitutive mKCNJ5 expression was the investigation of rapid kinetic responses in these models, post translational modification of steroidogenic enzymes and transcription factors. Characterization of the cell line demonstrated a time dependent increase in mKCNJ5 transgene transcript and protein expression. Corresponding increases in target gene CYP11B2 transcript levels as well as in aldosterone were also observed. This is also the first study demonstrating an increase in protein levels for aldosterone synthase mediated by mKCNJ5 using the novel antibody developed by Gomez-Sanchez C. and colleagues [250, 251].

The physiological regulation of aldosterone production in response to Ang II or elevated serum potassium involves two steps. The acute rate regulating step includes the activation of steroidogenic acute regulatory protein (StAR) (occurring within seconds to minutes of agonist treatment). StAR regulates the transport of cholesterol from the outer to the inner mitochondrial membrane. The chronic rate-limiting step involves CYP11B2 transcription which results from elevated transcription factor NURR1, as well as the activation by phosphorylation of members of the cAMP response element binding protein (CREB) including ATF2 [25, 39, 86, 239, 252]. Components of both, the acute and chronic rate limiting steps are regulated by elevated intracellular calcium and the activation of calcium-calmodulin kinases (CaMKs) [116, 117]. These kinases activate transcription factors which then translocate to the nucleus and bind the CYP11B2 promoter to initiate its transcription. *In vitro* studies defining the pathology involved in

mutated KCNJ5-mediated aldosterone excess in PA have also been reported to involve elevated calcium facilitated events. In agreement with all these studies, the HAC15-TRE-mKCNJ5 cells exhibited elevated transcription of calcium sensitive transcription factor NURR1 with doxy incubation in a time dependent manner. The NURR1 stimulatory response was not as pronounced as seen in previous studies of transient constitutive expression of KCNJ5 harboring G151R and L168R mutations [230]. However, this may be reflective of the differences in the pathology caused by mutations in different residues in KCNJ5. Certainly, this phenotypic difference has been noted between the G151R and G151E mutations in the same location [11]. It could also relate to the ability of this particular adrenocortical carcinoma cell line expressing the T158A mutation to compensate for elevated calcium signaling, such as VSNL1 as described by Williams and colleagues [253]. Of note, no toxicity was observed for up to 72 h after by doxy incubation and mKCNJ5 expression. Our studies also indicate elevated nuclear protein levels of phosphorylated ATF2. These results are in agreement with recent findings in APA which have also shown to involve the activation of CaMK II and elevated nuclear localization of active, phosphorylated CREB[254]. Temporally, the activation of NURR1 and ATF2 occurred prior to the stimulation of CYP11B2, as seen in the case of CYP11B2 stimulation by agonists Ang II and K^+ which activate the calcium pathway. In an elegant comparison of different pharmacological agents using an electrophysiological approach, Tauber and colleagues identified verapamil as an efficient blocker of mKCNJ5 mediated calcium influx in adrenal H295R cells[247]. However, effects on CYP11B2 mRNA expression and aldosterone production were not confirmed. Our study confirmed the efficiency of a relatively high dose of verapamil in

the inhibition of mKCNJ5 mediated stimulation of CYP11B2 mRNA and protein, aldosterone production and activation of transcription factors. In order to rule out any non-specific effects of doxy itself, wildtype HAC15 cells were incubated in doxy for 60 h and analyzed for changes in gene expression (*Supplemental Figure 3.2.S1)*. No increase in gene expression of KCNJ5, CYP11B2 or NURR1 was detected by real time qPCR. The positive control consisting of 18 mM K^+ treatment for 60 h increased CYP11B2 and NURR1 transcript levels.

Physiologically, StAR regulates the movement of cholesterol from the outer to the inner mitochondrial membrane, where cholesterol side chain cleavage (CYP11A1) catalyzes the synthesis of pregnenolone. Physiologically, StAR is regulated at the level of transcription, translation, as well as post-translational modification. The translational regulation of StAR controls the level of expression of the 37 kDa StAR. The N-terminal containing the mitochondrial targeting sequence is cleaved to yield the intra-mitochondrial 30 kDa StAR. Phosphorylation of StAR is also crucial for its activity [255-259]. Although the activation of cAMP/protein kinase A seems to be the most potent agonist for StAR, Ang II mediated elevated intracellular calcium levels have also shown to increase the StAR activation in the H295R cell line[260, 261] as well as in primary cultures of bovine adrenals [262, 263]. Sequencing of StAR has revealed three sites for phosphorylation by CaMKs [117]. *In vitro* studies indicate that incubation with CaMK inhibitor, KN93, does not decrease StAR transcript levels[261]. However, the effect of KN93 on CaMK mediated phosphorylation of StAR was not reported. Additionally, janus kinase (JAK)[264] and MAPK pathways[265, 266], which are also activated by Ang II, have also been reported as regulators of StAR activation.

Expression of mKCNJ5 in the HAC15-TRE-mKCNJ5 cells by doxy incubation did not increase StAR transcript levels (data not shown), although western analysis identified increase in total StAR protein with a molecular mass of approximately 30 kDa StAR. This suggests that mKCNJ5 mediated events may result in an increase in active StAR of lower molecular mass. StAR of approximately 37kDa molecular mass were also detected, without any noticeable changes in protein levels.

Our findings also indicate that mKCNJ5 expression increases post translational phosphorylation of StAR. It is widely known through *in vitro* and *in vivo* studies, that serine residue 195 (S195) in humans and the corresponding S194 residue in mice are essential phosphorylation sites in the regulation of StAR function in normal physiology[267, 268]. This site has been identified as a target for phosphorylation by the cAMP mediated pathway[103, 269]. However, the target residues for StAR phosphorylation by calcium signaling have not yet been identified. An antibody that detects phosphorylation at the S195 residue in human StAR (kind donation from Dr. Douglas Stocco) was used in this study. Thus, while it is clear that both mKCNJ5 and Ang II mediated pathways certainly activate human StAR at the S195 residue, the possibility of other additional target serine/threonine residues cannot be ruled out. Furthermore, whether StAR phosphorylation by mKCNJ5 was brought about by CaMKs alone, or in unison with different activated pathways remains unanswered. Finally, although the time point we focused on for StAR activation was 24 h of doxy incubation due to maximal activation of total and phosphorylated StAR levels, we detected increases in both events as early as 6 h in a reverse time course experiment (data not shown). Overall, the events of acute StAR activation and chronic events of activation of

transcriptional regulators of CYP11B2 was confirmed in a temporal manner similar to that observed in Ang II stimulated adrenal cells.

The mechanistic evaluation of mKCNJ5 in cell physiology of primary cultures of human adrenal glands is a unique aspect of this study, although it proved especially challenging. Utilizing constitutive viruses, GFP (control) and mKCNJ5 transgenes were expressed in primary zonal cultures of the human adrenal fasciculata-reticularis-medulla (ZF/ZR). While a dose dependent increase in mKCNJ5 mRNA was observed, similar increases in CYP11B2 mRNA or aldosterone production were not obtained. A positive control consisting of ZF/ZR cells were treated with ACTH (data not shown) indicated an increase in aldosterone production and CYP11B2 mRNA expression. However, these results have to be considered with certain caveats. Firstly, the basal CYP11B2 mRNA levels were almost undetectable, which causes a seemingly much higher stimulation by ACTH. Secondly, ACTH is a known agonist for CYP11B2 expression, acting via the cAMP pathway. We, therefore, raised the question of the possibility that the calcium pathway were 'muted' in ZF/ZR cells. However, pilot data of ZF/ZR cells treated with calcium ionophores (ionomycin and BAYK) (unpublished data) indicated lack of any increase CYP11B2 mRNA and aldosterone synthesis. This may confirm our hypothesis of a 'muted' calcium signaling pathway or a better ability of ZF/ZR cells to compensate and/or efflux the elevated intracellular calcium. Efficient intracellular calcium assays may indicate whether calcium levels are indeed elevated in primary ZF/ZR cells. A role for calcium regulating proteins other than calmodulin, such as PCP4, has been documented in APA[270] and may play a role in ZF physiology. Certainly, the levels of calcium induced transcription factors have been known to be more active in the

CYP11B2 expressing glomerulosa cell type versus the adrenal ZF/ZR [87]. Whether this phenotype of either resistance to calcium signaling or an inability to elevate intracellular calcium, is an inherent physiological feature of the ZF/ZR cell types, or is a phenotype adapted after cells are introduced into culture, remains to be investigated. These results, along with reports from other laboratories describing a ZF-like phenotype in APA harboring KCNJ5 mutations[232], may be a cause for speculation that mutations in KCNJ5 alone in ZF cells may not lead to the formation of APA. A second hit alongside the KCNJ5 mutation may be likely and/or necessary to lead to an APA phenotype. In a single pilot experiment to perform similar studies in cultures of human adrenal cortex, enriched for glomerulosa/capsular cells, we obtained only a modest (at best) stimulation of aldosterone production, and no increase in CYP11B2 mRNA with high doses of mKCNJ5 (data not shown). The inherent challenge herein is the ability to obtain Ang II responsive ZG cells due the normal adrenal not having a distinct ZG, but rather few, scattered CYP11B2 expressing cell clusters[31].

This study does have its limitations including the inability to differentiate between mutant and wildtype mRNA and protein for KCNJ5. Firstly, our model system was unable to differentiate between mutant and wildtype mRNA and protein for KCNJ5. A potential solution was using an approach of tagging the mKCNJ5 transgene. We considered this option as being potentially risky due to the possibility of interference of the tag in channel activity. While real time genotyping assays for mRNA allow for the identification of a point mutation in KCNJ5, they lack the ability to quantitate the percentage of the WT and mKCNJ5 mRNA. Secondly, in a dose dependent assay involving transduction of constitutive mKCNJ5 in HAC15 carcinoma cells (data not shown), a relatively large

dose (MOI 20) of the constitutive virus was required to cause any elevation in aldosterone production in HAC15 cells. This could cause speculation whether the observed increase in KCNJ5 mRNA and protein after doxy treatment, is also in part due to the cells increasing their endogenous WT KCNJ5 expression as a compensatory mechanism to attain resting membrane potential.

Overall, this study has defined the role of acute events (including a role for StAR) and more chronic transcriptional events (including a role for ATF2 and NURR1) in the pathology of PA caused by mutations in the KCNJ5 selectivity filter and confirmed the possibility of verapamil as an effective therapeutic strategy in the treatment for PA. Moreover, our data have re-emphasized the need for 1) development of better methods of enrichment of zonal cultures of human adrenal cells which preserve the intracellular calcium responses, 2) generation of animal models for the study of PA for further understanding the origin and development of APA and genetic mutations leading to PA, 3) *in vivo* validation of verapamil as a therapeutic inhibitor specific for mutated KCNJ5 and finally 4) the development of inhibitors specific to mutated KCNJ5 channel.

FIGURES

Figure 3.2.1. Doxy causes time dependent induction of (A) KCNJ5 mRNA and (B) KCNJ5 protein in HAC15-TRE-mKCNJ5 cells. Cells were were treated with 1 µg/mL for the indicated times. qPCR was used for mRNA transcript detection, and western analyses for protein quantification. Doxy incubation caused a time dependent increase in KCNJ5 mRNA **(A)** and protein **(B)**. Results represent the means ± S.E.M. of at least five independent experiments for mRNA (A) and three independent westerns (B) (*p<0.05 versus basal).

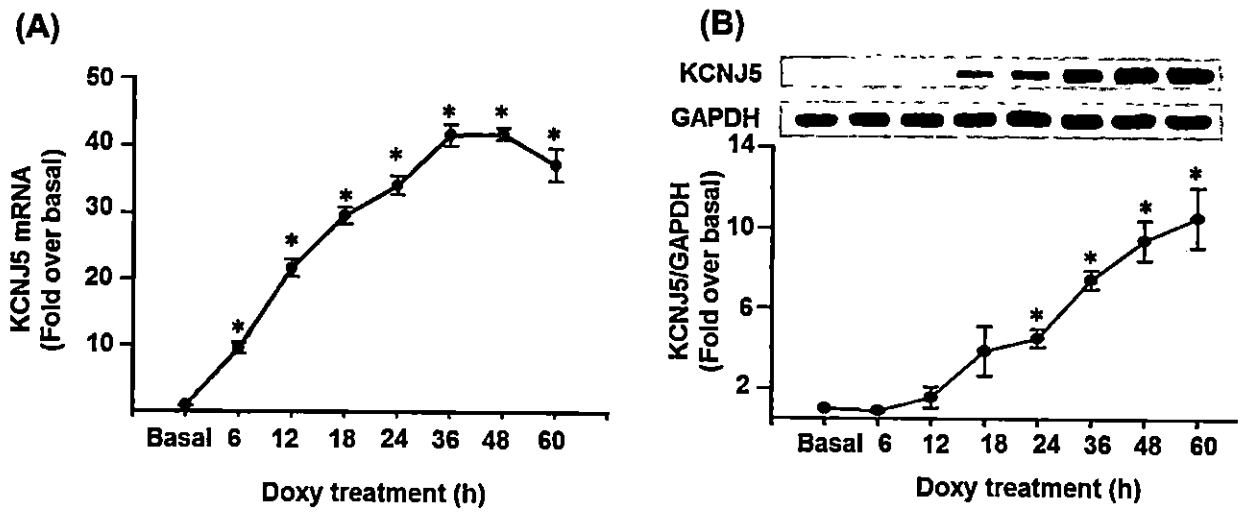
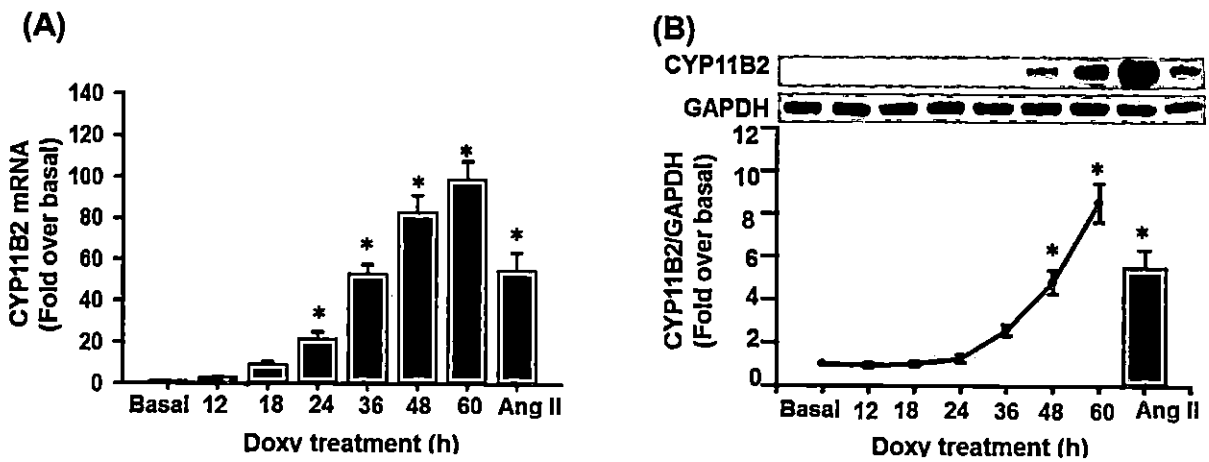


Figure 3.2.2. mKCNJ5 increases CYP11B2 mRNA and protein expression ,and aldosterone production through calcium mediated pathway. Cells were incubated in 1 µg/mL doxy at indicated times, causing a time dependent increase in CYP11B2 mRNA *(Panel A)* and protein *(Panel B)*. Pre-incubation with calcium channel blocker, verapamil (10 µM) inhibited mKCNJ5–mediated and Ang II-stimulated CYP11B2 mRNA and protein expression. Aldosterone levels in cells incubated in doxy or Ang II for 60 h, and the ability of verapamil to block these stimulatory effects. Black bars represent the absence of verapamil and the grey bars represent pre-incubation with verapamil (10 µM). Results represent the means ± S.E.M. of at least five independent experiments for mRNA *(Panels A and D)* and three independent westerns *(Panels B and C)*. The westerns *(Panels B and C)* are representative of three independent experimental analyses. Statistical analyses were performed using one-way ANOVA (*p<0.05 versus basal, §p<0.05 versus corresponding treatment in the absence of verapamil).



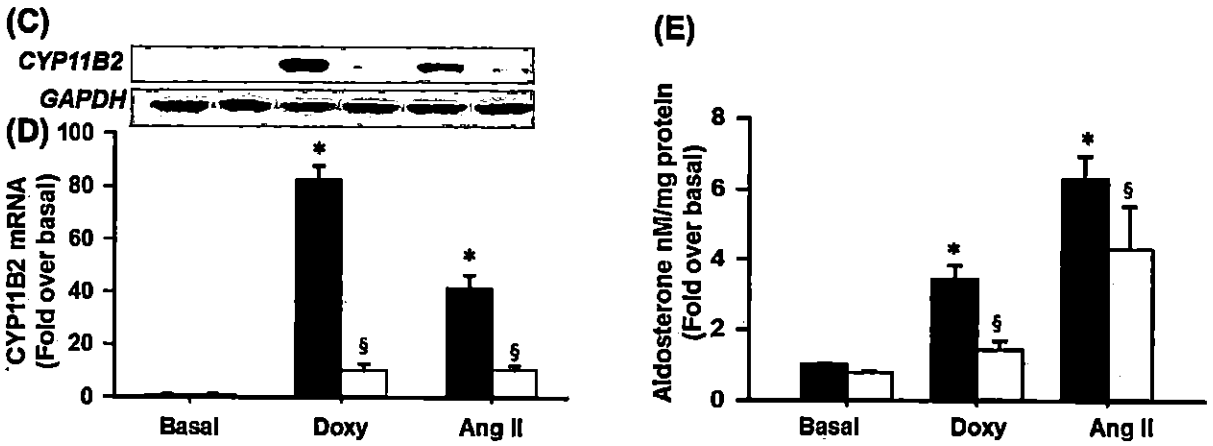


Figure 3.2.3. mKCNJ5 increases the activation of transcription factors required for CYP11B2 expression, including NURR1 (A,B,C) and ATF2 (D). Doxy incubation caused a time dependent increase in NURR1 mRNA *(Panel A)*. Pre-incubation with verapamil (10 μM) abrogates mKCNJ5–mediated and Ang II-stimulated NURR1 mRNA *(Panel B)* and nuclear protein expression *(Panel C)*. Expression of mKCNJ5 and Ang II treatment stimulates phosphorylation of ATF2 (normalized to total ATF2) *(Panel D)*. Ca^{2+} channel blocker, verapamil (10 μM), inhibited these stimulatory effects of mKCNJ5. The western for nuclear localized NURR1 and ATF2 *(Panels B and D)* are representative of three independent western analyses. Black bars represent the absence of verapamil and the grey bars represent pre-incubation with verapamil (10 μM) *(Panels C and D)*. Results represent the means ± S.E.M. of at least five independent experiments for mRNA (A,C) and three independent westerns (B,D). Statistical analyses were performed using one-way ANOVA (*$p<0.05$ versus basal, §$p<0.05$ versus corresponding treatment in the absence of verapamil).

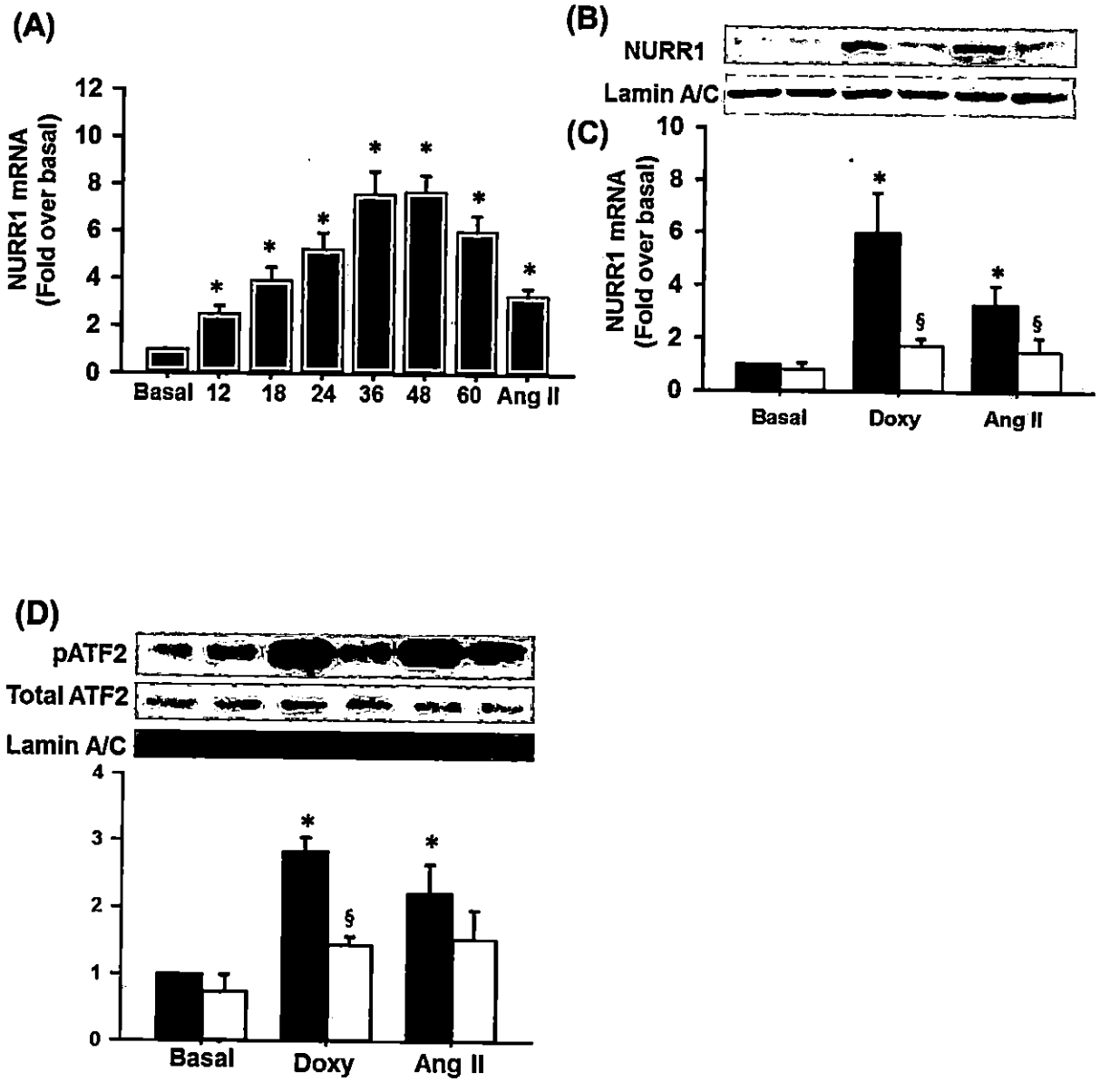

(A)
NURR1 mRNA (Fold over basal)
12
10
8
6
4
2
0
Basal
12
18
24
36
48
60
Ang II
(B)
NURR1
Lamin A/C
(C)
NURR1 mRNA (Fold over basal)
10
8
6
4
2
0
Basal
Doxy
Ang II
§
(D)
pATF2
Total ATF2
Lamin A/C
4
3
2
1
0
Basal
Doxy
Ang II
§

Figure 3.2.4. mKCNJ5 activates steroidogenic acute regulatory protein at the levels of translational and post-translational modification. Cells treated with 1 µg/mL for 24 h were analyzed for StAR induction by western analyses. A representative of three independent sets of protein analyses for total StAR, phosphorylated StAR and GAPDH (loading control) *(Panel A)*. Semi-quantification by densitometry of mKCNJ5 effects on total StAR levels and phosphorylation of StAR. Both, total and phosphorylated StAR levels are elevated by mKCNJ5 expression *(Panels B and C)*.

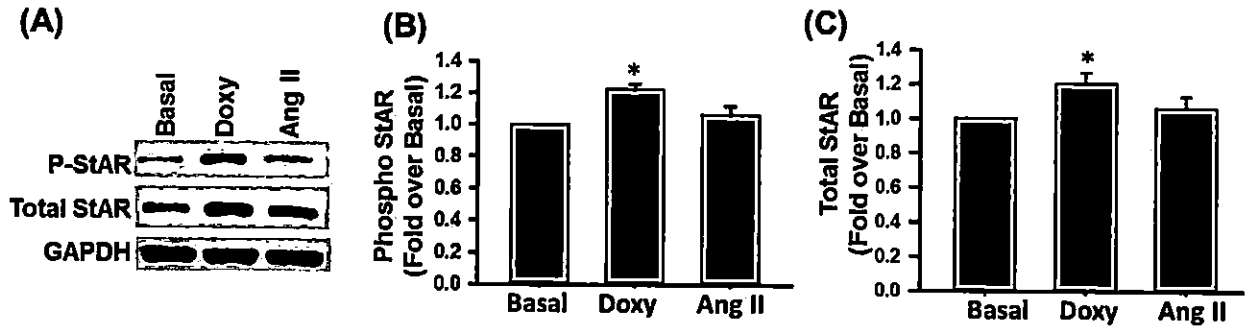
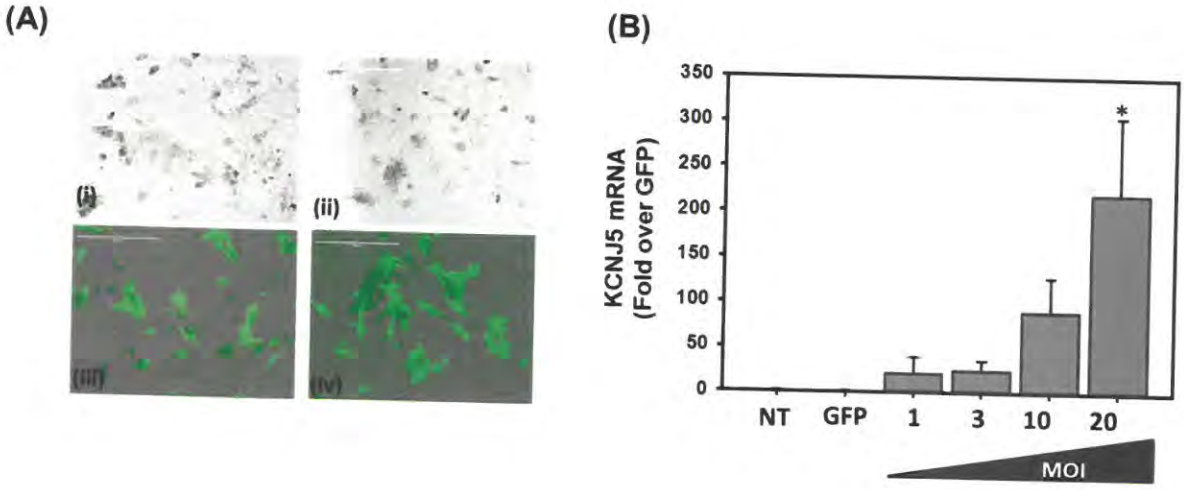
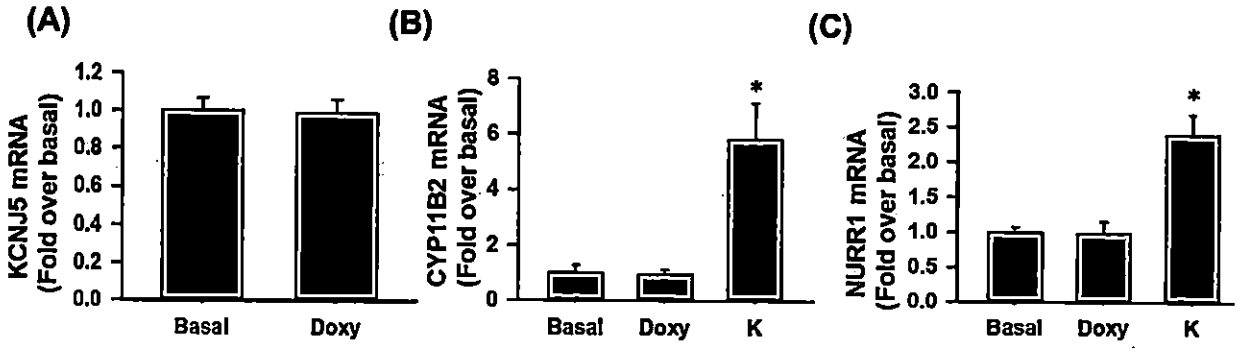


Figure 3.2.5. Effect of mKCNJ5 expression in primary human ZF/ZR cells. Primary zonal cultures of ZF/ZR cells were transduced with lentiviruses constitutively expressing GFP (control, multiplicity of infection or MOI 10) or mKCNJ5 (at indicated MOI doses). Images to view GFP fluorescence confirmed successful transduction. RNA from cells was used for transcript analyses. **(A)** GFP was detected in cells transduced with the GFP lentivirus *(iii, iv)*, but not in non-transduced cells *(i, ii)*. **(B)** Real time qPCR for KCNJ5 indicated a dose dependent increase in KCNJ5 mRNA. Results represent the means ± S.E.M. of three independent experiments. Statistical analyses were performed using one-way ANOVA (*p<0.05 versus basal).



Supplemental Figure 3.2.S1. Doxy does not have off target effects on HAC15 cell gene expression of (A) KCNJ5, (B) CYP11B2 or (C) NURR1. Control wildtype cells were incubated with1 µg/ml doxy were analyzed by qPCR. Doxy itself did not have any effect on KCNJ5 *(Panel A)*, CYP11B2 *(Panel B)* or NURR1 *(Panel C)* expression. CYP111B2 agonist K^+ was used as a positive control. Results represent the means ± S.E.M. of three independent experiments. Statistical analyses were performed using one-way ANOVA (*p<0.05 versus basal).



CHAPTER IV

Discussion

4.1. Primary aldosteronism and KCNJ5 mutations

Using whole exome sequencing in human aldosterone producing adenomas, Choi and colleagues, in 2011, described somatic (G151R and L168R) and germline (T158A) mutations in the selectivity filter of the gene encoding an inward rectifying potassium channel, KCNJ5[5]. The somatic mutations were prevalent in 34 % of APA, with higher prevalence in females. These genetic mutations are believed to cause renin independent aldosterone excess in primary aldosteronism. Subsequent studies by various research groups also identified additional APA somatic mutations in and around the selectivity filter of KCNJ5, and more recently, in aldosterone producing micronodules [3, 6, 8, 9, 11, 12, 227-230, 232, 241, 243, 271-275]. However, KCNJ5 mutations have not yet been reported in adrenal hyperplasia or adrenocortical cancer. Germline mutations in KCNJ5 lead to a hereditary form of primary aldosteronism, termed as familial hyperaldosteronism type III (FH III). Microarray analyses, quantitative RT-PCR transcript evaluation and immunochemical protein evaluation of APA and normal adrenal tissues have demonstrated elevated expression of CYP11B2 in APA harboring KCNJ5 mutations [9, 227, 230, 232].

4.2. Former studies and limitations

4.2.1. Former studies on somatic and familial KCNJ5 mutations in PA

Following original report by Choi and colleagues, numerous studies have identified additional somatic and germline mutations in the selectivity filter of KCNJ5. Mutations in regions other than the selectivity filter KCNJ5 evidently also interfere with normal KCNJ5 function and have been detected in APA and in FH III [228, 272]. So far, all reported

mutations in KCNJ5, both sporadic and familial, have been heterozygous. APA with mutated Kir 3.4 have also been shown to have disrupted adrenocortical zonation[231]. Unpublished data from our lab (Nishimoto et al, in preparation), has shown an increasing gradient in Kir 3.4 expression from the ZR to the ZG.

Perhaps one of the most interesting features of somatic KCNJ5 mutations is the variability in its prevalence. Prevalence studies (unpublished data, through Sanger sequencing) in our own adrenal APA cohort, consisting of APA obtained from Japan, Italy and various regions of the United States, aptly demonstrates this ethnic variation. The overall prevalence of KCNJ5 mutations has been found to be approximately 50 % in our APA cohort. Across ethnicities, the European and American cohorts have a prevalence of 47 % and 36 %, respectively, while the Japanese cohort exhibits a stark increase in prevalence at 63 %. Moreover, personal communication with various centers within Japan, also demonstrate a center-to-center difference in prevalence. While this may be related to diagnostic stringency, the potential effects of genetics, environment and lifestyles, cannot be ignored. Similar prevalence has also been reported by other research centers [5, 6, 9, 11, 232, 274]. A comparison of prevalence within genders demonstrates a female bias in most centers. Japan alone, however, reports almost equal prevalence in both females and males. There are also areas of controversy such as the levels of expression of KCNJ5 itself in APA with or without KCNJ5 mutations, the size of KCNJ5 mutation harboring APA and the actual origins of the mutations and/or APA [6, 9, 232].

Familial KCNJ5 mutations are rather rare in occurrence. The T158A mutation, which is the primary focus of one half of this dissertation, occurs as a germline mutation causing

FH III [5, 11], and also as a sporadic mutation in our cohort (unpublished data). The T158A-affected patients harboring the T158A KCNJ5 mutation has been reported to exhibit severe Mendelian hyperaldosteronism, accompanied by adrenocortical hypertrophy and hyperplasia. This family also exhibited unusually elevated levels of aldosterone and a hybrid steroid, 18-oxocortisol [5]. This dissertation identifies and characterizes a novel germline mutation (Y152C). Interestingly, congenital heterozygous G387R mutation in KCNJ5 has been reported in a cardiac pathology termed 'Long QT Syndrome' [224-226, 276]. A recent clinical report also suggests KCNJ5 mutation as a second hit, with KCNJ2 mutations forming the primary cause of Andersen-Tawil syndrome [277]. However, these reports have not described any adrenal pathology.

Alongside characterization studies on APA harboring WT or mutated KCNJ5, efforts towards defining the molecular mechanisms facilitated by the mutated KCNJ5 expression *in vitro*, are also underway.

4.2.2. Former *in vitro* studies utilizing constitutive mKCNJ5 expression

Following the description of KCNJ5 mutations in PA, numerous *in vitro* analyses defined the mechanistic effects of mutant KCNJ5 on adrenal cell function. Electrophysiological studies in kidney HEK as well as adrenocortical carcinoma cell lines defined a pathology entailing a disturbance in conductance of the K channel allowing for Na^+ influx. The resultant depolarization of the cell causes opening of voltage gated Ca^{2+} channels. This further stimulates the reverse potentiation of the Na^+/Ca^{2+} exchangers, leading to a cumulative elevation in intracellular Ca^{2+} levels [247]. Molecular findings employing

constitutive mutant KCNJ5 expression in adrenocortical carcinoma cells indicated an increase in the expression of CYP11B2 and aldosterone production via dysregulation of the calcium signaling pathway, involving calmodulin and CaMKs [13, 227, 230, 247]. The expression of calcium mediated transcriptional regulators of CYP11B2, including NURR1, has also been defined. However, these studies also have some pitfalls, as described in section 4.2.3.

Reports on the effects of mKCNJ5 on the expression of other steroidogenic enzymes have revealed an increase in expression of CYP11B1 (besides CYP11B2) and an inhibition in CYP17 mRNA. Corresponding increases in aldosterone, cortisol, as well as hybrid steroid 18-oxocortisol were also demonstrated. No changes were reported in the expression of enzymes CYP21, HSD3B2, CYP11A1 or StAR [13]. Furthermore, while calcium dysregulation is a common feature in mechanisms mediated by all KCNJ5 mutations [11, 228, 230, 247], each type of mutation may be accompanied by specific pathological phenotypes. Certainly, differences in toxicity and pathology have been noted in different amino acid substitutions in the same 151 residue (G151R and G151E) [11]. *In vitro* expression of mutated KCNJ5 has also exhibited the ability of the cells to respond and compensate the ionic changes and toxicity by upregulating survival pathways, such as those involving elevated expression of visilin 1 (VSNL1) [253].

Due to the large role of calcium dysregulation in the mechanisms facilitated by mutated KCNJ5, the use of calcium channel blockers has been explored. *In vitro* studies have indicated that nifedipine, a blocker of both T-type and L-type channels, at higher doses, can successfully block aldosterone production mediated by mutated KCNJ5 [13, 227, 230, 247]. In an elegant study by Tauber and colleagues, comparison of inhibitors of KCNJ5

channels, Ca^{2+} channels and the Na^+/Ca^{2+} and H^+/Cl^-exchangers indicated that verapamil could most potently inhibit mutant KCNJ5 mediated dysregulation of calcium [247]. The efficient use of calcium channel blockers in humans has also been well documented in cardiovascular disease, and more recently, in PA caused by mutations in calcium channels [234].

4.2.3. Limitations in former studies

Former reports on mutated KCNJ5 mediated aldosterone production have employed the use of constitutive expression of mutated KCNJ5 in adrenocortical carcinoma cells by transient methods such as electroporation or, more recently, by stable lentiviral transduction. These approaches have established a role for change in membrane polarity and calcium dysregulation in aldosterone production mediated by mKCNJ5. However, both these techniques are accompanied by their respective set of drawbacks. The technique of electroporation, although effective in transgene expression, relies on increasing permeability of the cell membrane by the external application of an electric field, for delivery of the expression vector in the host cell. The major challenges of this technique include low cell survival and limited flexibility in control of parameters of the system. External electric field may also alter the function of channels and transporters in the cell membrane. The increase in recovery time may be accompanied by simultaneous extrusion of the vector from the cell. Finally, mutated KCNJ5 expression itself specifically increases Na^+ influx which is itself toxic to cells, as previously reported. Differences in responses based on cell passage numbers are likely to produce a variation in cell responses in subsequent experimental sets. Stable lentiviral transduction of adrenocortical cells certainly circumvents the technical pitfalls of

electroporation. However, constitutive expression of mKCNJ5 makes the study of the acute events regulating aldosterone production challenging.

In terms of technique, one more concern in the use of constitutive expression of KCNJ5 has been selecting an appropriate control for comparison. The role of WT KCNJ5 in hyperpolarizing the cell, and its ability to repress Ang II and K stimulation (both involving elevated calcium signaling pathways) in adrenal cells, renders it a poor control. Currently used alternatives include the empty backbone vector/virus (as used for the generation of mKCNJ5 expression vector/virus). While these may be the only options in cells with reduced amenability to genetic alternation, (e.g., primary adrenal cultures) cell lines offer the scope of generating conditional expression systems that allow for endogenous 'self' controls.

As mentioned previously, no effects of mutated KCNJ5 have thus far been reported on transcript levels of steroidogenic enzymes CYP21, HSD3B2, CYP11A1 and StAR expression [13]. However, StAR activity is also heavily dependent its translational and post translational modifications (details in section 4.6). Thus far, the effects of mutated KCNJ5 on the translational and post translational modifications of StAR have not been reported. Finally, a major drawback of most *in vitro* studies has been the lack of validation in primary cultures of human adrenal cells. Primary cell confirmatory studies are necessary since the only available adrenocortical cell lines are of carcinoma origin, harboring a mutation in the beta-catenin gene. Finally, the use of calcium channel blockers as a therapeutic strategy in FH III or in place of adrenalectomy (in the case of somatic mutations in APA) has indicated the use of verapamil in electrophysiological studies [247]. While verapamil was shown to abrogate mKCNJ5 mediated calcium influx

by nearly 85 %, its ability to inhibit CYP11B2 expression and aldosterone production has yet to be confirmed. Thus, while *in vitro* methods of constitutive expression have been very helpful in understanding several aspects of the mechanisms involved in mKCNJ5 mediated PA, there are certain draw backs, that this dissertation has attempted to address.

4.3. Innovation, significance and summary of current study

This study is the first to 1) define a novel germline KCNJ5 mutation in PA, 2) develop a doxycycline-inducible system (HAC15-TRE-mKCNJ5 cells) for the expression of mutated KCNJ5 harboring the T158A mutation (mKCNJ5) and 3) demonstrate the effects of mutated KCNJ5 expression in primary cultures of human adrenal cells.

The novel mutation was identified as an index case in a female patient and consisted of a tyrosine-to-cysteine substitution at the 152 residue of the selectivity filter (Y152C). *In vitro* molecular and electrophysiological analyses indicated Na^+ influx, intracellular dysregulation, elevated CYP11B2 expression and aldosterone production. Massive adrenal hyperplasia and nodular phenotype were also observed by immunostaining.

The primary advantage of the doxycycline inducible expression system is the ability to have an endogenous control, consisting of cells that are not incubated with doxycycline. Specifically, a major advantage of the conditional expression of mKCNJ5 is the ability to investigate time-dependent responses that include post translational modification of steroidogenic enzymes and transcription factors. The T158A mutation that was selected for expression in adrenal cells is one that occurs in both germline and somatic cases. Characterization of the HAC15-TRE-mKCNJ5 cell line demonstrated time dependent

increases in transcript levels of mKCNJ5, CYP11B2 and NURR1. A concomitant increase in aldosterone production was also observed. This is also the first study demonstrating an increase in protein levels for aldosterone synthase mediated by mKCNJ5 using the novel antibody developed by Gomez-Sanchez C. and colleagues[250, 251]. HAC15-TRE-mKCNJ5 cells also exhibited a time-dependent increase in the expression of the calcium regulated transcription factor NURR1. Nuclear protein levels of NURR1 protein as well as phosphorylated ATF2 were also elevated. These effects of mKCNJ5 were abrogated by the pretreatment with verapamil. Expression of mutated KCNJ5 in primary cells using lentiviruses indicated a dose-dependent increase in KCNJ5 mRNA. However, increases in CYP11B2 mRNA or aldosterone production were not detected. A high dose of verapamil inhibited these mutated KCNJ5 stimulated effects. Further discussions on these points can be found in sections 4.5, 4.6 and 4.7.

4.4. Novel germline mutations in PA

In contrast to sporadic somatic mutations which occur in almost 50 % of APA, germline mutations are far less prevalent. Thus far, besides the Y152C mutation defined in our study, very few germline mutations have been described, including T158A, G151E, G151R, I157S, R52H, E246K, and E282Q [5, 8, 11, 243, 272]. The G247R mutation which was also detected as germline, is functionally indistinguishable from wildtype KCNJ5. Of these germline mutations, only T158A, G151R, G151E and I157S have been shown to cause FH III [5, 8, 11, 243, 272, 278]. These germline mutations have shown variation in phenotype in *in vitro* analyses as well as in pathogenesis observed in patients.

The Y152 mutation was identified as an index case in a 62-year old female patient who presented with resistant hypertension and hypokalemia. Unilateral adrenalectomy was

performed on the basis of adrenal CT findings. Sanger sequencing of the adrenal and blood DNA sequencing for KCNJ5 revealed a point c.455A>G mutation, Tyr152Cys substitution. Tyrosine 152 belongs to the Gly-Tyr-Gly (GYG) motif of the K selectivity filter of the channel and is highly conserved among orthologs and paralogs.

4.4.1. Phenotypic differences in patients with different KCNJ5 mutations

A striking difference between the patient with the Y152C mutation harboring patient and other FH III cases was the late age (48 years) at which PA was diagnosed. Most FH III cases are diagnosed before 7 years of age. However, the patient exhibited severe target organ damage, including left ventricular hypertrophy and impaired renal function, causing us to speculate that the patient had been hypertensive for a considerable time prior to being diagnosed with PA.

Studies on FH III have suggested differences in pathology based on the type of KCNJ5 mutations. The T158A mutation harboring patients exhibited massive adrenal hyperplasia, severe aldosteronism and required bilateral adrenalectomy[5]. In contrast, the G151E-affected patients had a relatively milder phenotype, with normal appearing adrenals, treatable with medical therapy [11]. Interestingly, our patient affected by the germline Y152C mutation, demonstrated pathological features that are common to both the mild G151E and severe T158A mutations. As in the case of the T158A mutation, the Y152C harboring adrenal presented hyperplasia. Of note, several nodular structures were observed in the adrenalectomized tissue. These nodules also exhibited elevated levels of aldosterone synthase as well as KCNJ5 protein, as detected by immunochemical analyses. Similar to the G151E mutation, the phenotype of the Y152C-

affected patient was also relatively mild. CT scanning indicated minimal changes in adrenal physiology. Furthermore, cortisol, but not aldosterone, was suppressed following dexamethasone administration (excluding Glucocorticoid remediable hyperaldosteronism). The *in vitro* analysis of the different KCNJ5 mutations by various research groups has helped shed light on the mechanisms underlying PA.

4.4.2. *In vitro* analyses of the Y152C mutation in KCNJ5

Our studies also focused on expressing the Y152C mutation in adrenocortical cells *in vitro,* by electroporation for molecular analyses. Electrophysiological studies in HEK293 cells indicated that Y152C mutation caused Na^+ influx, similar to most of the KCNJ5 mutations. However, barium treatment studies suggested that the Y152C mutation retained some conductance to K^+, in contrast to G151R and G151E mutations that are Ba^{2+} insensitive [5, 11]. Calcium influx was also elevated by Y152C as compared to WT KCNJ5, albeit not as much as G151E. Electrophysiological studies showed that G151E was associated with a much larger Na^+ conductance compared to other mutated channels and consequent Na-dependent cell lethality. This may explain why the G151E mutation, which has a severe impact on the channel function, and the Y152C mutation which has reduced effect on Na^+ conductance, are both associated with a mild clinical phenotype. Expression of Y152C elevated CYP11B2 and NURR1 transcript levels compared to the control. When compared to the G151R effects (from our previous electroporation studies), Y152C was unable to elevate NURR1 expression as much as G151R. This may, however, be related to differences in phenotype, as seen between G151R and G151E mutations. Alternatively, it may indicate a change in temporal events, causing us to miss the initial sharp increase NURR1 expression. Besides the

limitations of the technique of electroporation (as discussed in section 4.2.3), one of the major limitations was the inability to confirm an increase in aldosterone levels *in vitro,* although diagnosis of PA in the patient did reveal renin independent aldosterone production (plasma aldosterone=24 ng/dL and plasma renin activity < 0.6 ng/mL/h).

4.5. Effect of T158A on the transcriptional regulation of CYP11B2

As discussed in section 4.3, the primary advantages of the doxy-inducible HAC15-TRE-mKCNJ5 cell line included 1) comparison against an endogenous control and 2) ability to investigate temporally rapid events such as post translational regulation of steroidogenic enzymes and transcription factors.

4.5.1. Physiological regulation of CYP11B2 transcription

As described in section 1.6.2., the transcriptional regulation of CYP11B2 is controlled at the chronic rate limiting step of aldosterone production. Elevation of transcription of CYP11B2 by physiological agonists results from elevation in transcription factor NURR1, as well as from the activation by phosphorylation of members of the cAMP response element binding protein (CREB), including ATF2 [25, 39, 86, 239, 252]. These events rely heavily on the elevation of intracellular calcium and the activation of calcium-calmodulin kinases (CaMKs) [40, 80-85]. These kinases activate transcription factors which then translocate to the nucleus and bind the CYP11B2 promoter and initiate its transcription [40, 80-85].

4.5.2. Transcriptional regulation of CYP11B2 by mKCNJ5

Numerous *in vitro* studies defining the pathology involved in mutated KCNJ5-mediated aldosterone excess in PA have also been reported to involve cellular calcium facilitated events. In agreement with all these studies, the HAC15-TRE-mKCNJ5 cells exhibited elevated transcription of calcium sensitive transcription factor NURR1 with doxy incubation in a time dependent manner. The NURR1 stimulatory response was not as pronounced as seen in previous studies of transient constitutive expression of KCNJ5 harboring G151R and L168R mutations [230]. However, this may be reflective of the differences in the pathology caused by mutations in different residues in KCNJ5. Certainly, this phenotypic difference has been noted between the G151R and G151E mutations in the same location [11]. It could also relate to the ability of this particular adrenocortical carcinoma cell line expressing the T158A mutation to compensate for elevated calcium signaling, such as VSNL1 as described by Williams and colleagues [253]. Of note; no toxicity was observed for up to 72 h after doxy incubation and mKCNJ5 expression. Our studies also indicate elevated nuclear protein levels of phosphorylated ATF2. These results are in agreement with recent findings in APA which demonstrate elevated levels of phosphorylated CaMK II and nuclear localization of active, phosphorylated CREB[254]. Temporally, the activation of NURR1 and ATF2 occurred prior to the stimulation of CYP11B2, as seen in the case of CYP11B2 stimulation by agonists Ang II and K^+ which activate the calcium pathway.

4.5.3. Calcium channel blockers as inhibitors of mutated KCNJ5 pathogenesis

Several electrophysiological and molecular analyses, by our group as well as many others, have defined a role for calcium dysregulation in mutated KCNJ5 mediated aldosterone excess. In an elegant comparison of different phamacological agents using an electrophysiological approach, Tauber and colleagues identified verapamil as being a more efficient blocker of mKCNJ5 mediated calcium influx in adrenal H295R cells [247]. However, effects on CYP11B2 mRNA expression and aldosterone production were not confirmed. Our study confirmed the efficiency of a relatively high dose of verapamil in the inhibition of mKCNJ5 mediated stimulation of CYP11B2 mRNA and protein, aldosterone production and activation of transcription factors[247]. Comparing our findings from this study to those on G151R and L168R [230], we observed that verapamil seemed to have better inhibitory effects as compared to that of nifidipine. These results were also in accordance with reports by Tauber and colleagues[247].

As speculated by other groups, a high dose of verapamil in combination with useful MR blockers such as spironolactone may be an effective therapeutic strategy, and may perhaps serve a replacement for adrenalectomy. A caveat is that there are no methods of diagnosis of somatic KCNJ5 as a cause for PA without tissue analysis post adrenalectomy. Progress in the area of diagnostic tests for markers of KCNJ5 mediated PA is therefore essential. The use of calcium channel blockers may, however, be useful in the treatment of FH III.

4.5.4. Potential off target effects of doxycycline

In order to rule out any non-specific effects of doxy itself, wildtype HAC15 cells were incubated in doxy for 60 h and analyzed for changes in gene expression (*Supplemental*

Figure 1). No increase in gene expression of KCNJ5, CYP11B2 or NURR1 was detected by real time qPCR. The positive control consisting of 18 mM K^+ treatment for 60 h increased CYP11B2 and NURR1 transcript levels.

4.6. Effect of mKCNJ5 on the steroidogenic acute regulatory protein (StAR)

Physiologically, StAR regulates the movement of cholesterol from the outer to the inner mitochondrial membrane, where cholesterol side chain cleavage (CYP11A1) catalyzes the synthesis of pregnenolone. This is the acute rate limiting step in aldosterone production and typically occurs within seconds to minutes after agonist treatment.

4.6.1. Physiological regulation of StAR

StAR is regulated at the level of transcription, translation, as well as post-translational modification. The translational regulation of StAR controls the level of expression of the 37 kDa StAR. The N-terminal containing the mitochondrial targeting sequence is cleaved to yield the intra-mitochondrial 30 kDa StAR. Furthermore, the post translational modification of StAR by phosphorylation is also crucial for its activity. [255-259]. Although the activation of cAMP/protein kinase A seems to be the most potent agonist for StAR, Ang II mediated elevated intracellular calcium levels have also shown to increase the StAR activation in the H295R cell line[260, 261] as well as in primary cultures of bovine adrenals [262, 263]. Sequencing of StAR has revealed three sites for phosphorylation by CaMKs [117]. *In vitro* studies indicate that incubation with CaMK inhibitor, KN93, does not decrease StAR transcript levels[261]. However, the effect of KN93 on CaMK mediated phosphorylation of StAR was not reported. Additionally, janus kinase (JAK)[264] and

MAPK pathways[265, 266], which are also activated by Ang II, have also been reported as regulators of StAR activation.

4.6.2. Effects of mKCNJ5 on StAR activation

Expression of mKCNJ5 in the HAC15-TRE-mKCNJ5 cells by doxy incubation did not increase StAR transcript levels (data not shown), although western analyses identified an increase in total StAR protein with a molecular mass of approximately 30 kDa. This suggests that mutated KCNJ5 mediated events may result in an increase in active StAR. StAR of 37kDa molecular mass was also detected, without any noticeable changes in protein levels. Therefore, it may be difficult to indicate the role of mKCNJ5 in the peptidic cleavage of the 37 kDa StAR peptide.

Our findings also indicate that mKCNJ5 expression increases post translational phosphorylation of StAR. It is widely known through *in vitro* and *in vivo* studies, that serine residue 195 (S195) in humans and the corresponding S194 residue in mice are essential phosphorylation sites in the regulation of StAR function in normal physiology [267, 268]. This site has been identified as a target for phosphorylation by the cAMP mediated pathway[103, 269]. However, the target residues for StAR phosphorylation by calcium signaling have not yet been identified. An antibody that detects phosphorylation at the S195 residue in human StAR (kind donation from Dr. Douglas Stocco) was used in this study. Thus, while it is clear that both mKCNJ5 and Ang II mediated pathways certainly activate human StAR at the S195 residue, the possibility of other additional target serine/threonine residues cannot be ruled out. Furthermore, whether StAR phosphorylation by T158A was brought about by CaMKs alone, or in unison with

different activated pathways, remains unanswered. Finally, although the time point we focused on for StAR activation was 24 h of doxy incubation due to maximal activation of total and phosphorylated StAR levels, we detected increases in both events as early as 6 h in a reverse time course experiment (data not shown).

Overall, the events of acute StAR activation and chronic events of activation of transcriptional regulators of CYP11B2 was confirmed in a temporal manner similar to that observed in Ang II stimulated adrenal cells.

4.7. Mutated mKCNJ5 expression in primary human adrenal cell cultures

The mechanistic evaluation of mKCNJ5 in cell physiology of primary cultures of human adrenal glands is a unique aspect of this study, although it proved especially challenging.

4.7.1. Effects of T158A expression in primary human adrenal cell cultures

Utilizing constitutive viruses, GFP (control) and mKCNJ5 transgenes were expressed in primary zonal cultures of the human adrenal fasciculata-reticularis-medulla (ZF/ZR) cells. While a dose dependent increase in mKCNJ5 mRNA was observed, similar increases in CYP11B2 mRNA or aldosterone production were not obtained. A positive control consisting of ZF/ZR cells treated with ACTH (data not shown) indicated an increase in aldosterone production and CYP11B2 mRNA expression. However, these results have to be considered with certain caveats. Firstly, the basal CYP11B2 mRNA levels were almost undetectable, which causes a seemingly much higher stimulation by ACTH. Secondly, ACTH is a known agonist for CYP11B2 expression, acting via the cAMP pathway. We, therefore, raised the question of the possibility that the calcium

pathway is 'muted' in ZF/ZR cells. However, pilot data of ZF/ZR cells treated with calcium ionophores (ionomycin and BAYK) (unpublished data) indicated lack of any increase CYP11B2 mRNA and aldosterone synthesis. This may confirm our hypothesis of a 'muted' calcium signaling pathway or a better ability of ZF/ZR cells to compensate and/or efflux the elevated intracellular calcium. Efficient intracellular calcium assays may indicate whether calcium levels are indeed elevated in primary ZF/ZR cells. A role for calcium regulating proteins other than calmodulin, such as PCP4, has been documented in APA[270] and may play a role in ZF physiology. Certainly, the levels of calcium induced transcription factors have been known to be more active in the CYP11B2 expressing glomerulosa cell type versus the adrenal ZF/ZR [87]. Whether this phenotype of either resistance to calcium signaling or an inability to elevate intracellular calcium, is an inherent physiological feature of the ZF/ZR cell types, or is a phenotype adapted after cells are introduced into culture, remains yet to be investigated. These results, along with reports from other laboratories describing a ZF-like phenotype in APA harboring KCNJ5 mutations[232], may be a cause for speculation that mutations in KCNJ5 alone in ZF cells may not lead to the formation of APA. A second hit alongside the KCNJ5 mutation may be likely and/or necessary to lead to an APA phenotype. In a single pilot experiment in cultures of human adrenal cortex, enriched for glomerulosa/capsular cells, we obtained only a modest (at best) stimulation of aldosterone production, and no increase in CYP11B2 mRNA with high doses of mKCNJ5 (data not shown). The inherent challenge herein is the ability to obtain Ang II responsive ZG cells due the normal adrenal not having a distinct ZG, but rather few, scattered CYP11B2 expressing cell clusters[31].

4.7.2. Alternatives to primary human adrenal cells

An obvious alternative to the use of primary cultures of human adrenals would be bovine adrenal cells. Bovine adrenals yield much larger amounts of cells, and have been used successfully in numerous studies on the regulation of adrenal steroidogenesis. However, these cells have two main caveats; 1) a common CYP11B isozyme performs the activities of both CYP11B1 and CYP11B2 [62-65], and 2) similar to human adrenal cells, bovine cells also seem to lose glomerulosa-like phenotype rather quickly in culture. However, bovine adrenals have been used to efficiently study other steroidogenic enzymes, such as StAR [260-263, 279]. Rodent adrenals express ZG specific CYP11B2 and ZF localized CYP11B1, similar to humans. Previous studies have standardized techniques to isolate rat capsular/glomerulosa cells [85, 134, 280]. However, a single experiment would require adrenals from numerous rats, which is a major limitation. Furthermore, rat adrenals also differ from human adrenals in that they do not express CYP17, and therefore, do not possess a functional androgen-producing zona reticularis. Rabbit adrenals which exhibit physiology similar to that of humans (similar in zonation and steroid production) would be a useful alternative, albeit expensive. Finally the decision for primary cultures of adrenal cells has to be made with at least some knowledge regarding the expression of KCNJ5 and other K channels such as TWIK-related acid-sensitive K (TASK) channels, TASK 1-3, due to potentially differing functions of these channels across species. As such, knock out models for TASK 1 and/or TASK 3 have exhibited primary aldosteronism with gender bias (as described in section 1.6.1.) [281-283]. More recently, an inverse relationship has been described between TASK 2 expression and aldosterone production [284]. Currently, studies

comparing the expression of K^+ and Ca^{2+} channels across humans, rats and mice by our group are underway (Nishimoto et al, in preparation). A similar study to include rabbits would define the possibility of rabbit adrenals being used in place of human adrenals.

4.8. Limitations of the study

4.8.1. Novel Y152C mutation

The description of the Y152C mutation was useful in better understanding germline mutations in PA. However, a major drawback was our inability to obtain DNA samples from family members to confirm the hereditary nature of the mutation. Access to the single index case with the Y152C mutation also did not permit statistical tests in micrcoarray analyses. Finally, the *in vitro* analyses also included the technical drawbacks that come with electroporation strategy of gene expression. It was in recognition of those caveats that the HAC15-TRE-mKCNJ5 cell line was developed.

4.8.2. Limitations of *in vitro* analyses of T158A mutation

The study utilizing the HAC15-TRE-mKCNJ5 had its limitations. Firstly, our model system was unable to differentiate between mutant and wildtype mRNA and protein for KCNJ5. This limitation arises from the lack of antibodies that are specific to wildtype and mutated KCNJ5 channel proteins. A potential solution was the use of an approach of tagging the T158A transgene. We considered this option as being potentially risky due to the possibility of interference of the tag in channel activity. While real time genotyping assays for mRNA allow for the identification of a point mutation in KCNJ5, they lack the ability to quantitate the percentage of the WT and mKCNJ5 mRNA. Secondly, in a dose

dependent transduction of constitutive mKCNJ5 in HAC15 carcinoma cells (data not shown), a relatively large dose (MOI 20) of the constitutive virus was required to cause any elevation in aldosterone production in HAC15 cells. This could cause speculation whether the observed increase in KCNJ5 mRNA and protein after doxy treatment, is also, in part, due to cells increasing their endogenous WT KCNJ5 expression as a compensatory mechanism to attain resting membrane potential.

4.9. Summary and conclusion

Overall, this study has: 1) defined a novel germline mutation in PA that underlies aldosterone excess, 2) demonstrated the role of acute events (including a role for StAR) and 3) defined a role for chronic transcriptional events (including a role for ATF2 and NURR1) in the pathology of PA caused by mutations in the KCNJ5 selectivity filter and 4) confirmed the possibility of verapamil as an effective therapeutic strategy in the inhibition of mKCNJ5 mediated aldosterone production.

Moreover, our data have re-emphasized the need for 1) development of better methods for enrichment of zonal cultures of human adrenal cells which preserve the intracellular calcium responses, 2) generation of animal models for the study of PA for further understanding the origin and development of APA and genetic mutations leading to PA, 3) in vivo validation of verapamil as a therapeutic inhibitor of PA caused by mutated KCNJ5 and finally 4) the development of inhibitors specific to mutated KCNJ5 channel.

CHAPTER V

Appendix

(Additional Publications)

INTRODUCTION TO APPENDIX

The Appendix constitutes some of my contributions in projects other than those that are directly related to my main thesis work.

Along with my studies on the mechanisms of mutated KCNJ5 in primary aldosteronism, I also worked on the microarray comparison of aldosterone producing adenoma (APA) with and without KCNJ5 mutations. During this transcriptome analysis, we identified sporadic elevated expression of the gonadotropin releasing hormone receptor (GnRHR) in APA, suggesting a potential role of GnRH in the regulation of aldosterone. While numerous studies have detailed the expression of GnRHR and various G-protein coupled receptors in pathologies that involve dysregulation of steroidogenesis, thus far, these reports have not defined the functional significance of GnRHR in the causation of disease. The manuscript in **Section I**, was a study performed in co-operation with our long standing collaborators from Tohoku University, Sendai Japan. This focused on mechanistic definitions of GnRHR activation in aldosterone production. By the generation of doxycycline-inducible GnRHR cell line, we demonstrate that chronic activation of GnRHR leads to increased CYP11B2 expression and aldosterone production, by a mechanism involving the activation of calcium signaling. Activation of the GnRH receptor may constitute a distinct pathogenesis and phenotype in some cases of PA.

In addition to studying the regulation of aldosterone in pathological conditions, I also had the opportunity to work on a project aimed at defining the regulation of CYP11B2 expression in normal physiological conditions. Interestingly, steroidogenic factor-1 (SF-1), which is a known activator of most steroidogenic enzymes in the adrenal, has been found to repress the expression of only CYP11B2. The research in **Section II** of Chapter V, defines the mechanisms underlying these inhibitory effects of SF-1. Herein we demonstrate that SF-1 inhibition of CYP11B2 expression occurs at multiple levels, including the blockade of NURR1 expression. However, this mechanism may not include the repression of intracellular calcium levels. Other factors such as post-translational modification of SF-1 are potential key players in the repression of CYP11B2 transcription. Further studies in this direction are currently underway and will

add to our understanding of adrenal zonation, aldosterone biosynthesis and aldosterone excess disorders.

Section III of the Appendix is a review article under the guidance of Drs. Rainey and Bollag, which provided me the opportunity to appreciate years of *in vitro* and *in vivo* studies defining the regulation of aldosterone production. In this review we specifically focus on summarizing the key molecular events involved in the acute and chronic phases of aldosterone secretion induced by physiological agonists: Angiotensin II (Ang II), potassium (K+) and adrenocorticotropin (ACTH). Imbalance in any of these processes may lead to several disorders of aldosterone excess.

The manuscript **Section IV** was one of the first few studies to define the effects of mutated KCNJ5 in APA and in adrenal cells. My contribution in this article was the comparison of transcriptomes of APA with and without KCNJ5 mutations by microarray analyses, followed by confirmation of candidate genes by real time quantitative PCR on APA tissue. In this study, we report a 38 % (18/47) prevalence of KCNJ5 mutations in APA. *In vitro* expression of the then defined G151R and L168R mutations indicated an increase in transcript levels of CYP11B2 and NURR1. This research was also the first study that initiated me into my dissertation studies on the role of mutated KCNJ5 in sporadic and familial primary aldosteronism (PA). During the process of working on this manuscript, I also learnt to appreciate the caveats of this study, and have accordingly planned my methodologies in my dissertation project.

Finally, the manuscript in **Section IV** has allowed me to continue to build on the prevalence studies. Since this the publication of this manuscript in 2012, we have increased our cohort to include more than 150 APA. I have screened these APA for mutations in KCNJ5 as well as other recently described genetic mutations such as those in ATPase pumps and calcium channels. A current side project now focuses on demonstrating the transcriptome differences between APA harboring the various different mutations for better understanding of the pathogenesis of PA.

SECTION I

ABERRANT GONADOTROPIN-RELEASING HORMONE RECEPTOR (GNRHR) EXPRESSION AND ITS REGULATION OF CYP11B2 EXPRESSION AND ALDOSTERONE PRODUCTION IN ADRENAL ALDOSTERONE-PRODUCING ADENOMA (APA).

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ABSTRACT

Aberrant expression of gonadotropin-releasing hormone receptor (GnRHR) has been reported in human adrenal tissues including aldosterone-producing adenoma (APA). However, the details of its expression and functional role in adrenals are still not clear. In this study, quantitative RT-PCR analysis revealed the mean level of GnRHR mRNA was significantly higher in APAs than in human normal adrenal (NA) (P=0.004). GnRHR protein expression was detected in human NA and neoplastic adrenal tissues. In H295R cells transfected with GnRHR, treatment with GnRH resulted in a concentration-dependent increase in CYP11B2 reporter activity. Chronic activation of GnRHR with GnRH (100 nM), in a cell line with doxycycline-inducible GnRHR (H295R-TR/GnRHR), increased CYP11B2 expression and aldosterone production. These agonistic effects were inhibited by blockers for the calcium signaling pathway, KN93 and calmidazolium. These results suggest GnRH, through heterotopic expression of its receptor, may be a potential regulator of CYP11B2 expression levels in some cases of APA.

Key words: Gonadotropin-releasing hormone receptor (GnRHR), aldosterone, aldosterone producing adenoma (APA)

INTRODUCTION

Aldosterone-producing adenoma (APA) represents the major cause of hyperaldosteronism. Growing evidence suggests that many adrenal tumors have aberrant expression of G-protein coupled receptors that lead to inappropriate growth and steroid hormone production[94]. The gonadotropin-releasing hormone receptor (GnRHR), also known as the luteinizing hormone-releasing hormone receptor (LHRHR), is a member of the G protein-coupled receptor (GPCR) family. GPCR is known to play roles in many biological processes, including cell proliferation and metabolism[285]. Recently, it was demonstrated that GnRHR mRNA is highly expressed in some cases of APA [94, 180]. Ziegler *et al.* reported GnRHR expression in human normal adrenal gland, adrenocortical adenoma and the SW-13 adrenocortical tumor cell-line[286].

A number of studies involving gonadotropin-releasing hormone (GnRH)-mediated signal transduction indicate that GnRHR activates both adenylate cyclase and phospholipase C via G-protein coupled receptors[287, 288]. However, the role of GnRHR in the regulation of adrenal function is still not clear. Herein, we examined the expression of GnRHR protein in human normal and neoplastic adrenal tissues. In addition, we performed quantitative RT-PCR (qPCR) analysis in order to confirm the statistical significance of the mRNA levels transcribed. In addition, we developed an adrenocortical cell line with doxycycline (doxy)-inducible GnRHR to examine the functional role of GnRHR in regulation of CYP11B2 expression and aldosterone production.

MATERIALS AND METHODS

Subjects and tissues

APA, cortisol-producing adenoma (CPA), non-functioning adenoma lacking clinical hormonal abnormalities (NFA), adrenocortical carcinoma (ACC) and normal adrenal gland (NA) resected with renal carcinoma were retrieved from the surgical pathology files of Tohoku University Hospital. For immunohistochemistry, the specimens were fixed in 10% formalin for 24-48 h at room temperature and embedded in paraffin wax. Western or immunoblotting analysis was performed using lysates from frozen tissue specimens. In qPCR analysis, NA and APA were obtained through the Department of Medicine at UT Southwestern, the Cooperative Human Tissue Network (Philadelphia, PA) and Clontech (Palo Alto, CA). Adrenal samples were acquired from autopsy (NA) performed within 6 h after death where the cause of death was unrelated to adrenal functions. Adenoma samples were obtained at the time of surgery. Specimens for RNA isolation were snap-frozen and stored at -80 °C until use. Research protocols for this study were approved by the ethics committee at Tohoku University School of Medicine, the Institutional Review Boards of the University of Texas Southwestern Medical Center (Dallas, TX, USA) and Georgia Health Sciences University (Augusta, GA).

RNA extraction and reverse transcription reaction

Total RNA from NAs (n=16) and APAs (n=20) was extracted using TRIzol Reagent (50-100mg/ml) (Invitrogen, Carlsbad, CA). The purity and integrity of the RNA were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo

Alto, CA) and its quantity was determined by the NanoDrop spectrophotometers (ND1000, NanoDrop Technologies, Wilmington, DE). Total RNA of 2 µg was reverse transcribed using the High Capacity cDNA Archive Kit (Life Technologies, CA) according to the manufacturer's recommendations and incubated at 25 °C for 10 min and 37 °C for 2 h. The synthesized cDNA was subjected to 1:10 dilution and stored at -20 °C.

qPCR analysis

The primer and probe set for human CYP11B2 was designed using Primer Express 3.0 (Life Technologies, CA) and purchased from IDT (Integrated DNA Technologies Inc., Coralville IA, USA) as published previously (Saner-Amigh *et al.* 2006). Taqman gene expression assays used for GnRHR RNA detection was commercially obtained from Life Technologies, CA (Ye *et al.* 2007). For the GnRHR reaction, the 20µl total volume consisted of TaqMan Fast Universal PCR Master Mix (2×) (Life Technologies, CA) 10µl, 900nM of each primer, 400nM of the probe and 5µl of each first-strand cDNA sample. CYP11B2 reaction mix consisted of 10µl TaqMan PCR Master Mix (2×) (Life Technologies, CA), 100nM of primer/probe mix and 5µl of each first-strand cDNA sample (20ng). Each reaction included 10µl of TaqMan PCR Master Mix (2×) (Life Technologies, CA), 100nM probe and 50nM each primer (Ye *et al.,* 2007)._Quantitative normalization of cDNA in each tissue-derived specimen was performed using expression of18S rRNA (tissue) or cyclophilin (PPIA) (for transduced cells) as an endogenous internal controls. 18s mRNA was detected and quantified using TaqMan Ribosomal RNA Control Reagents (Vic Probe) (Applied Biosystems) (Ye *et al.,* 2007). PPIA was detected

and quantified using commercially available TaqMan gene expression assay (FAM Probe) (Life Technologies, CA) (Monticone *et al.*, 2012). Negative controls contained water instead of first-strand cDNA. The generated C_t value of each gene was normalized by its respected C_t value of 18S rRNA or PPIA (ΔC_t). Each gene was subsequently normalized using the average ΔC_t value of the normal adult adrenal or basal group ($\Delta\Delta C_t$). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$ [289].

Western blotting analysis

Human adrenal specimens were homogenized in a fivefold volume of the triple detergent lysis buffer, containing 50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml Nonidet P40, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml antipain and 0.5% sodium deoxycholate. The homogenates were centrifuged at 15,000 × *g* for 10 min, and the supernatant was subjected to western blot analysis. The protein extracts (15µg/lane) were electrophoresed on SDS polyacrylamide gels (10%) and then transferred to Immobilon-P transfer membrane (Millipore Corporation, Billerica, MA, USA). The membrane was incubated for 1 h with the antibody against GnRHR (sc-8682, Santa Cruz Biotechnology Inc.) (1:500). The membrane was washed several times with TBST-20 buffer and then incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (1:20,000; sc-2004, Santa Cruz Biotechnology Inc.) for 30 min. Immune complexes were visualized using an Immobilon western chemiluminescent HRP substrate (Millipore Corporation). Expression of □-actin was utilized as an internal control using anti-□-actin monoclonal

antibody (1:20,000; sc-81178, Santa Cruz Biotechnology Inc.) and its secondary antibody (goat anti-mouse IgG-HRP, 1:20,000; sc-2005, Santa Cruz Biotechnology Inc.).

For quantification of protein expression, the relative immunointensity of the bands at 38 kDa for GnRHR (NCBI Reference Sequence: NP_000406.2) or at 42 kDa for □-actin were determined using NIH Image software.

Immunohistochemistry

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan) and goat polyclonal antibody for GnRHR (sc-8682) (Santa Cruz Biotechnology, Inc.). Antigen retrieval was performed by heating the slides in an autoclave at 120 C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of GnRHR antibody used in this study was 1:300. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Normal goat IgG was also used in place of the primary antibodies as a negative control.

Cell culture

H295R human adrenocortical tumor cells were cultured in Dulbecco's modified Eagles/Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT) and antibiotics. The adrenocortical H295R-TR cells expressing the tet repressor were transduced with the doxy-inducible GnRHR lentiviru[94].

Cells were cultured as previously described (Ye et al 2009). Cells were subcultured and treated with agonists Ang II (10 nM) (Sigma-Aldrich, St. Louis, MO) or GnRH alone or with inhibitors KN93 (1 μM) (EMD Millipore USA, Billerica, MA) calmidazolium (0.3 μM) (Sigma-Aldrich, St. Louis, MO). Doxy (0.25 μg/ml) was present throughout the experiment.

Plasmids

The subcloned chimeric construct containing the human *CYP11B2* 5'-flanking region from -1521 to +2 relative to the transcription start site fused upstream of the luciferase cDNA (pGL3-Basic, Promega, Madison, WI) was used for the transient transfection study[249]. The expression construct of human GnRHR, encoding the full-length human GnRHR, was purchased from the UMR cDNA Resource Center (Rolla, MO). The human GnRHR was subcloned into the pLenti-TO-V5/DEST vector system (Life Technologies, Carlsbad, CA) to generate a lentivector expressing doxy-inducble GnRHR. Lentiviruses were prepared in HEK293T cells as previously described [239].

Transient Transfection assays of CYP11B2-luciferase reporter plasmid and GnRHR expression plasmid

For transfection experiments, cells were subcultured onto 12 well dishes at a density of 400,000 cells per well. Transfections were carried out, for 6 hours, using the transfection reagent Transfast (Promega, Madison, WI) according to manufacturer's directions. To normalize luciferase activity, cells were co-transfected with 0.05μg/well of β-galactosidase plasmid (Promega). Following recovery of the

cells, for 20-24 hours, they were treated with increasing concentrations of human GnRH (Sigma, St. Louis, MO) (3nM, 30nM, 300nM and 3,000nM) for 6 hours prior to being lysed and assayed for activity using the Luciferase assay system (Promega).

Effect of GnRHR activation on endogenous CYP11B2 and aldosterone production in H295R-TR/GnRHR cells

Cells were plated at a density of 50,000 cells per well in a 48 well dish in growth medium containing 2% NuSerum and 1% ITS and incubated at 37°C for 48 h (6 replicates). Eighteen hours prior to starting the experiments, the cells were changed to a low-serum experimental medium (DMEM/F12 supplemented with 0.1% NuSerum and antibiotics). The cells were subsequently treated in the same low-serum experimental medium with indicated agonists and/or calmodulin inhibitor (calmidazolium) or calcium calmodulin kinases inhibitor, KN93. Cells stimulated with 10 nM Ang II served as a positive control, and non-treated cells were considered as the basal (control). At the end of 4 days of incubation, media was collected for aldosterone measurement, protein was isolated from one well per treatment, and RNA was isolated from the remainder of the replicates. cDNA was generated from the RNA and analyzed for gene expression using qPCR. Each bar represents the fold increase ± S.D. over basal (no treatment, control) by the $2^{-\Delta\Delta Ct}$ method, using human PPIA for normalization. Aldosterone measurements were determined as described earlier[94], using the aldosterone Coat-A-Count RIA kit from Siemens (New York, NY), following the manufacturer's guidelines. Aldosterone levels were normalized to protein.

Statistical analysis

For qPCR results, the data obtained were analyzed and compared with control values (mean of normal adrenal samples) using the Mann–Whitney Rank Sum test with the SigmaStat 3.0 software package (SPSS, Chicago, IL, USA). Results of other *in vitro* experiments were analyzed by ANOVA followed by post hoc Tukey test. The results were considered significantly different when the *P* value was ≤ 0.05.

RESULTS

GnRHR mRNA expression in APA VS NA

The results of qPCR analysis demonstrated that GnRHR mRNA expression in all of the 16 NAs (100%) were within the normal range (defined as±2SD from the mean of the normal adrenals) but only 9 of 20 APA samples (45%) within the normal range (Figure 5.1.1). Two APA samples had more than a 120-fold elevation in GnRHR expression over the normal controls (Figure 1). The mean level of GnRHR mRNA was significantly higher in APAs than in NAs (p=0.004) (Figure 5.1.1).

GnRHR protein expression in adrenocortical tissues

GnRHR protein expression in human adrenal tissues was studied using Western blot analysis (Figure 5.1.2, Panel A). The human adrenal tissue extracts, including APA, CPA, NFA, ACC and NA, demonstrated two bands with a size of about 38 kDa. Since the presumed molecular weight of human GnRHR is 37,600 (NCBI Reference Sequence: NP_000406.2), either of these two bands presumably

represents GnRHR protein and the other may be a GnRHR protein with some modification, such as glycosylation. Semi-quantitative analysis of the bands corresponding to GnRHR and □-actin did demonstrate that relative expression levels of GnRHR protein in 4APAs were 1.2-fold higher than those in 4 NAs, but not statistically significant (P=0.20) (Figure 5.1.2, Panel B).

As predicted from the Western results, immunoreactivities for GnRHR were sparsely detected in all the adrenal cases including NA (in the zona glomerulosa (ZG) and fasciculata (ZF)), APA, CPA, NFA and ACC (Figure 5.1.3). The immunopositivities were found in the cell membrane as well as the cytoplasm, compatible with a previous report[290, 291]. Immunohistochemical analysis revealed that GnRHR positive tumor cells were sparsely distributed in APA, and the mean ratio of the positive cells was approximately 10% in the whole tumor area of the cases examined (data not shown).

Concentration-dependent effects of GnRH on CYP11B2 reporter gene activity and in H295R cells

In H295R cells co-transfected with CYP11B2 and GnRHR, treatment with GnRH significantly increased CYP11B2 promoter activity starting at 30nM compared to basal levels (approximately 4-fold) (P<0.05) (Figure 5.1.4). As a positive control, we also confirmed that angiotensin II (Ang II) (100nM) stimulated CYP11B2 reporter activity (approximately 8-fold) in this experimental model (data not shown).

Effect of GnRHR activation on endogenous CYP11B2 transcript levels and aldosterone production

H295R-TR/GnRHR cells treated doxy elevated GnRHR mRNA by 23-fold (P<0.001) (Figure 5A). Treatment with GnRH (100 nM) demonstrated significant increase in GnRHR mRNA by 8-fold (P<0.001) (Figure 5.1.5, Panel B), CYP11B2 mRNA by over 100-fold (P<0.001) (Figure 5.1.6), and aldosterone production by 50-fold (P<0.001) (Figure 5.1.7.). In addition, the treatment with calcium signaling inhibitors calmidazolium and KN93, inhibited the stimulatory effects of GnRH on CYP11B2 mRNA expression by 30% and 60 %, respectively. Similarly the stimulation in aldosterone production was inhibited by KN93 and calmidazolium by 95% and 40%, respectively. The positive control consisting of the cells treated with 10 nM Ang II for 4 days, demonstrated a 4-fold increase in aldosterone production.

DISCUSSION

GnRHR is abundantly expressed in pituitary gonadotropic cells, where it can trigger the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) activated by GnRH secreted from the hypothalamus[292]. In addition, GnRHR has also been detected in many extra-pituitary tissues including some tumor cells[293-299]. Results of our present study demonstrated that GnRHR mRNA and protein were expressed in human normal and neoplastic adrenal tissues including APA, consistent with those in previous reports[180, 286, 290, 291]. In the results of Western blotting analysis, GnRHR protein levels tended to be higher in APA than NA although statistically not significant possibly due to limited number of adrenal cases examined in

this study. However, we could not deny the possibility that increased expression of GnRHR mRNA did not result in increased GnRHR protein expression in APA. It awaits further study for clarification in the future.

In human tissues, the ligand for GNRHR, GnRH, has two forms: GNRH1 and GNRH2[293]. It has been reported that GNRH1 is mainly expressed in the hypothalamus, and GNRH2 is expressed at significantly higher levels outside the brain, including the kidney, bone marrow and prostate [290, 300]. On the other hand, we have previously reported that in human adult adrenal, predominant isoform of GNRH is GnRH1, and the mRNA level is approximately 8-fold lesser compared to human adult hypothalamus [94]. Taken together, it is postulated that the aberrant GNRHR in human adrenal tissue may be controlled by the paracrine/autocrine action of GNRH1 expressed in the adrenal gland.

It was previously reported that three patients with APA showed increased serum aldosterone levels following GnRH stimulation, suggesting the expression of GnRHR in these adrenal tissues [180]. Albiger and colleagues were the first to report a case of primary aldosteronism (PA) during pregnancy with response to GnRH and human chorionic gonadotropin (hCG) administration, which also suggested the roles of aberrant expression of non-adrenal GPCRs as one of the sporadic causes of PA [290]. They further demonstrated, in a series of non-pregnant PA patients, that aberrant GnRHR was expressed and several of these patients increased aldosterone secretion in response to GnRH administration [290]. Therefore, the ability of GnRHR to regulate intracellular signaling pathways known to activate aldosterone production made GnRHR as a potential candidate for regulator of APA steroidogenesis. Results of our present *in vitro*

study also demonstrated that GnRH ligand elevated the promoter activity and expression level of CYP11B2 in H295R cells transfected with the GnRHR vector. In this study, a second adrenocortical cell line, H295R-TR/GnRHR with doxy-inducible GnRHR was also used to study the effect of GnRHR on endogenous CYP11B2 regulation in order to further confirm the results from reporter analyses in H295R cells. Chronic GnRH (100 nM) treatment increased CYP11B2 mRNA expression and aldosterone production An inhibition of this stimulatory effect by inhibitors calmidazolium and KN93 confirmed the involvement of calcium signaling in CYP11B2 transcript stimulation through GnRHR activation. This stimulation in CYP11B2 expression and aldosterone production may be attributed in part to the observed increase in cell proliferation caused by GnRHR activation (data not shown). It may be speculated that APA with elevated GnRHR levels may exhibit increased cell proliferation, CYP11B2 expression and aldosterone production. Indeed, GnRH stimulation has shown to have anti-apoptotic effects in ovarian cancers[301, 302]. Furthermore, the increase in aldosterone production was also paralleled with decrease in the production of androstenedione by 50% without stimulation of cortisol production with 4 days GnRH treatment (data not shown), indicating a possible divergence of pathways towards mineralocorticoid production. It may therefore be speculated that chronic activation of GnRHR in adrenocortical cells triggers several different signaling cascades, including calcium signaling pathways that increase aldosterone production and/or pathways that may increase cell proliferation. The somatic mutations of the KCNJ5 potassium channels have been recently reported to result in the loss of ion selectivity by the cellular membrane, and hyper-activation of voltage gated Ca^{2+} channels, causing the increment of the intracellular levels of calcium

in APA [3, 5]. In addition, somatic mutations in ATP1A1 and ATP2B3 have been very recently reported in APA, both of which were considered to increase intracellular calcium ion concentrations, stimulating calcium-dependent signaling and aldosterone output [233]. Therefore, it becomes pivotal to examine the association between GnRHR expression levels and these somatic mutations. However, in this study, we could not perform the analysis due to the limitation of tissue availability, and it awaits further study for clarification in the future.

In summary, our current study demonstrates that human normal and neoplastic adrenal tissues expressed GnRHR. The increased GnRHR mRNA expression was also detected in APAs compared to normal adrenal cortex. We also demonstrated that GnRHR activation increased CYP11B2 promoter activity, CYP11B2 mRNA expression and aldosterone production. These findings all suggest novel and important roles of GnRHR in the regulation of adrenocortical functions.

FIGURES

Figure 5.1.1. Relative GnRHR expression in normal adrenals versus aldosterone producing adenomas. A vertical point scatterplot comparing GnRH receptor expression in normal adrenals *vs* aldosterone-producing adenomas. All 16 normal adrenals (100%) fell within the normal range (defined as ±2 SD from the mean of the normal adrenals) as opposed to only 9 out of 20 APA samples (45%). The difference in the mean values between the two groups is statistically significant (*$P < 0.05$).

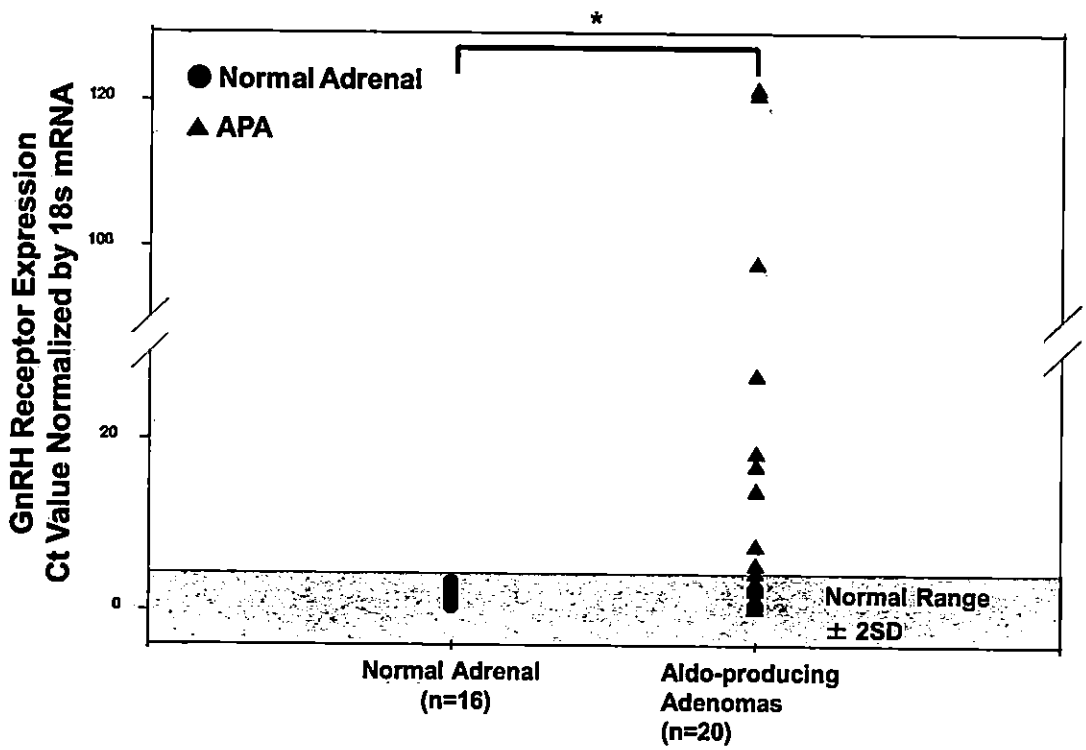
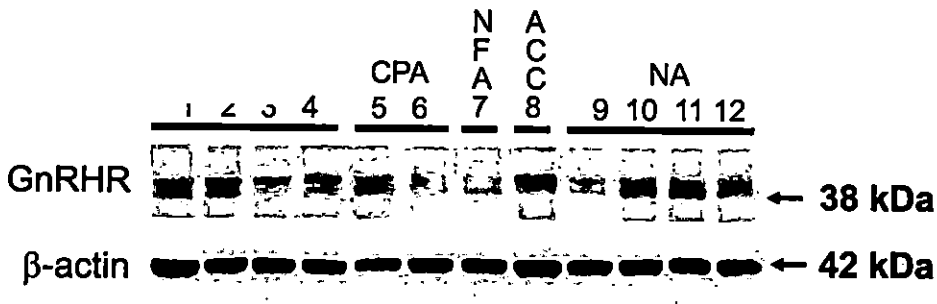


Figure 5.1.2. GnRHR protein expression in aldosterone-producing adenoma (APA), cortisol-producing adenoma (CPA), non-functioning adenoma (NFA), adrenocortical carcinoma (ACC) and normal adrenal gland (NA). (A) Western blotting analysis of GnRHR and β-actin in aldosterone-producing adenoma (APA), cortisol-producing adenoma (CPA), non-functioning adenoma (NFA), adrenocortical carcinoma (ACC) and normal adrenal gland (NA). Western blot analysis was repeated twice and similar results were obtained. (B) Relative expression levels of GnRHR protein in tissues of APA (n=4) and NA (n=4). The relative immunointensity of the band representing GnRHR protein was normalized with that of □-actin protein. The ratio of each normalized value to that of NA was demonstrated as the relative expression levels of GnRHR. Data are shown as mean + SEM.

(A)



(B)

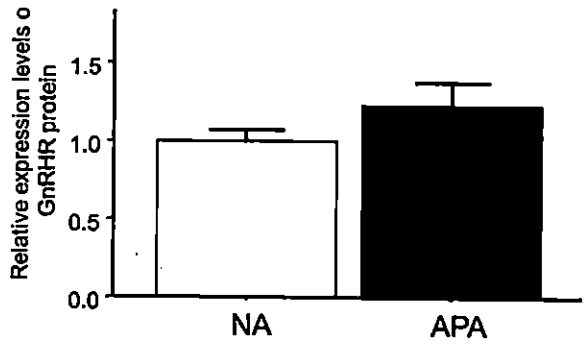


Figure 5.1.3. Immunohistochemistry for GnRHR in the cortex of normal adrenal gland (NA), aldosterone-producing adenoma (APA), cortisol-producing adenoma (CPA), non-functioning adenoma (NFA) and adrenocortical carcinoma (ACC). We performed the immunohistochemical analysis at least in five cases. In the representative figures, the immunopositive cells were sparsely detectable in the zona glomerulosa (ZG) and fasciculate (ZF) of NA, and these tumors. The percentage of GnRHR-positive cells in the representative cases were approximately 5% in NA, 10% in APA, 5% in CPA, 10% in NFA and 5% in ACC, respectively. *Bar*, 100 μm.

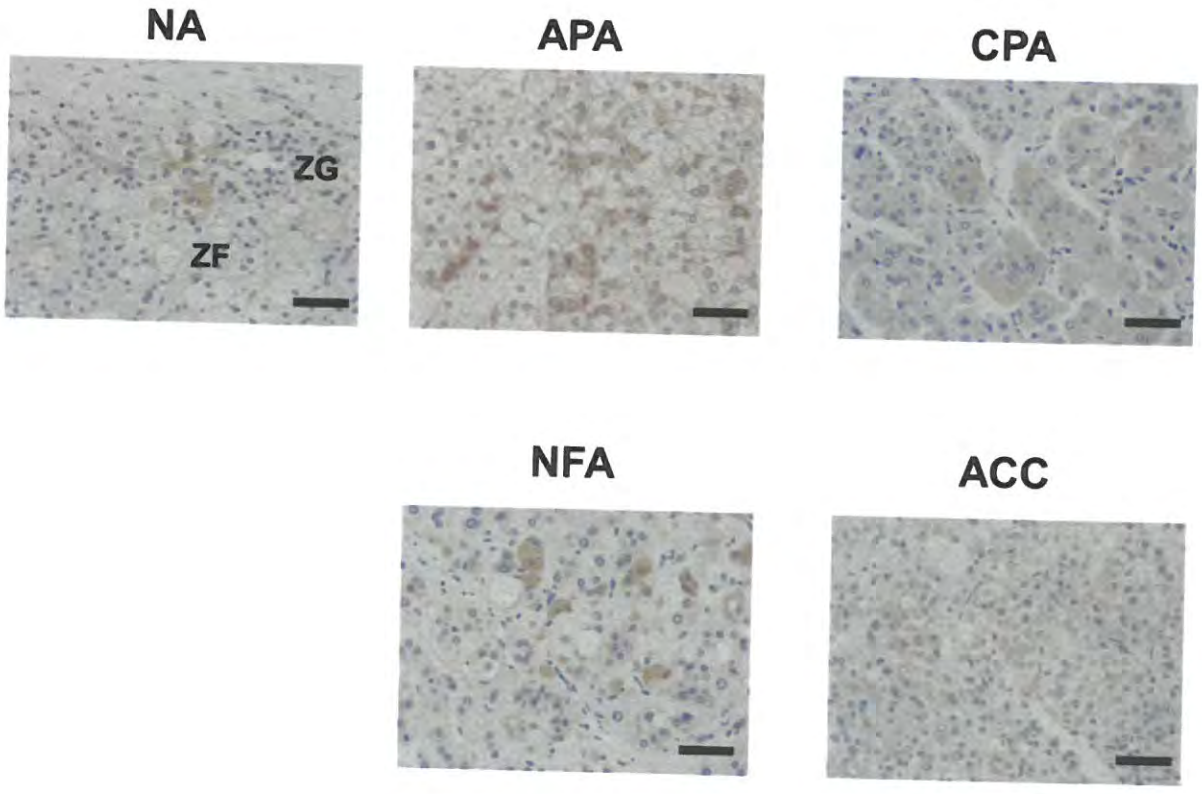


Figure 5.1.4. Concentration-dependent effects of GnRH on CYP11B2 reporter gene activity in H295R cells co-transfected with GnRHR-containing vector. H295R cells were co-transfected with CYP11B2 luciferase reporter constructs at a concentration of 1 µg/well, along with GnRH receptor expression plasmid, at a concentration of 0.3 g/well. Cells were allowed to recover overnight at 37°C then treated with the indicated amounts of GnRH for 6 h and then lysed and assayed for luciferase activity. Data were normalized to co-transfected β-galactosidase. Data shown were expressed as fold induction over basal reporter plus GnRH receptor. Results represent the mean +/- SEM of data from at least three independent experiments, each performed in triplicate (* $P < 0.05$, *vs.* Basal).

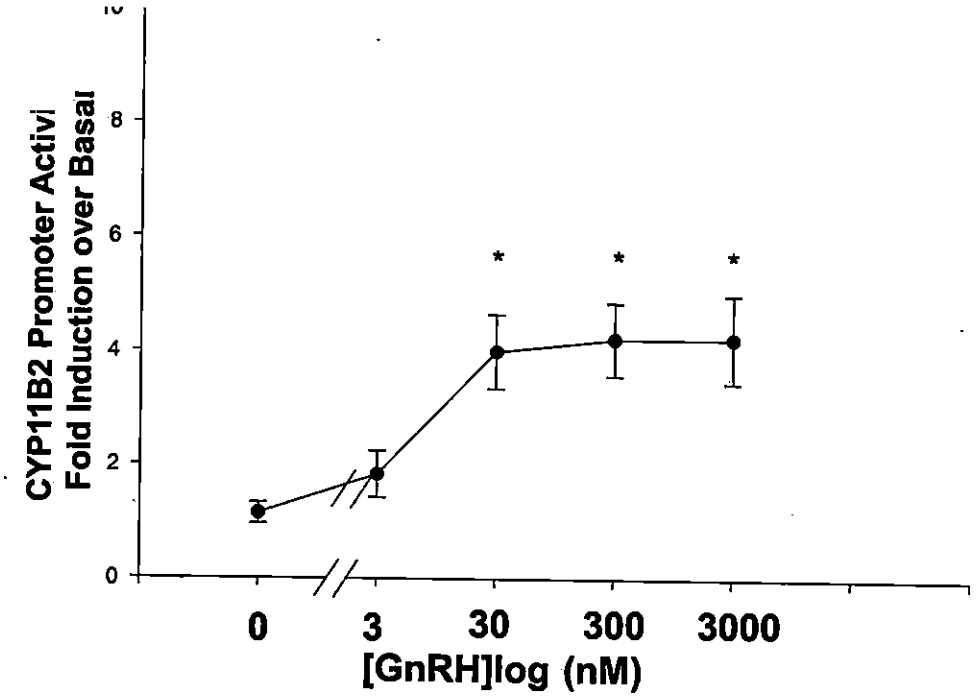


Figure 5.1.5. Comparison of GnRHR expression in H295R-TR/GnRHR cells (A) H295R-TR/GnRHR cells (6 replicates) were plated in growth media without (Basal) or with (Basal+Doxy) the presence of doxycycline for 48 h. Cells were treated with low serum (LS) media for 4 days. RNA was then isolated for cDNA generation and quantification of mRNA expression levels of GnRHR. **(B)** H295R-TR/GnRHR cells (6 replicates) were plated for 48 h. Media was then replaced with fresh LS GnRH (100 nM). After 4 days, RNA was isolated for cDNA generation and quantification of GnRHR mRNA expression levels. Media also contained 0.25µg/mL doxy, throughout this experiment. Bars represent the Mean ± S.D. of fold increase in mRNA levels versus basal and normalized to PPIA, (*P <0.001 *vs.* Basal, ¥ P <0.001, *vs.* Basal + Doxy).

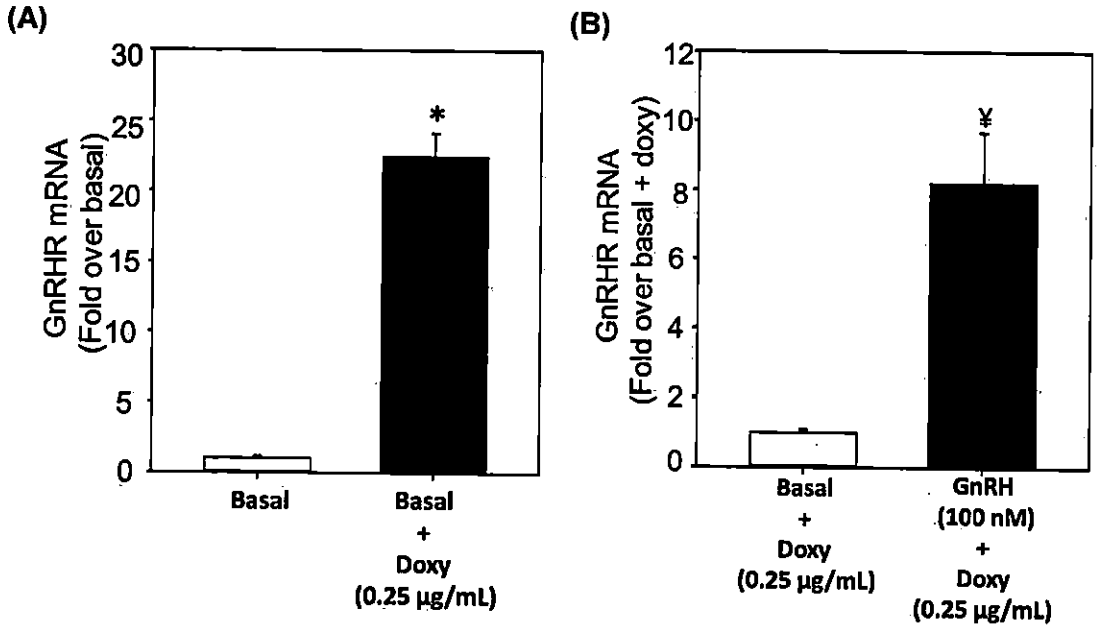


Figure 5.1.6. Effect of chronic GnRH treatment on CYP11B2 expression. H295R-TR/GnRHR cells (6 replicates) were pre-treated with 0.3 µM calmidazolium (calmodulin inhibitor) or 1 µM KN93 (calcium calmodulin kinases inhibitor). Media was then replaced with fresh LS containing inhibitors and agonists Ang II (10 nM) or GnRH (100 nM). After 4 days RNA was isolated for cDNA generation and quantification of mRNA expression levels of CYP11B2. Bars represent the Mean ± S.D. of fold increase in mRNA levels *vs* Basal and normalized to PPIA. Media also contained 0.25µg/mL throughout the experiment (**P* <0.001 *vs.* Basal, §*P* <0.001, *vs.* respective agonist).

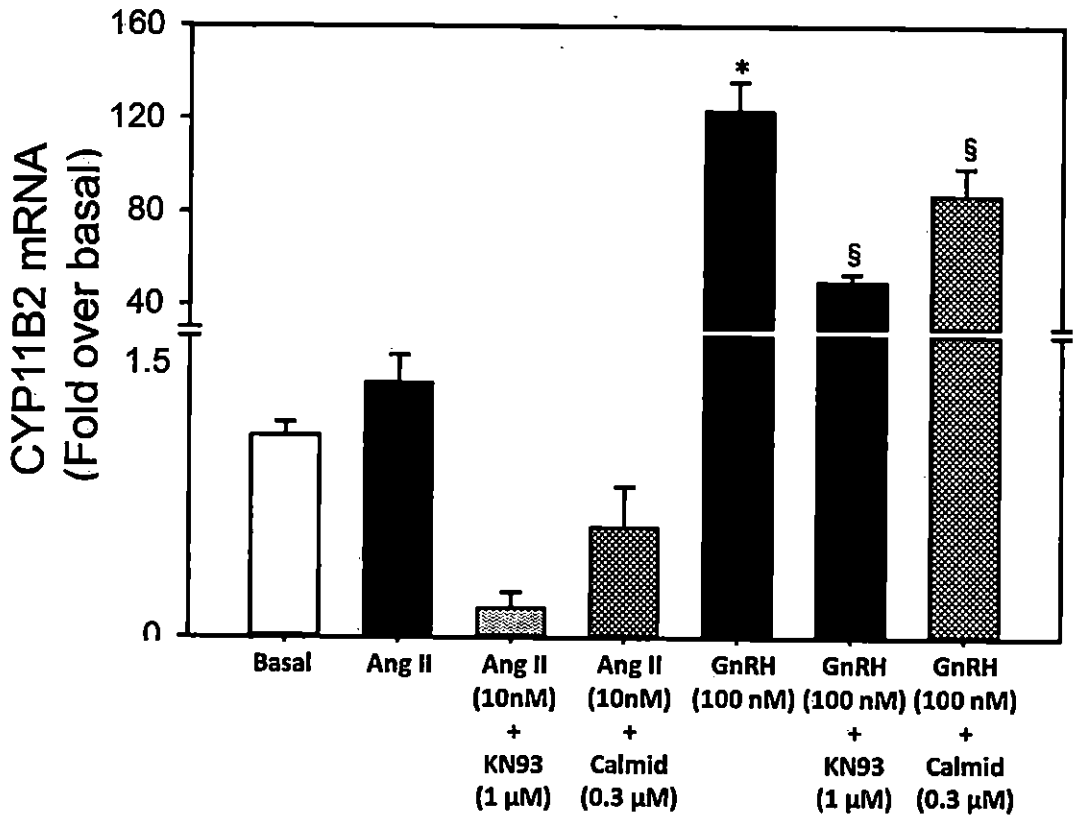
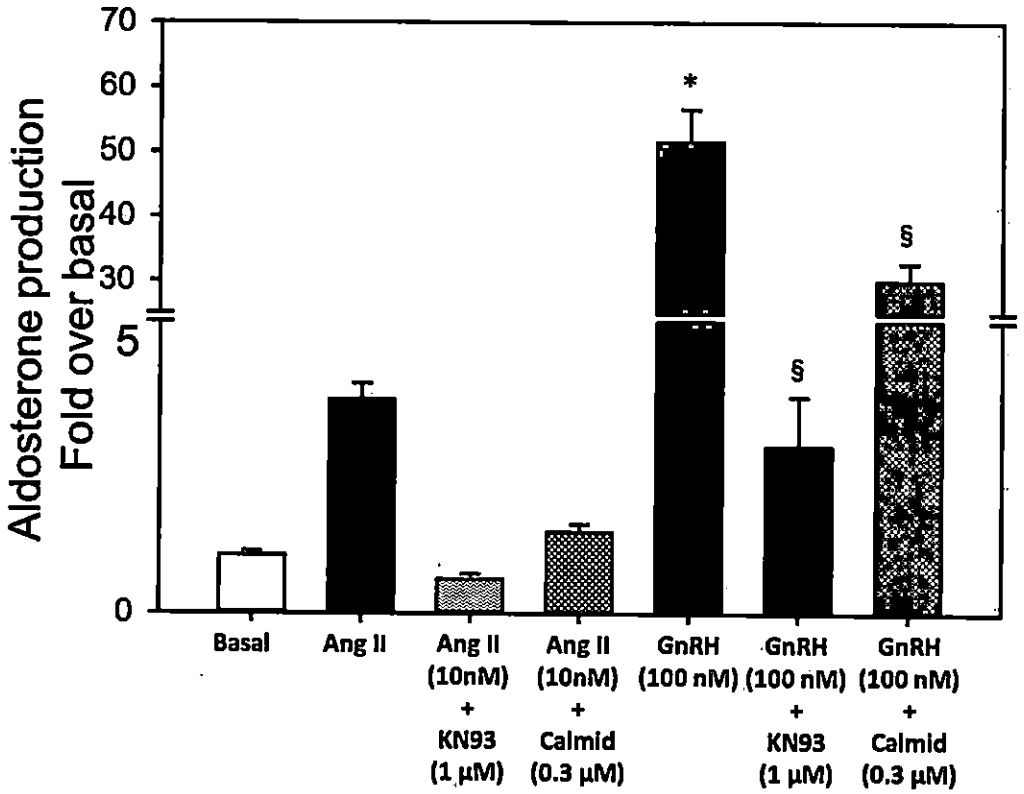


Figure 5.1.7. Effect of chronic GnRHR activation on aldosterone production. H295R-TR/GnRHR cells (6 replicates) were initially pre-treated with 0.3 µM calmidazolium (calmodulin inhibitor) or 1 µM KN93 (calcium calmodulin kinases inhibitor). Media was then replaced with fresh LS containing inhibitors and agonists Ang II (10 nM) or GnRH (100 nM). After 4 days, media was collected for aldosterone assay, and protein lysates from each treatment group were collected for normalization of steroid estimation. Bars represent the Mean±S.D. of fold increase in aldosterone levels per mg protein (*vs* Basal). Media also contained 0.25µg/mL doxy, throughout the experiment (*P<0.001 *vs.* Basal, §P<0.001, *vs.* respective agonist).



SECTION II

THE MOLECULAR MECHANISMS UNDERLYING STEROIDOGENIC FACTOR-1 (SF-1) MEDIATED REPRESSION OF ALDOSTERONE SYNTHASE (CYP11B2) EXPRESSION

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Running title: Repressive effects of SF-1 on CYP11B2

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Abstract

Background: CYP11B2 (aldosterone synthase) is required for the synthesis of aldosterone and is expressed mainly in the adrenal glomerulosa. Because dysregulation of aldosterone production is one of the major causes of endocrine hypertension, we have studied mechanisms regulating CYP11B2. We recently demonstrated that steroidogenic factor 1 (SF-1) increases the expression of most adrenal steroids, but represses that of CYP11B2.

Hypothesis: SF-1 represses CYP11B2 expression at the transcriptional level.

Methods: A doxycycline (doxy) inducible SF-1 adrenal cell model (H295R/TR/SF-1) was used. Microarray and quantitative RT-PCR were used to study transcription factor expression. The effect of SF-1 on CYP11B2 promoter activity and on adrenal calcium signaling involved in CYP11B2 expression was also studied.

Results: Doxy incubation caused time-dependent induction of SF-1 protein and mRNA that plateaued after 6 h. Microarray analysis indicated that elevated SF-1 (72 h) inhibited Ang II stimulated expression of CYP11B2 and an essential transcription factor, NURR1. The agonistic effects of Ang II, K^+ and forskolin on mRNA levels of CYP11B2 (6 h) and NURR1 (1 h) were repressed by elevating SF-1 levels. Nuclear protein levels of NURR1 were also inhibited by SF-1, although other transcription factors such as cFOS and ATF2 (data not shown) were not repressed. Thus, SF-1 mediated repression of NURR1 may be the mechanism in CYP11B2 regulation. Agonist stimulated elevation in intracellular calcium was also unaffected by elevated SF-1.

Conclusions: These studies indicate that SF-1 inhibition of CYP11B2 expression occurs at multiple levels including blockade of NURR1 expression, which may not include the repression of intracellular calcium levels. Other factors such as post-translational modification of SF-1 might be key players in the repression of CYP11B2 transcription. Further studies in this direction will add to our understanding of adrenal zonation, aldosterone biosynthesis and aldosterone excess disorders.

Key words: steroidogenic factor 1 (SF-1), aldosterone synthase (CYP11B2), adrenocortical zonation

INTRODUCTION

The mammalian adrenal cortex is divided into three concentric zones – the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR), each with different steroidogenic functions. The ZG synthesizes mineralocorticoids, the ZF produces glucocorticoids, and the ZR produces adrenal androgens. In humans, the gene CYP11B2, encoding the enzyme aldosterone synthase, is selectively expressed in the glomerulosa [28, 30, 303] where it catalyses the 11β- and 18-hydroxylation of deoxycorticosteorne, followed by 18-oxidation to produce aldosterone. Aldosterone maintains the blood pressure in normal and pathological conditions under the regulation of the renin-angiotensin-aldosterone system (RAAS). This zone specific transcription of CYP11B2 depends on the presence of various cis-elements and transacting factors that lie within the proximal 1000 basepairs upstream of the transcriptional start site. These include three key regulatory cis-elements: one cAMP response element (CRE) and two distal cis-elements (Ad5 and NBRE) that are able to bind members of the nerve growth factor-induced clone B family of transcription factors (NGFI-B or NR4A family).[82, 83, 85, 86, 249, 304]. These transcription factors are activated by the physiological regulators of aldosterone production that include, Ang II, K^+ and ACTH through distinct signaling cascades that increase binding to the regulatory elements and CYP11B2 transcription.

Inappropriate adrenal CYP11B2 expression is a cause of the adrenal disease, primary aldosteronism (PA) that also causes hypertension. PA, represents a form of low-renin hypertension that is characterized by high aldosterone but with suppressed renin. 35% of PA cases are due to the presence of a unilateral aldosterone producing adenoma

(APA) [305]. Hence defining the molecular mechanisms underlying CYP11B2 regulation proves important in understanding disorders of excess aldosterone secretion.

SF-1 plays a pivotal role in adreno-gonadal development and reproductive function [306, 307] as well as in the transcriptional regulation of almost all steroidogenic and reproductive enzymes [93, 94, 308-314]. SF-1 knock-out mice have adrenal and gonadal agenesis, impaired gonadotropin expression and abnormalities in the ventromedial hypothalamus [308, 314]. SF-1 primarily binds to variations of AGGTCA motif on the promoters of most steroidogenic enzymes [94, 308, 310] and activates the expression of most adrenal steoridogenic genes [93, 308, 314]. Recent studies by our group demonstrated that SF-1 enhances the expression of CYP11B1 while repressing that of CYP11B2 under basal and Ang II stimulated conditions [93, 94]. On the other hand, a basal level of SF-1 appears to be essential for the activation of enzymes required for steps of steroidogenesis upstream of CYP11B2. Thus the relative SF-1 expression seems to decide the role of SF-1 as inhibitory or stimulatory [94]. The molecular mechanism of this process is, as yet, unknown.

Herein, we define the molecular mechanism underlying SF-1 inhibition of aldosterone production and CYP11B2 transcription. We hypothesize that SF-1 blocks adrenal cell aldosterone production by inhibiting the agonist-mediated activation of key transcription factors required for CYP11B2 expression. Our study supports the regulation of SF-1 activity in the zonal expression of mineralocorticoids versus glucocorticoids in the human adrenal gland.

MATERIALS AND METHODS

Cell Culture

The development of the SF-1-inducible cell line H295R/TR/SF-1 has been previously described [315]. Cells were cultured in growth media consisting of DMEM/ F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 2% NuSerum (BD Biosciences, San Jose, CA), 1% insulin/transferrin/selenium Premix (BD Biosciences), and 1% penicillin/streptomycin (Life Technologies) and 0.1% gentamicin (Sigma-Aldrich, St. Louis, MO). Briefly, two cell lines were generated following transfection with the plasmid encoding the tet repressor (pcDNA6/TR) to establish the H295R/TR cells. These cells were then transfected with a doxycycline (doxy) responsive promoter driven SF-1 expression vector (pcDNA4/TO/SF-1). Antibiotic selection with blasticidin (5μg/ml) and zeocin (100μg/ml) was used to isolate clones over-expressing SF-1 in a doxy-inducible fashion. For the time dependent SF-1 curve, cells were plated at a 200,000 cells/well in 24-well Costar dishes for 48 hours, and after overnight starvation, the media was replaced with fresh media with or without (basal) doxy (0.25 μg/ml) for 0.5, 1, 3, 6 and 24 h. RNA and protein were isolated for real time quantitative PCR and western blot as described below. For other experiments, H295R/TR/SF-1 cells were subcultured, plated at 400,000 cells per well in 12 well costar plates for 48 h, starved overnight and treated with or without doxy (0.25 μg/ml) for 6 h. This was followed by treatment with agonists Ang II (10 nM), K^+ (18 mM) and forskolin (10 μM) for 6 h.

Real-time quantitative PCR

Total RNA isolation was conducted by using an RNeasy Mini Kit (QIAGEN, Valencia, CA). The purity and concentration was determined by the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two µg of the RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The synthesized cDNA was diluted 1:5 and stored at -20^0 C. The primer and probe sets for human CYP11B1 and CYP11B2 were designed using Primer Express 3.0 (Applied Biosystems) and purchased from Integrated DNA Technologies (Coralville, IA) as published previously [94]. A total volume of 20 µl used per reaction and consisted of 10 µl TaqMan Fast Universal PCR Master Mix (2X) (Applied Biosystems), 5 µl of the respective primer-probe and 5 µl of each first-strand cDNA sample. Real-time quantitative PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). As per the manufacturer guidelines, denaturation was carried out at 95^0 C for 20 sec followed by amplification for 40 cycles (3 sec at 95^0 C and 30 sec at 60^0 C, fluorescence measurement). For CYP11B1 and CYP11B2, the primer-probe mix consisted of 900 nM of each primer and 400 nM of the probe. 18s rRNA was similarly detected and quantified using the TaqMan Ribosomal RNA Control Reagents (Vic Probe) (Applied Biosystems), and used for normalization of gene expression. Negative controls contained water instead of first strand cDNA. Primers-probes for NURR1 and cFOS were purchased from Applied Biosystems. The fold change was calculated over the basal sample, which served as the internal calibrator, using the $2^{-\Delta\Delta Ct}$ method, previously described in several studies by the same research group [94, 239, 316].

Transient transfection assays

Transient transfections in H295R/TR/SF-1 cells were performed as described [94]. In brief, on day 0, 400,000 H295R/TR/SF-1 cells per well were plated in 12-well Costar dishes in a volume of 1.0 ml of growth media consisting of 2% NuSerum, 1% insulin/transferrin/selenium Premix and 1% penicillin/streptomycin and 0.1% gentamicin. After overnight starvation in 0.1% low serum media, on day 2, cells were incubated in low serum media with/without 0.25 µg/ml doxy for 6 h. The media was then changed to serum free DMEM/F12 (without supplements and antibiotics) and cells were then transfected with a mixture of plasmid DNA consisting of CYP11B2-pGL3 Basic (1.0 µg/well), and 100 ng/well of transcription factors NURR1 (pCMV-XL5-NURR1) and/or a constitutively active form of ATF2 (pCMX-VP16-ATF2) using the transfection reagent Transfast (Promega, Madison, WI) for 6 h. As controls, two wells per experiment were transfected with empty vectors pGL3 Basic, pCMV-XL5 and/or pCMV-VP16. To normalize luciferase activity, cells were co-transfected with renilla (pRL-CMV) (50 ng/well) (Promega). After 6 h, transfected cells were allowed to recover for 24 h in media with or without 0.25 µg/ml doxy. In the final 6 h, cells were treated with Ang II (10 nM) (Sigma-Aldrich) for 6 h and then lysed and assayed for luciferase activity and renilla activity (Promega) using a Fluostar Optima Microplate Luminometer (BMG Labtech, Inc., Durham, NC). Relative level of transactivation was calculated by normalizing luciferase units using renilla activity.

Luciferase and renilla assays

Trasfected cells were lysed in a Renilla-luciferase lysis reagent (Promega). For the luciferase assay, 20 µl/well volume of the cell lysates were assayed following the manufacturer's instructions. The microplate was read immediately using the FLUOstar Optima bioluminometer (BMG Labtech, Durham, NC). For the renilla assay, similarly, 20 µl/well of the cell lysates were loaded on Costar assay plates. Relative level of transactivation was calculated by dividing luciferase units by renilla units.

Western analysis

H295R/TR/SF-1 cells were plated at a 200,000 cells/well in 24-well Costar dishes for 48 h, and, after overnight incubation in low serum medium, the media was replaced with fresh media without (basal) or with doxy (0.25 µg/ml) for 0.5, 1, 3, 6 and 24 h. Cells were lysed in 1X sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, 2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue) following recommendations from Cell Signaling Technology (Danvers, MA). Twenty microliters of cell lysate were run in a 10% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane. After transfer, the membranes were blocked for 1 h at room temperature with a 5% BSA solution in a 1X Tris-buffered saline/0.01% Tween 20 (TBS-T) solution. Then membranes were incubated for 1 h at room temperature with the primary antibody against SF-1, a generous donation by Dr. Morohashi, at a 1:5000 dilution in 5% BSA/TBS-T. The membranes were then washed thrice, for 5 minutes each, with TBS-T before incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3000 dilution in 5% BSA/TBS-T)

obtained from Santa Cruz Biotechnology. After short washes in TBS-T, the immunoreactive bands were visualized using the ECL Western Blotting Substrate from Pierce Thermo Scientific (Rockford, IL). Immunoreactivity for β-actin was similarly used as a loading control.

Microarray analysis

On Day 0, 400,000 H295R/TR/SF-1 cells per well were plated onto 12-well dishes in growth medium consisting of 2% NuSerum, 1% insulin/transferrin/selenium Premix and 1% penicillin/streptomycin and 0.1% gentamicin. After 48 h, cells were incubated in low serum medium overnight followed by treatment without or with doxy (0.25 μg/ml) for 72 h. In the last 6 h, cells were treated with Ang II (10 nM). Total RNA isolation was conducted by using an RNeasy Mini Kit (QIAGEN, Valencia, CA). The purity and integrity of the RNA was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA concentration was determined by the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Microarray analysis was performed using Genespring (Agilent Technologies, Palo Alto, CA).

Intracellular calcium assays

H295R/TR/SF-1 cells were grown in a 96 well plate until 80-90% confluence and incubated in media with/without 0.25 μg/ml doxy for 6 h. Cells were then incubated with 100 μL/well Fura2 fluorescent dye for 40 mins and then used to examine the effect of elevated SF-1 on Ang II (10 nM) and K^+ (18 mM) stimulated adrenal cell calcium

signaling. Fluorescence intensity was recorded for 5 sec before the addition of agonist and continued for 70 seconds after the addition of agonists.

Statistical analyses

Data were calculated as mean ± S.E.M. and compared by one way ANOVA using the SigmaStat 3.1 software package (SPSS, Chicago, IL). Results were considered significantly different when *P* value was ≤0.05.

RESULTS

Doxy causes time dependent induction of SF-1

In 2009, Ye et al. used the H295R/TR/SF-1 cell model to determine the confirm the opposing effects of elevated SF-1 on CYP11B1 and CYP11B2 [94]. H295R/TR/SF-1 cells were then incubated for 72 h with doxy to induce SF-1 expression. Herein, we first examined the time dependent effects of doxy (0.25 µg/ml) on H295R/TR/SF-1 cell expression of SF-1. SF-1 protein levels (Fig 5.2.1, Panel A) also followed a time dependent induction, which increased significantly after 3 h of doxy treatment and plateaued thereafter. Real time qPCR analysis also showed a significant increase in SF-1 transcript levels at 3 h (Fig 5.2.1, Panel B).

Elevated SF-1 represses CYP11B2 expression

In Fig 5.2.1, Panel C, we demonstrated that CYP11B2 repression occurred after only 6 h of SF-1 induction. Treatment with agonists Ang II, K+ and forskolin increased CYP11B2 expression by 13.7, 4.7 and 3.5 fold, respectively. 6 h doxy incubation

resulted in inhibition of the agonistic effects of all agonists - Ang II by 55.5% (4.8 fold versus basal, $p<0.05$), K^+ by 53.7% (1.7 fold versus basal, $p<0.05$) and forskolin by 52.5% (1.5 fold versus basal, $p<0.05$). In accordance to observations by Ye *et al.*, 6 h doxy incubation and the resulting elevation of SF-1 levels significantly decreased Ang II (10 nM) stimulated CYP11B2 transcript levels. In addition, we showed that elevated SF-1 also inhibited K^+ (18mM) and forskolin (10 μM) stimulated CYP11B2 mRNA expression. SF-1 that elevated over 6 h was also sufficient to significantly increase agonist stimulated CYP11B1 activity (Fig 5.2.1, Panel D), a phenomenon shown by Bassett *et al.* as well as Ye *et al.* [93, 94]

In order to exclude potential off target effects of doxycycline treatme itself, we replicated the Ang II (10 nM) and doxy treatment in the parental H295R/TR cells that do not contain the doxy inducible SF-1 (Fig 5.2.1, Panel E). As expected, doxy treatment neither inhibited Ang II stimulation of CYP11B2 expression in H295R/TR cells, nor elevated SF-1 expression. This suggests that the actions of doxy treatment result from the elevation of SF-1 and not a non-specific drug effect .

Elevated SF-1 inhibits the expression of transcription factor NURR1, but not cFOS or ATF2 activation

RNA isolated from H295R/TR/SF-1 cells after 72 h doxy treatment followed by 6 h Ang II (10 nM) stimulation was subjected to microarray analysis as an initial method of screening genes affected by elevated SF-1. Microarray analysis in Fig 5.2.2, Panel A revealed that elevated SF-1 inhibited the expression of a rapid response transcription factor NURR1 by over 3-fold.

H295R/TR/SF-1 cells were also incubated with/without 0.25 µg/ml doxy followed by agonists Ang II (10 nM), K^+ (18 mM) and forskolin (10 µM) to determine the effect of elevated SF-1 on the expression on the transcription factor NURR1. Real time RT-PCR analysis indicated that treatment with agonists Ang II, K^+ and forskolin significantly stimulated the expression of NURR1 mRNA by 46, 31 and 136 fold ($p<0.05$) respectively, in comparison with basal (Fig 5.2.2, Panel B). Further, incubation with doxycyline for 1 h caused a significant decrease in all agonist stimulated NURR1 transcript levels. Elevated SF-1 significantly repressed the stimulation of NURR1 mRNA by agonists Ang II by 63.9% (16.9 fold versus basal, $p<0.05$), K+ by 65.4% (10.2 fold versus basal, $p<0.05$) and forskolin by 57.7% (57.4 fold versus basal, $p<0.05$). Similar repressive effect on Ang II – stimulated NURR1 nuclear protein level was also observed (Fig 5.2.2, Panel C). However elevated SF-1 did not seem to have inhibitory effects on on agonist stimulated cFOS transcript levels (Fig 5.2.3, PanelD), or on agonist stimulated activation (by phosphorylation) ATF2, a transctiptional regulator of CYP11B2 from the cyclic AMP responsive element binding protein (CREB) family .

Elevated SF-1 has no effect on agonist–stimulated adrenal cell calcium signaling

Calium infux is essential for CYP11B2 expressions in adrenal glomerulosa cells. We wanted to determine if elevated SF-1 blocked agonist-stimulated calcium signalling. H295R/TR/SF-1 cells were grown in a 96 well plate and assayed for the effects of elevated SF-1 on agonist–stimulated calcium flux. The calcium assay results indicated that increasing SF-1 by incubation with doxy had no significant effect on Ang II (10 nM) (5.2.3. Panel A) and K^+ (18 mM) (5.2.3. Panel B) induced calcium flux.

DISCUSSION

Adrenocortical zonation results from selective expression of aldosterone synthase (CYP11B2) and 11-βhydroxylase (CYP11B1) in these zones, respectively. This zone specificity is an interesting phenomenon since both genes have very high sequence similarity; their promoters, especially, having several common *cis*-regulatory elements [78]. However, the molecular mechanisms conferring the specificity of CYP11B2 to the ZG has not been completelely understood. We were particularly intruiged by the fact that SF-1, a known steroidogenic gene activator, had contrasting effects on CYP11B1 and CYP11B2. This inhibtion of CYP11B2 expression and aldosterone secretion, depends on the relative level of SF-1 expression levels because complete knock down of SF-1 impairs expression of both CYP11B1 and CYP11B2 [94].

The purpose of the current study was to extend these findings and determine if SF-1 plays a role in the zonation of CYP11B2 expression. Previous studies by Ye *et al.* encompassed the incubation of a unique adrenocortical cell line, the H295R/TR/SF-1 cells [315], that contains a doxy-inducible SF-1 transgene [94]. We speculated that such prolonged exposure to elevated SF-1 might skew the cellular millieu to a predominantly fasciculata phenotype, making studies of the resultant low CYP11B2 transcript levels a challenge. This was especially required since the H295R/TR/SF-1 cells being studied express much lower CYP11B2 than it's parent cell lines, the H295R/TR and H295R cells. This rationale led us to designing a time dependent induction assay for SF-1 mRNA and protein. Doxy caused a significant 6 fold increase in SF-1 mRNA after 3 h of incubation. SF-1 transcript levels peaked at 6 h (~7 fold increase compared to basal)

and plateaued thereafter. This was supported by time dependent increase in SF-1 protein levels. Short-term induction of SF-1 expression allowed a level of expression of CYP11B2 mRNA that was easily detectable by real time RT-PCR and was hence used in the experiments that followed.

The Ye *et al.* study showed that elevated SF-1 inhibited agonistic effects of Angiotensin II (Ang II) on CYP11B2 expression. The effect of elevated SF-1 on other agonist stimulation, however, was not addressed. This would be an essential finding since the major CYP11B2 agonists - Ang II, K^+ and forskolin (a cAMP analog) - follow distinct cellular signaling pathways [40]. We observed that elevated SF-1 inhibited the stimulatory effects of all agonist-stimulated CYP11B2 mRNA by ~50 %. To confirm that the SF-1 inhibition on CYP11B2 mRNA was selective, we also determined the effect of elevated SF-1 on CYP11B1, in the same samples. The possibility of the observed results being related to doxy rather than elevated SF-1 could be ruled out based on the result that doxy incubation did not repress CYP11B2 or stimulate SF-1 expression in the parental H295R/TR cells.

Since elevated SF-1 seemed to inhibit stimulation of agonists that follow different cellular signaling pathways, it led us to hypothesize that the mechanism of the repressive effects SF-1 is probably through the inhibition of transcription factors essential for CYP11B2 expression. Ang II and K^+ mediated stimulation of CYP11B2 expression has been shown to result from the activation of several transcription factors such as the members of the nerve growth factor-induced clone B family (NGFI-B or NR4A family) [40, 82-86, 316] as well as by ATF1, ATF2 and CREB. Microarray analysis of

RNA obtained from studies by Ye *et al.* indicated that increase in SF-1 for 72 h caused a 3 fold decrease in NURR1 transcript levels. In order to confirm this observation, we once again utilized the H295R/TR/SF-1 cells. Since NURR1 is a rapid response gene, the cells were incubated with agonists for only 1 h after 6 h pre-incubation with doxy. As expected, Ang II, K^+ and forsolin stimulated NURR1 mRNA was significantly supressed (50%, $p<0.05$) by elevated SF-1. Similar effects were confirmed on the active nuclear protein levels of NURR1 by western analyses. Other transcription factors such as ATF2, are mainly regulated at the post translational level by phosphorylation under agonist stimulated conditions. The effects of elevated SF-1 on agonist-stimulated activation of ATF2 (by phosphoryalation) was also investigated as early as 30 min (maximal activation by Ang II) (data not shown). However, these unpublished findings suggest that elevated SF-1 does not repress Ang II mediated phosphorylation of ATF2. Similary, cFOS mRNA expression at the same time point was also unaffected by elevated SF-1 in basal or agonist stimulated conditions. However, since cFOS is also a rapid response transcription factor, we cannot discount the possibility that we may have missed the time point. Overall, the study of transcription factors indicated that one mechanism of SF-1 repression of CYP11B2 is the inhibition of NURR1 stimulation of CYP11B2 expression.

Activated transcription factors transcolate to the nucleus where they can bind the CYP11B2 promoter to enhance its transcription. To investigate whether SF-1 repression of CYP11B2 included interference of transcriptional activators from binding promoter, we utilized a simple promoter/reporter approach (data not shown). H295R/TR/SF-1 cells were co-transfected with CYP11B2 reporter construct (B2-Luc)

and expression vectors with either transcription factors NURR1 and/or constitutively active ATF 2 in the presence or absence of elevated SF-1. The combined effect of NURR1 and ATF 2 stimulated CYP11B2 promoter activity. However, elevated SF-1 did not inhibit the stimulatory effects of all the transcription factors on CYP11B2 promoter activity. For the purpose of identifying a potential repressive site for SF-1, pilot reporter studies were conducted using deletion constructs of the CYP11B2 promoter with/without NURR1 expression (unpublished data). However, the repressive effects of SF-1 in presence and absence of constitutive NURR1 were not relieved in any of the deletion constructs. Thus a specific repressive site of SF-1 in the CYP11B2 promoter was not defined. Together, these data suggest that the effects of SF-1 repression may not be localized to the nuclear events.

Finally, it is well established that elevation in calcium influx has a pivotal role in Ang II and K^+ stimulated signaling events in CYP11B2 expression in adrenal glomerulosa cells. Numerous studies have demonstrated that inhibition of calcium signaling has been shown to decrease CYP11B2 expression and aldosterone secretion [82, 83, 86, 145, 148]. Activation of transcription factors also appears to be mediated through phosphorylation by agonist-activated Calcium calmodulin kinases (CaMK) [40, 83, 86, 249]. These activated transcription factors then localize to the nucleus and facilitate CYP11B2 transcription. Therefore, we investigated whether increased SF-1 blocked calcium signaling stimulated by Ang II and K^+. Fluorescence assay for intracellular calcium suggested that elevated SF-1 expression did not affect calcium signaling in agonist stimulated adrenal cells.

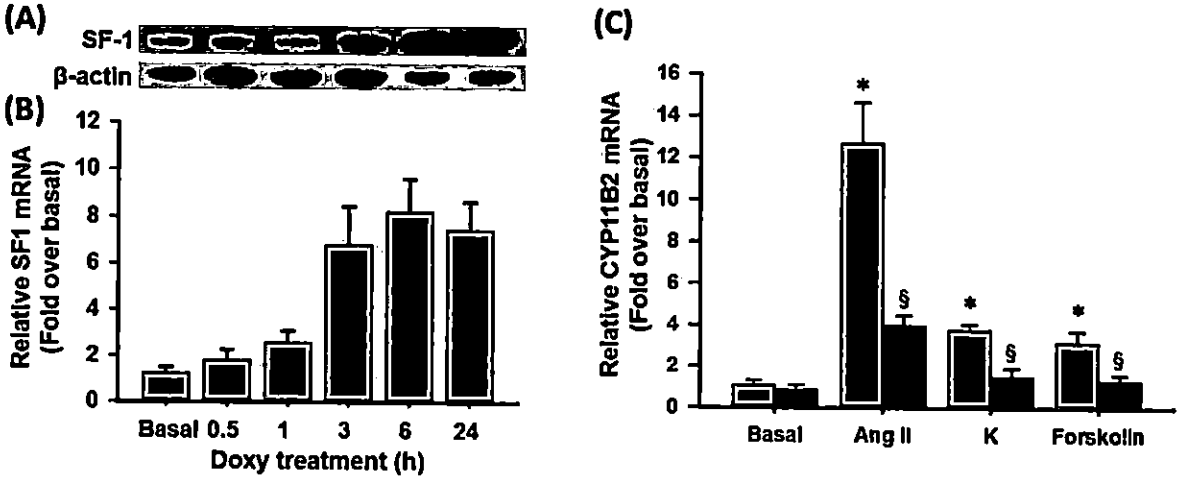
Besides the pathways analysed in our studies, SF-1 can be also be regulated by post translational modifications such as phosphorylation and sumoylation [307, 317]. In order to define a role for phosphorylation of SF-1 in its ability to repress CYP11B2, current *in vitro* studies by our group in a similar doxy-inducible SF-1 cell line harboring a S203A phosphorylation mutant form of SF-1 are under way. Besides phosphorylation, SF-1 sumoylating enzymes Ubc9 and PIAS1 which are expressed at higher levels in the ZG [89, 90] could also contribute towards conferring zone specificity in SF-1 activity. Ubc9 and PIAS1 also regulate transcription modulators of CYP11B2 such as Chicken Ovalbumin Upstream Promoter-Transcription Factor I (COUP-TF I). COUP-TF has been shown to compete with SF-1 to bind a common cis-regulatory site on the CYP11B2 promoter [88, 90].

The repressive action of SF-1 also depends on its abiltiy to compete with other CYP11B2 activators such as DAX-1 (NR0B1). DAX-1 has been shown to inhibit SF-1 mediated expression of several steroidogenic genes [318, 319]. A higher expression of DAX-1 in the ZG might maintain low levels of SF-1 to direct the steroidogenic biosynthetic pathway towards aldosterone production [320]. DAX-1 has been reported to be expressed at higher levels in the rat ZG. However, a similar zonal expression of DAX-1 in humans has not yet been reported [321]. The possibility that SF-1 modulates the dimerization of transcription factors such as the members of the NR4A family, an important nuclear event involved in CYP11B2 expression also needs to be addressed. Finally, SF-1 could act through a definite 'repressive site' in the CYP11B2 promoter that has not yet been defined.

To summarize, in our study, our studies demonstrate that the repressive effects of SF-1 occur at the level of NURR1 mediated activation of CYP11B2 transcription, without affecting calcium signaling in this process. Thus, the effects of SF-1 mediated repression of CYP11B2 seem to be broad based and at multiple levels. Investigation of molecular signaling in CYP11B2 expression would be essential for better understanding the overall regulation of SF-1 in aldosterone production. Study of the regulation of SF-1 might provide insight into adrenocortical zonation, aldosterone biosynthesis and aldosterone excess disorders.

FIGURES

Figure 5.2.1. Elevated SF-1 expression inhibits all agonists-stimulated CYP11B2 expression. Cell lysates were obtained from cells incubated in doxy at the indicated times and the SF-1 protein levels were examined by immunoblotting. β-actin was used as a loading control. Data are representative of 3 independent experimental sets *(Panel A)*. Real time RT-PCR was used to quantify SF-1 transcript levels *(Panel B)*. Elevated SF-1 inhibited stimulatory effects of agonists Ang II, K^+ and forskolin *(Panel C)* but elevated the expression of CYP11B1 *(Panel D)*. For Panels C and D, gray bars indicate no doxy, and black bars indicate presence of doxy. Doxy does not have off target effects in parental H295R-TR cells (*Panel E)*. Data represent the means ± S.E.M. of at least four independent experimental sets of H295R/TR/SF-1 cells incubated with doxy for the indicated times. The fold difference was calculated as a relative measure in comparison the basal. *p≤0.05 compared with basal by one way ANOVA, §p p≤0.05 versus respective agonist.



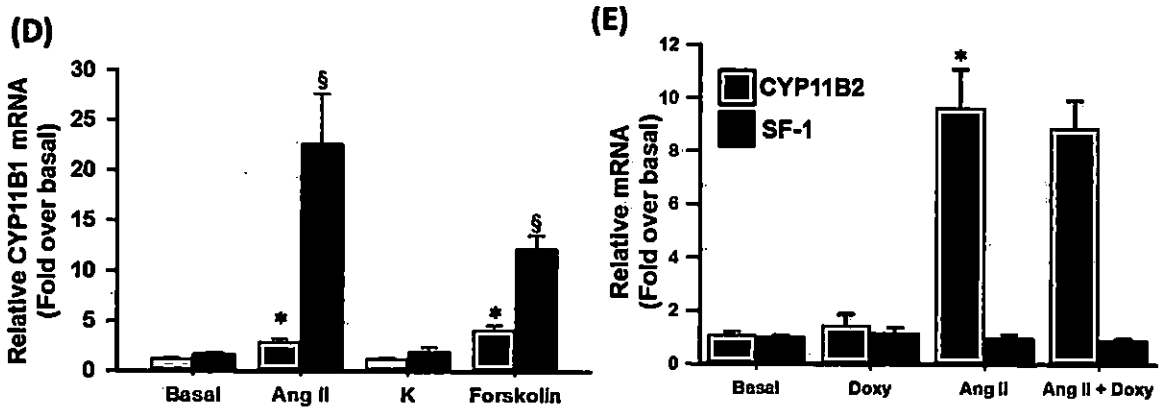
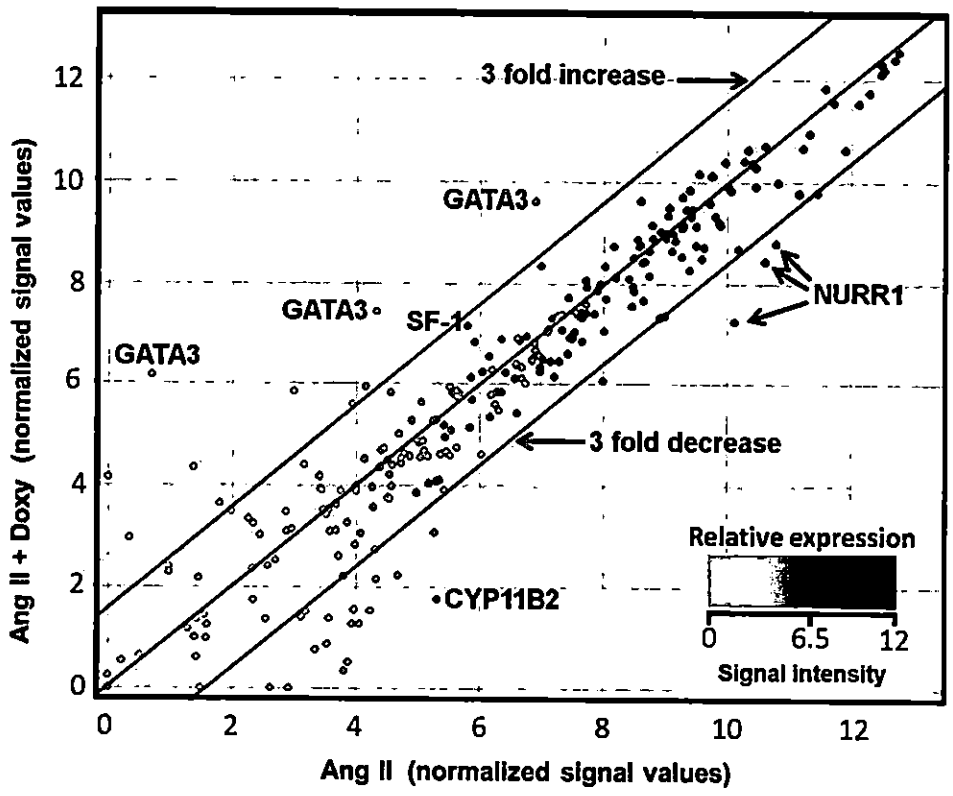
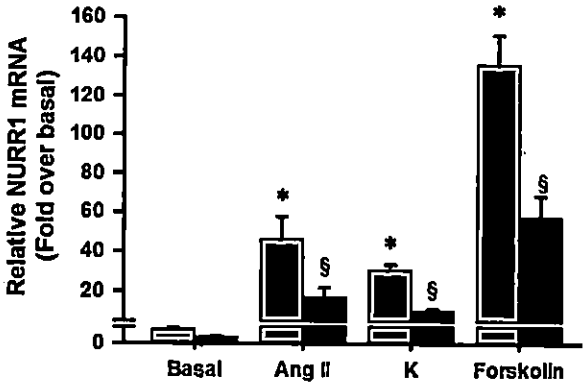


Figure 5.2.2. Effect of SF-1 on transcription factors expression. Microarray analysis of cells incubated with Ang II with/without doxy pre-incubation (single experimental set) is represented in the scatter plot *(Panel A)*. Real time RT-PCR was used to determine transcript levels of NURR1 mRNA *(Panel B)* and cFOS *(Panel D)*. Representative blot for the effect of elevated SF-1 on nuclear localized NURR1 protein expression *(Panel C)*. The fold difference is a relative measure in comparison to the basal. Data for NURR1 represent the means ± S.E.M. from six independent experiments. *$p<0.05$ compared to respective agonist treatments (in the absence of doxy), $^{\S}p<0.05$ compared to basal gene expression. Data for cFOS represent the means ± S.E.M. from three independent experiments. *$p<0.05$ compared to the absence of doxy (treatment with the respective agonist alone). Statistical analyses were performed by one way ANOVA.

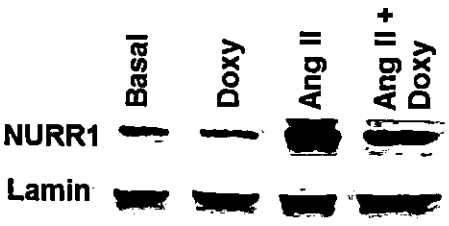
(A)


3 fold increase
GATA3
GATA3
SF-1
GATA3
NURR1
3 fold decrease
CYP11B2
Relative expression
0
6.5
12
Signal intensity
Ang II + Doxy (normalized signal values)
Ang II (normalized signal values)
0
2
4
6
8
10
12

(B)


Relative NURR1 mRNA (Fold over basal)
160
140
120
100
80
60
40
20
0
Basal
Ang II
K
Forskolin
*
§

(C)


Basal
Doxy
Ang II
Ang II + Doxy
NURR1
Lamin

(D)

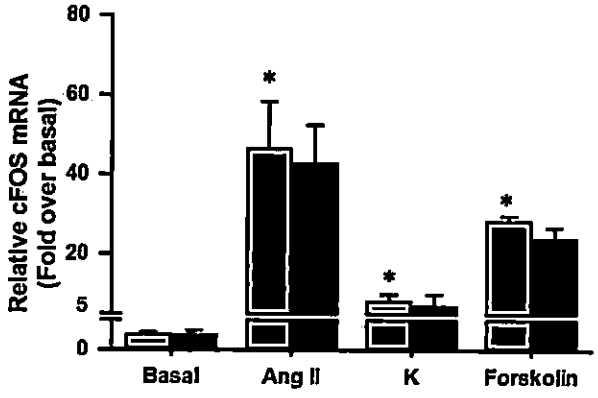
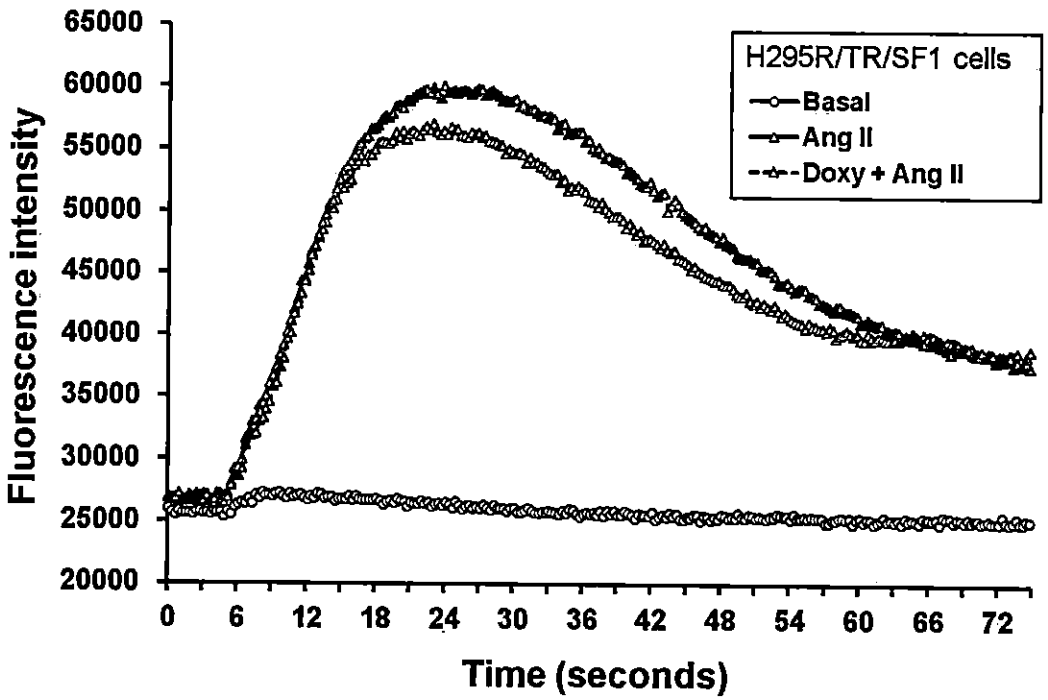
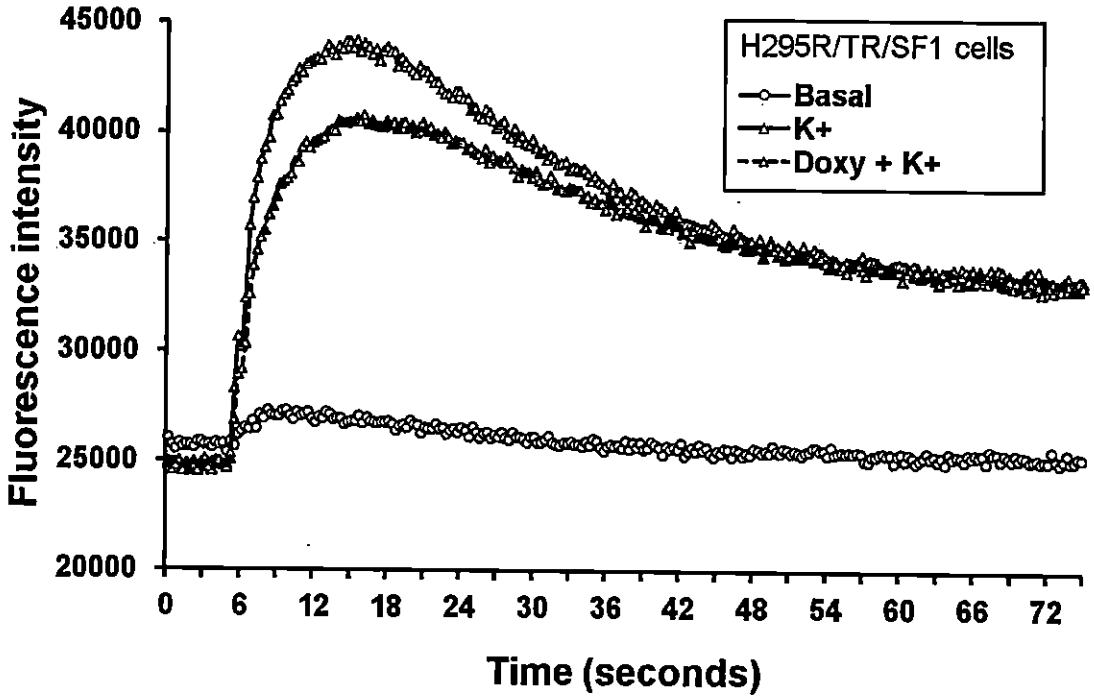

Relative cFOS mRNA (Fold over basal)
80
60
40
20
5
0
Basal
Ang II
K
Forskolin
*

Figure 5.2.3. Elevated SF-1 did not inhibit calcium flux stimulated by Ang II (A) or K (B). Cells were treated with/without 0.25 µg/ml doxy for 6 h and then incubated with 100 µL/well Fura2 fluorescent dye for 40 mins to determine SF-1 effects on intracellular Ca^{2+} levels stimulated by Ang II *(Panel A)* or K^{+} (18 mM) *(Panel B)*. Fluorescence intensity was recorded for 5 sec before the addition of agonist and continued for 70 seconds after the addition of agonists. The figures are data representative of 3 independent experimental sets.

(A)



(B)


H295R/TR/SF1 cells
Basal
K+
Doxy + K+
Fluorescence intensity
45000
40000
35000
30000
25000
20000
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (seconds)

SECTION III

ACUTE AND CHRONIC REGULATION OF ALDOSTERONE PRODUCTION

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Abstract

Aldosterone is the major mineralocorticoid synthesized by the adrenal and plays an important role in the regulation of systemic blood pressure through the absorption of sodium and water. Aldosterone production is regulated tightly by selective expression of aldosterone synthase (CYP11B2) in the adrenal outermost zone, the zona glomerulosa. Angiotensin II (Ang II), potassium (K^+) and adrenocorticotropin (ACTH) are the main physiological agonists which regulate aldosterone secretion. Aldosterone production is regulated within minutes of stimulation (acutely) through increased expression and phosphorylation of the steroidogenic acute regulatory (StAR) protein and over hours to days (chronically) by increased expression of the enzymes involved in the synthesis of aldosterone, particularly CYP11B2. Imbalance in any of these processes may lead to several disorders of aldosterone excess. In this review we attempt to summarize the key molecular events involved in the acute and chronic phases of aldosterone secretion.

Key words: Aldosterone, aldosterone synthase (CYP11B2), acute regulatoin, chronic regulation, Angiotensin II (Ang II), serum K, adrenocorticotropin (ACTH)

Introduction

Aldosterone is the major mineralocorticoid involved in maintaining fluid and electrolyte balance in all mammals. In humans, excessive secretion of this hormone results in hypertension, contributes to cardiac fibrosis, congestive heart failure, and exacerbates the morbidity and mortality associated with these disorders [183, 190]. Although the signal transduction processes regulating aldosterone production are as yet incompletely understood, ongoing research has identified several important pathways mediating steroidogenesis. Aldosterone production (equivalent to secretion in the case of this steroid hormone) is primarily regulated by angiotensin II (Ang II), serum potassium, as well as adrenocorticotropic hormone (ACTH).

Steroidogenesis (Aldosterone production)

In mammals, aldosterone biosynthesis occurs almost solely in the adrenal zona glomerulosa. Aldosterone is derived through a series of enzymatic steps that involve three cytochrome P450 enzymes and one hydroxysteroid dehydrogenase (Figure 5.3.1). The enzymes cholesterol side-chain cleavage (CYP11A1), 21-hydroxylase (CYP21) and aldosterone synthase (CYP11B2) belong to the cytochrome P450 family of enzymes. CYP11A1 and CYP11B2 are localized to the inner mitochondrial membrane, while CYP21 is found in the endoplasmic reticulum. Cytochrome P450 enzymes are heme-containing proteins that accept electrons from NADPH via accessory proteins and utilize molecular oxygen to perform hydroxylations (CYP21 and CYP11B2) or other oxidative conversions (CYP11A1). The fourth enzyme, type 2 3β-hydroxysteroid dehydrogenase (HSD3B2), is a member of the short-chain

dehydrogenase family and is localized in the endoplasmic reticulum. Aldosterone and cortisol share the first few enzymatic reactions in their biosynthetic pathways (cholesterol to progesterone); however, adrenal zone-specific expression of CYP11B2 (aldosterone synthase) in the glomerulosa and that of CYP11B1 (11β-hydoxylase) in the fasciculata leads to the functional zonation observed in the adrenal cortex [78].

Like all steroid hormones, the glomerulosa cell uses cholesterol as the primary precursor for steroidogenesis. The cholesterol needed for adrenal steroidogenesis can come from several sources, which include de novo synthesis from acetate or cholesteryl esters stored in lipid droplets or up-take by the low-density lipoprotein (LDL) receptor (for LDL) or scavenger receptor-BI (for high-density lipoprotein or HDL). Movement of cholesterol from the outer mitochondrial membrane, across the aqueous intra-membranous space, to the inner mitochondrial membrane must occur for CYP11A1 to access the molecule for cleavage to pregnenolone. Because steroid hormones are secreted upon synthesis, the initial reaction involving mitochondrial conversion of cholesterol to pregnenolone is tightly controlled and represents the rate-limiting step in aldosterone synthesis. This step is regulated by the expression and phosphorylation of steroidogenic acute regulatory protein (StAR) [101, 103, 104]. Pregnenolone passively diffuses into the endoplasmic reticulum and is converted to progesterone by HSD3B2. Progesterone is hydroxylated to deoxycorticosterone by CYP21. Finally, aldosterone biosynthesis is completed in the mitochondria, where deoxycorticosterone undergoes 11β- and 18-hydroxylation, followed by 18-oxidation, which in humans can be mediated by a single enzyme, CYP11B2. Although the last step of cortisol production also involves the 11-hydroxylation of 11-deoxycortisol to cortisol by 11β-hydoxylase, this

enzyme only poorly catalyzes the 18-hydroxylation reaction and does not catalyze 18-oxidation.

There are several factors regulating aldosterone production in the adrenal zona glomerulosa. First, the selective expression of CYP11B2 in the glomerulosa creates a tightly controlled zone-specific ability to make aldosterone and limits production of the steroid outside of this relatively small adrenal zone [28, 30, 303]. In rats and mice CYP11B2 is expressed in a tight zonal pattern that circles the adrenal [28, 30]. A recent study revealed a variation in human adrenal glomerulosa zonation characterized by the presence of relatively few subcapsular cell clusters expressing CYP11B2 [31]. This phenotype may relate to the relatively high sodium diet and resultant suppression of the renin-angiotensin system in most human populations. It is hypothesized that these CYP11B2-expressing cortical cell clusters may be the precursors to aldosterone-producing adenomas (APA) [31]. The absence of CYP17 in glomerulosa cells is a second mechanism resulting in diversion of the steroidogenic pathway toward aldosterone [32]. Finally, the centripetal blood flow in the adrenal cortex, itself, prevents the precursors of aldosterone in the fasciculata cells from accessing CYP11B2 in the zona glomerulosa, thereby helping to maintain the functional specificity of the adrenocortical zones.

The regulation of glomerulosa aldosterone biosynthesis is divided into two key events in the steroidogenic pathway [99, 100]. Acutely (minutes after a stimulus), aldosterone production is controlled by rapid signaling pathways that increase the movement of cholesterol into the mitochondria where it is converted to pregnenolone. This has been called the "early regulatory step" and is mediated by increased expression and phosphorylation of StAR protein (Figure 5.3.2) [101-104]. Chronically (hours to days),

aldosterone production is regulated at the level of expression of the enzymes involved in the synthesis of aldosterone (Figure 5.3.3) [40]. This has been called the "late regulatory step" and is particularly dependent on increased expression of CYP11B2. The goal of this review article is to summarize our current understanding of the signaling pathways that regulate the early and late regulatory steps of aldosterone biosynthesis.

Acute effects of Ang II

As mentioned above, the initial enzymatic step in aldosterone biosynthesis is the hydrolysis of cholesterol to pregnenolone by the cholesterol side-chain cleavage complex found in the inner mitochondrial membrane. Thus, initiation of aldosterone production in response to agonists such as Ang II, elevated extracellular potassium concentrations or ACTH requires two major steps: first, cholesterol mobilization from lipid droplets to the mitochondria is thought to require cytoskeletal rearrangements [322-324] and activation of cholesteryl ester hydrolase by, e.g., extracellular signal-regulated kinase-1 and -2 (ERK-1/2) [325]. Mobilization is followed by movement of the cholesterol from the outer to the inner mitochondrial membrane, a process requiring active StAR protein [326-330]. As discussed below, StAR appears to be regulated both at the level of transcription [104, 105] and post-translationally, in that phosphorylation appears necessary for its full activity[101, 103, 104, 106].

Both of these processes are triggered, in the case of Ang II, by the binding of the hormone to the type 1 Ang II (AT_1) receptor and the activation of several signaling pathways (Figure 5.3.2). One such pathway is a phosphoinositide-specific phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the two second messengers, inositol 1,4,5-trisphosphate (IP_3) and

diacylglycerol (DAG) [109-115]. IP_3 is thought to initiate aldosterone secretion by eliciting a transient increase in the cytosolic calcium concentration and activating calcium/calmodulin-dependent protein kinases (CaMK). CaMK activity is clearly important in mediating aldosterone secretion, as inhibition of this enzyme decreases Ang II-induced aldosterone secretion [118-120]. On the other hand, DAG functions to stimulate protein kinase C (PKC), the activity of which has been suggested by some groups to underlie sustained aldosterone secretion from bovine glomerulosa cells [111, 115, 123, 125, 126]. This idea is supported by the ability of phorbol esters [127, 331], a DAG-generating enzyme [123] and synthetic DAGs [127] to elicit aldosterone secretion and of a selective PKC inhibitor to decrease Ang II-induced aldosterone secretion [126]. However, other groups have suggested an inhibitory role of PKC in the aldosterone secretory response as discussed below [332-334].

There are two additional pathways activated by Ang II binding to the AT_1 receptor, through mechanisms that are as yet incompletely defined. The first is an increase in calcium influx, in part through CaM kinase II [335-338] and GTP-binding proteins [339], with this enhancement acting synergistically with PKC-activating agents to stimulate secretion. Indeed, this calcium influx is essential for a continued aldosterone secretory response [109, 113], as well as for regulating PKC activity [340], since inhibition of calcium influx with calcium channel antagonists decreases Ang II-induced aldosterone secretion [120, 341-343]. Calcium influx occurs through voltage-dependent T- and L-type calcium channels, as well as store-operated calcium channels or capacitative calcium influx [120, 344-346]. The activity of the voltage-dependent channels is maintained by appropriate membrane polarization, maintained by proper functioning of potassium channels, such

as TWIK-related acid-sensitive K (TASK) channels [97, 108]. Emptying of the endoplasmic reticulum store by IP_3 also results in capacitative calcium influx, as discussed above. The importance of this calcium signal to acute aldosterone secretion, functioning largely through activation of CaMK, is well appreciated and has been reviewed thoroughly [120].

On the other hand, the role of DAG effector enzymes such as PKC, in modulating aldosterone synthesis remains somewhat controversial, with some reports showing that activation of the PKC pathway enhances aldosterone secretion, and others that this pathway inhibits aldosterone production [115, 128, 129, 333, 347, 348]. Thus, some investigators have shown that mimicking the PKC and calcium influx signals with pharmacologic agents essentially reproduces Ang II responses including aldosterone secretion [115, 123, 341] and the protein phosphorylation pattern [349]. These results suggest that the aldosterone secretory response to Ang II requires both a PKC activation event and a calcium influx stimulus. In contrast, there is evidence to indicate that the DAG signal and its effectors oppose aldosterone secretion acutely in rat glomerulosa cells, with inhibition of PKC by pretreatment with the non-selective inhibitor staurosporine enhancing subsequent Ang II-induced aldosterone secretion [333]. This result has been confirmed in experiments with the selective PKC inhibitors bisindolymaleimide I and Gödecke 6976 [348]. Other experiments have observed essentially no effect of DAG-mimicking phorbol esters on more chronic aldosterone secretion from the human adrenocortical carcinoma cell line, H295R [144]. One possible explanation arises from the lack of selectivity/specificity of phorbol esters as mimics of the DAG pathway and/or PKC inhibitors as blockers of the pathway (see below). Clearly, additional studies are

required to define the role of DAG-mediated signaling in Ang II-induced aldosterone synthesis.

Another signaling system activated by Ang II is phospholipase D (PLD), which can also increase DAG (indirectly) and presumably activate PKC [123, 350, 351]. PLD, of which there are two well-characterized mammalian isoforms PLD1 and PLD2, hydrolyzes phospholipids, primarily phosphatidylcholine, to yield phosphatidic acid (phosphorylated DAG), which can then be converted to DAG by the action of lipid phosphate phosphatases [121, 122]. Phosphatidic acid is a second messenger itself and is proposed to function as a slow-release reservoir of diacylglycerol for sustained cellular responses [121, 123, 124]. Phosphatidic acid can also be deacylated by phospholipase A_2 to produce lysophosphatidic acid (LPA), a lipid message that activates G protein-coupled LPA receptors. The released fatty acid can also be metabolized to additional lipid signals, such as eicosanoids and 12-hydroxyeicosatetraenoic acid (12-HETE), which has been reported to stimulate aldosterone secretion [352, 353]. Treatment of glomerulosa cells with exogenous PLD alone or in combination with the calcium channel agonist, BAY K8644, induces a sustained increase in aldosterone secretion without an increase in phosphoinositide hydrolysis [123], suggesting that PLD activity may be sufficient to stimulate aldosterone secretion. PLD activity also appears to be necessary for Ang II-induced aldosterone secretion, as demonstrated using the primary alcohol 1-butanol, which diverts production away from phosphatidic acid and DAG [354] and instead forms phosphatidylbutanol (versus the control tert-butanol, which does not affect PLD-generated lipid signals). 1-Butanol was found to inhibit the Ang II-induced increase in DAG and phosphatidic acid levels [350], as well as Ang II-elicited aldosterone secretion, in

bovine adrenal glomerulosa cells [350] and in H295R cells [351], whereas tert-butanol did not [351]. More direct evidence arises from studies in which PLD was overexpressed using adenovirus-mediated transduction. Overexpression of wildtype PLD1 or PLD2, but not the lipase-inactive isoforms, increased PLD activity [122]. However, only wildtype PLD2 enhanced Ang II-stimulated aldosterone secretion [122], suggesting that PLD2 is the isoform mediating aldosterone secretion in response to Ang II, although the lipid signal(s) mediating this effect is unclear.

Thus, as discussed above, DAG can be produced by both Ang II-activated phospholipase C and PLD and, in turn, can activate DAG effector enzymes, which include PKC isoenzymes and protein kinase D (PKD), as well as Ras guanine nucleotide exchange factors (Ras-GRP1 through 3), which are upstream of the mitogen-activated protein kinase pathway involving Ras, Raf, MEK and ERK-1/2, and chimaerins (GTPase-activating proteins for Rac) [355]. PKC isoenzymes constitute a multigene family that belongs to the cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) family [356]. Members of the family play an important role in mediating numerous intracellular signaling events, including those involved in cell growth and differentiation, membrane trafficking, secretion, and gene expression [120, 356]. The PKC family is divided into three groups based on their second messenger requirements: the conventional (also known as classical), novel, and atypical, with all members requiring acidic phospholipids such as phosphatidylserine for activation. Conventional and novel PKCs also require DAG and in addition conventional PKCs require calcium. Alpha, beta-I, beta-II and gamma comprise the conventional PKC subfamily, whereas novel PKCs include the delta, epsilon, eta, and theta isoforms. The novel isoforms have been shown

to activate protein kinase D (PKD) via phosphorylating serines in the catalytic domain [357] (see below).

PKCs are also involved in regulating adrenal steroidogenesis through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on substrate proteins [126, 331, 358-360]. There is evidence to suggest that DAG activation of PKC coincides with the expression of the rate-limiting StAR protein [104] as well as StAR phosphorylation [361]. In turn, phosphorylation of StAR has been proposed to be required for its full activity [101, 103, 104]. For example, using a mouse Leydig cell line, Stocco and colleagues [104] showed that activation of the PKC pathway can increase StAR expression but has a minimal effect on steroidogenesis, unless small concentrations of dibutyryl-cAMP are also included. Thus, these low doses of cAMP act synergistically with a PKC-activating phorbol ester to stimulate StAR phosphorylation and maximal steroid hormone production. Bollag and colleagues have also demonstrated that treatment of H295R cells with the DAG-mimicking phorbol ester, phorbol 12-myristate 13-acetate (PMA) increases mitochondrial cholesterol levels and aldosterone secretion [362], providing evidence for a possible role of PKC (and/or other DAG effector enzymes) in cholesterol transport and aldosterone production.

The 18 kDa mitochondrial protein peripheral type benzodiazepine receptor (PBR), also known as translocator protein (TSPO), has also been implicated in steroidogenesis [363, 364]. PBR has been proposed to functionally interact with StAR, among other proteins, to mediate mitochondrial cholesterol uptake into the mitochondria [365]. PBR is located on the outer mitochondrial membrane and is elevated in steroidogenic cells upon hormone stimulation [365]. Introduction of a null mutation of the PBR gene into R2C rat Leydig

tumor cells inhibited pregnenolone production in response to 22R-hydroxycholesterol, a precursor that bypasses the need for StAR [364] supporting an important role for this protein in steroidogenesis. Recently, the DAG-sensitive PKC isozyme, PKC-epsilon was shown to mediate PBR gene expression [366] in MA-10 Leydig cells using PKC inhibitors and small interfering (si)RNA targeting PKC-epsilon. These results may provide insight into the possible role of the PKC pathway in mediating aldosterone secretion, although additional studies are certainly required to fully define the role(s) of this large family of serine/threonine protein kinases.

The PKD family of enzymes, composed of PKD1 (also known as PKC-μ), PKD2 and PKD3 (also known as PKC-η), represent DAG-activated serine/threonine kinases downstream of novel PKCs and a Src family tyrosine kinase pathway. An involvement of PKD family members has been proposed for a number of important cellular functions, including Golgi trafficking, hypertrophy, immune response, proliferation, migration, invasion and survival. [367, 368], Importantly, PKD is activated in response to Ang II in primary bovine adrenal glomerulosa cells [369]. Moreover, adenovirus-mediated overexpression of a constitutively active PKD1 mutant enhances, and a dominant-negative PKD1 mutant inhibits acute Ang II-induced aldosterone secretion in these cells [369]. PKD performs a similar role to enhance chronic Ang II-induced aldosterone secretion and aldosterone synthase expression in the H295R cell model [370], a result which was corroborated by a study showing that knocking down PKD levels with RNA interference decreased Ang II-stimulated aldosterone secretion from these cells [371]. Thus, PKD appears to mediate Ang II-elicited aldosterone secretion acutely as well as to regulate aldosterone biosynthetic capacity of the adrenal. Also in the H295R cells,

PKD activation in response to Ang II resulted from PKC-epsilon-mediated transphosphorylation [370]. While the mechanism by which PKD enhances aldosterone secretion is unclear, it is known that StAR gene transcription is influenced by several transcription factors that are targets for PKD, including cAMP response-element (CRE)-binding protein (CREB)/CRE modulator (CREM)/activating transcription factor (ATF) family members and activator protein-1 (AP-1, Fos/Jun) [80, 106, 372, 373]. Indeed, Stocco and colleagues recently showed that PKD regulates StAR levels via CREB and AP-1 in MA-10 Leydig cells [374].

Acute effects of Potassium

Similar to Ang II, small increases in extracellular potassium levels also stimulate calcium influx, via depolarization of the glomerulosa cell plasma membrane and activation of the voltage-dependent calcium channels, transient T-type and long-lasting L-type. Also as with Ang II, this influx is required for the response to potassium, since inhibition of calcium influx abolishes potassium-stimulated aldosterone secretion [120, 343, 375-377]. Related to calcium influx, an interesting finding is the fact that decreasing potassium levels to 2 mM inhibits Ang II-stimulated aldosterone production, presumably by inhibiting Ang II-induced calcium influx [127]. It can be speculated that this mechanism serves under conditions of low serum potassium levels to prevent Ang II-stimulated aldosterone secretion, which could otherwise cause excessive excretion of potassium and subsequent severe, and possibly fatal, hypokalemia.

Other signaling pathways have also been proposed to be involved in potassium-induced aldosterone production, although controversy remains concerning these other events. For instance, some investigators have observed an ability of potassium to increase

cAMP levels and others have not [378-381]. Potassium has also been reported to stimulate PLD activity through voltage-dependent calcium channels in bovine glomerulosa cells [382], although whether and how this PLD activation contributes to potassium-elicited aldosterone production is unclear. Thus, there are still several areas related to the acute effects of potassium on aldosterone secretion in adrenal glomerulosa cells that still need to be studied and better understood.

Acute effects of ACTH

ACTH is able to stimulate aldosterone production acutely, both *in vivo* and *in vitro*. It does so by binding to the ACTH receptor (MC2R) which activates adenylate cyclase via the heterotrimeric G protein, G_s. Adenylate cyclase produces the second messenger cAMP, thereby stimulating the activity of cAMP-dependent protein kinase or protein kinase A (PKA). PKA can then phosphorylate and activate StAR [361], to increase cholesterol delivery to the inner mitochondrial membrane. In addition, PKA can regulate the transcriptional activity of CREB family transcription factors [105, 374, 383]. Since binding sequences for CREB/ATF transcription factors are found in the promoter of StAR [81, 373, 384-386], PKA can also rapidly increase expression of StAR protein levels and further enhance acute steroid production. In addition to stimulating cAMP-induced PKA activation, ACTH is capable of promoting calcium influx, likely through PKA-mediated phosphorylation of L-type calcium channels [387], thereby increasing cytosolic calcium concentration and further enhancing adenylate cyclase production of cAMP and aldosterone secretion [388].

Finally, there may be some PKA-independent effects of cAMP on aldosterone secretion, in that, a recent report has suggested the involvement of the guanine nucleotide

exchange factor, exchange protein directly activated by cAMP (Epac), in cAMP-mediated aldosterone production, via effects on CaMK activation [389].

Chronic effects of Ang II

As noted earlier, the capacity for aldosterone production is also regulated through the chronic action of the same factors that acutely stimulate its biosynthesis. The chronic actions involve changes in the size of the zona glomerulosa as well as glomerulosa cell capacity to produce aldosterone. This review focuses on the second issue (glomerulosa cell differentiation) because of recent advances in our understanding of the mechanisms regulating steroidogenic enzymes.

Ang II increases the expression of enzymes required for aldosterone synthesis, particularly CYP11B2. *In vivo* models have been crucial in describing such Ang II effects. Sodium restriction experiments in rats indicate that activation of the renin-Ang II system (most often by low sodium diets) induces the expression of CYP11B2 in glomerulosa cells without affecting that of CYP11B1 [152, 390, 391]. This result confirms the capacity of Ang II to specifically increase the production of aldosterone but not that of glucocorticoids [152, 391]. One explanation for the zone-specific effects of Ang II is the greater expression of AT_1 receptors in glomerulosa versus fasciculata cells [392], and the inhibition of CYP11B2 expression by angiotensin inhibitors or AT_1 receptor blockers has been demonstrated [393]. Ang II can also increase CYP11B2 expression by increasing glomerulosa cell sensitivity to Ang II through upregulation of its own receptor. This effect has been demonstrated *in vivo* in rats on a low-sodium diet [394, 395]; further studies have shown that pharmacological blockers of the AT_1 receptors, such as losartan [394, 395] and

angiotensin-converting enzyme (ACE) inhibitors [396, 397], reduce the effect of the RAS on the adrenal.

In vitro cell models have been particularly useful in defining the intracellular signaling mechanisms that lead to the chronic effects of Ang II. As with acute secretion, the best characterized pathway regulating Ang II-induced chronic production of aldosterone is the PLC-mediated generation of DAG and IP_3, which increases intracellular calcium and acts via CaMK. Calcium signaling appears to be the primary regulator of CYP11B2 transcription. However, since inhibitors of calmodulin and CaMK cannot completely block Ang II's stimulation of CYP11B2 [116], other signaling mechanisms are also likely involved. DAG-activated PKC, on the other hand, does not appear to increase CYP11B2 transcript levels, but does play a role in producing an aldosterone-secreting glomerulosa cell phenotype by inhibiting the expression of CYP17 [130, 131]. This effect has been recently attributed to Fos-mediated repression of the CYP17 transcriptional activator, steroidogenic factor 1 (SF-1) [33]. On the other hand, there is evidence suggesting that certain PKC isoforms, such as PKC-epsilon may actually inhibit CYP11B2 expression via activation of ERK-1/2 [128, 129]. However, PKC-epsilon has been shown to activate PKD, a kinase that can increase CYP11B2 expression [370] (see below), indicating that the role of the PKC family in regulating chronic aldosterone production is as yet unclear.

Ang II appears to increase CYP11B2 expression through the activation of its transcription. A number of studies involving promoter deletion and mutation analyses have revealed that cis-elements in the CYP11B2 promoter are essential for basal as well as Ang II-mediated CYP11B2 promoter activity. These include three key regulatory

cis-elements: one cAMP response element (CRE)/Ad1 and two distal cis-elements (Ad5 and NBRE) that are able to bind members of the nerve growth factor-induced clone B family of transcription factors (NGFI-B or NR4A family) [40, 80-85]. The over-expression of one of these transcription factors, NURR1 (NR4A2), has in fact been implicated in the development of aldosterone-producing tumors [87]. Using multiple glomerulosa cell models, microarray studies have defined additional transcription factors that are regulated by Ang II [132-134]. The role of these newly defined factors requires further study. Conversely, other transcriptional regulators such as SF-1 (also called AD4BP and NR5A1) have been shown to repress basal and Ang II-stimulated CYP11B2 expression [93, 398]. However, it appears that the relative level of SF-1 may decide its ability to repress CYP11B2 expression, since complete knock down of SF-1 impairs the entire steroidogenic synthetic pathway [398]. The underlying mechanism, however, is yet to be elucidated. As mentioned previously, yet another Ang II-mediated mechanism regulating chronic aldosterone secretion seems to be via PKD [370]. The pathway involved is undetermined in adrenal cells; although PKD activation of the transcription factor CREB has been demonstrated in kidney HEK 293 cells [399] and in MA-10 Leydig cells [374]. In the Leydig cell model PKD was shown to increase StAR levels via effects on CREB and AP-1 transcription factors [374]. At the level of cholesterol uptake in glomerulosa cells, Ang II has been shown to up-regulate the expression of LDL and HDL receptors [135] and of enzymes involved in cholesterol synthesis [136].

Finally, Ang II also increases aldosterone production by expansion of the zona glomerulosa via hypertrophy and hyperplasia. This effect has been demonstrated *in vivo*, in rats on low-sodium diets, which display glomerulosa cell hypertrophy and

proliferation in AT_1R-dependent and -independent processes [131]. *In vitro,* primary cultures of bovine glomerulosa cells also showed similar responses to Ang II [400, 401]. These effects may be attributed to the ability of Ang II to induce the expression of cyclin D1 [137]. Mitogenic and hypertrophic effects of Ang II have also been demonstrated in epithelial cells, vascular smooth muscle cells [402, 403], fibroblasts, cardiac myocytes [404] and rat intestinal cells [405]. It can thus be speculated that the predisposition to cardiovascular damage resulting from activation of the renin-angiotensin-aldosterone system can be attributed to Ang II stimulation of not only aldosterone production pathways but also glomerulosa cell hyperplasia and pathological growth of cardiovascular cells. Worth noting is the fact that in mice with targeted deletion of the renin/Ang II system, potassium can substitute for the effects of Ang II to increase adrenal expression of CYP11B2 and synthesis of aldosterone [138, 139]. This result suggests that the mechanisms of potassium and Ang II stimulation of CYP11B2 expression likely overlap.

Chronic effects of potassium

Besides inducing early events to increase aldosterone production, potassium also regulates later/chronic events. It has been well documented that high potassium diets in rats increase the expression of aldosterone synthase (CYP11B2) and aldosterone production [140, 141]. A recent study in mice also reported a slight increase in the thickness of the zona glomerulosa and suggested the role of several genes in this process. These genes, including Mtus 1, Smoc 1 and Grp 48, were observed to be upregulated with 28 days of a high potassium diet, although the *in vitro* experiments did

not completely parallel these microarray results [142]. However, attributing these observed changes solely to the chronic effects of potassium on aldosterone production is particularly challenging, since physiological potassium levels are tightly regulated by the renin-angiotensin system. Indeed, serum potassium levels following high potassium diets were not reported to be abnormally high. Other *in vitro* studies using primary cultures of rat glomerulosa cells and the human adrenocortical H295R cell line have demonstrated increased CYP11B2 mRNA levels, promoter activation and aldosterone biosynthesis in response to elevated potassium levels in the growth media [144-147]. These results may help to explain the finding that in transgenic mice with targeted deletion in the renin-angiotensin system, potassium can induce CYP11B2 expression in the adrenal as well as the synthesis of aldosterone [138, 139].

As mentioned earlier, the mechanism of potassium signaling in glomerulosa cells involves depolarization of the cells to allow extracellular calcium influx through the T- and L-type calcium channels. This increase in calcium influx upregulates CYP11B2 expression. Consistent with these findings, increasing calcium influx with the pharmacological calcium channel agonist BAYK8644 increases CYP11B2 mRNA expression in the H295R cell model [82, 116]. Further, this potassium-induced calcium influx elevation is abrogated by the calcium channel blocker nifidipine [145, 146], which also inhibits Ang II-induced CYP11B2 upregulation. As in the case of Ang II, potassium-induced calcium signaling occurs through the binding of calcium to the protein calmodulin. The calcium-calmodulin (CaM) complex activates several enzymes and kinases, many of which are expressed in a tissue-specific manner. Amongst the different CaM kinases (CaMK), types I and IV are more likely to be involved in chronic

stimulation of aldosterone secretion by Ang II and potassium [148]. Immunohistochemistry was used to demonstrate that CaMKI expression is elevated in the glomerulosa of the human adrenal gland [148]. The CaMK antagonist KN93 and the calmodulin inhibitor calmidazolium effectively inhibit potassium-induced CYP11B2 mRNA upregulation [40, 116] and promoter activation [40, 148].

Another similarity that potassium shares with Ang II is the ability to activate several transcription factors, such as of NURR1, ATF1, ATF2 and CREB, which bind the proximal promoter of CYP11B2 at key cis-elements to enhance transcription. Activation of these transcription factors appears to be mediated through phosphorylation by potassium-activated CaMK [40, 80, 81, 83]. Supporting these data is a recent study in which knock down of these transcription factors by siRNA technology reduced potassium-induced CYP11B2 promoter activity and mRNA levels [83]. This idea is also supported by the observation that within the adrenal cortex, the transcription factor NURR1 has higher expression in the zona glomerulosa and in aldosterone-producing tumors compared to the adjacent zona fasciculata [87].

As mentioned earlier, a small but interesting effect of a high-potassium diet in mice is the observed slight increase in the thickness of the zona glomerulosa [406]. A more significant increase in the thickness of the zona glomerulosa was found in rats on a high potassium diet for 2 – 7 weeks [143]. Also observed in the 2009 study by Gao et al. was elevated expression of Gpr48, a G protein-coupled receptor that has been implicated in increasing tumor invasiveness and metastasis in the HeLa cervical carcinoma cell line [406, 407]. Moreover, Gpr48 is also associated with down-regulation of cyclin-dependent kinase inhibitor p27 (Kip1) [407]. It is hence tempting to ascribe the observed increased

glomerulosa thickness to the effects of Gpr48. Chronic high potassium can thus regulate long-term aldosterone production, sodium retention and ultimately blood pressure via chronic mechanisms involving increased glomerulosa cell size and/or number as well as the cells' aldosterone synthetic capacity. However, a better understanding of chronic effects of potassium on the molecular pathways underlying secretion of aldosterone by adrenal glomerulosa cells is required.

Chronic effects of ACTH

While thought of primarily as the regulator of adrenal cortisol production, ACTH is considered a secondary regulator of zona glomerulosa aldosterone production. It is clear that adrenal glomerulosa cells (both *in vivo* and *in vitro*) can acutely increase aldosterone production in response to ACTH. However, over time ACTH causes cultured glomerulosa cells to switch their phenotype to that of a cortisol-producing fasciculata cell [149, 150]. *In vivo* studies by Allen et al. demonstrated that ablation of the pituitary pre-proopiomelanocortin-secreting cells that produce ACTH, and the resultant low ACTH level, was accompanied by a steep decrease in the transcript levels of CYP11B1, but not of CYP11B2 [151]. In agreement with this observation, treatment with ACTH causes an initial increase in mRNA levels of CYP11B2 in the first 3 h; however, chronically, CYP11B2 expression decreases in response to ACTH *in vitro* in isolated rat adrenal cells [152]. Similarly, chronic low-dose infusion of ACTH in human subjects results in an initial increase in plasma renin activity and plasma aldosterone levels during the first 12 – 36 h, but a slow decline in these values over the next several days [153]. While the H295R adrenocortical cell lines express low levels of ACTH receptors, treatment of these cells with cAMP analogs preferentially increases the expression of CYP11B1 over

that of CYP11B2 [145]. The mechanism for chronic ACTH-mediated repression of aldosterone production and CYP11B2 expression remains unknown. An interesting observation has been that cAMP signaling reduces the sensitivity of adrenocortical cells to Ang II by down-regulating the expression of Ang II receptors [154, 155]. Another possible mechanism for the reduction in aldosterone production with chronic ACTH stimulation could be via the hormone's direct induction of CYP11B1 and CYP17, the activities of which direct the precursors of the steroidogenic pathway away from the production of aldosterone, and towards that of cortisol [130].

Since CYP11B2 has a cAMP-regulatory element (CRE) in its 5' promoter region [78], the mechanism preventing glomerulosa cells from responding to ACTH with increased CYP11B2 and excessive aldosterone production is not clear, but two possible mechanisms have been suggested thus far. First, at least in bovine glomerulosa cells, there is high expression of the inhibitory guanine nucleotide-binding protein G_i. Ang II signaling through the AT_1 receptor couples through G_i to inhibit ACTH-stimulated cAMP formation [156, 157]. Second, adrenal glomerulosa cells appear to express adenylyl cyclases 5 and 6, isoforms which are inhibited by a rise in intracellular calcium, a signaling mechanism common to Ang II and potassium stimulation of aldosterone secretion [158] The above provide evidence for a supportive, but not an obligatory, role of ACTH in aldosterone production.

Lessons from Primary Aldosteronism

Primary aldosteronism (PA) is the major endocrine cause of hypertension and is characterized by normal or elevated aldosterone levels in the presence of low

circulating plasma renin levels [4]. The most common cause of PA is idiopathic hyperaldosteronism (IHA), which occurs in approximately 60% of PA patients. The second most abundant cause of PA is unilateral adrenocortical adenoma, which is a curable form of PA that can be resolved by adrenalectomy. There are also rare forms of PA that include glucocorticoid-remediable aldosteronism (GRA), or familial hyperaldosteronism 1 (FH1), which is caused by unequal crossover between the CYP11B1 and CYP11B2 genes resulting in a hybrid gene with the CYP11B1 promoter driving CYP11B2. In these cases CYP11B2 expression and aldosterone secretion are regulated by ACTH in the adrenal zona fasciculata. Recently, several mouse models have been developed in an attempt to study PA.

Two recent studies using different mouse models, both with a deletion of genes encoding TWIK-related acid-sensitive K (TASK) channels, have provided interestingly different and complex primary aldosteronism phenotypes [97, 98]. As mentioned earlier, TASK channels maintain the membrane potential of the glomerulosa cell at a polarized ~ -70mV by being constitutively open and acting as a K+ leak channel. Inhibition of these channels by the AT_1R or by increased serum K^+ levels depolarizes glomerulosa cells and increases calcium influx to drive aldosterone secretion. In the study by Heitzmann et al., deletion of the TASK-1 channel resulted in a phenotype similar to the pathology of GRA, with characteristics such as salt-insensitive hyperaldosteronism, hypokalemia and dexamethasone-suppressible aldosterone secretion. The deletion of TASK-1 also seemed to change adrenal zonation and expression of CYP11B2, which was absent in the outermost zona glomerulosa but was expressed to a large extent in the zona fasciculata. Furthermore, this expression pattern seemed to be restricted only

to females and to males prior to puberty. On the other hand, Davies and colleagues found that deletion of both TASK-1 and TASK-3 created a model with a phenotype resembling the pathology of IHA. Male mice showed increased aldosterone secretion that was not suppressible with a high-salt diet or the AT_1R blocker candesartan [97]. Further studies will be required to understand the mechanism by which the different types of TASK channels interact and regulate adrenal function.

Besides TASK channels, Choi et al. have also identified both germ-line and somatic mutations that occur near the selectivity filter of the inward rectifying potassium channel *KCNJ5* (Kir3.4) to result in PA [5]. The amino acid substitutions resulting from these mutations modified channel ion selectivity, such that the channel became permeable to both sodium and potassium, which led to increased depolarization of adrenocortical cells. This depolarization is believed to cause elevated intracellular calcium and thereby the production of aldosterone and cell proliferation [5]. These findings are particularly relevant in that almost 40% of aldosterone-producing adenomas appeared to have such a mutation in KCNJ5. Additional studies will be needed to determine the exact mechanisms through which these mutations cause expansion of aldosterone-producing cells and formation of adenomas.

Summary

Aldosterone is an essential hormone with key roles in the regulation of electrolyte balance and blood pressure. Its normal physiological regulators include Ang II, K^+ and ACTH which can increase aldosterone secretion both acutely, by increasing StAR expression and phosphorylation, as well as chronically, by action on the steroidogenic

pathway, mostly through increasing gene expression of CYP11B2. Dysregulation in aldosterone secretion, as is seen in primary aldosteronism, leads to pathologies such as hypertension and cardiovascular disease. Several animal and cell culture models are being developed to better understand aldosterone secretion under normal and pathological conditions. The recent development of unique mouse models of primary aldosteronism and the discovery of the KCNJ5 mutations as a cause of human PA have been particularly helpful in providing new clues to the mechanisms controlling aldosterone production and the adrenal glomerulosa cell phenotype. However, additional studies will be needed to define completely the detailed pathways that activate and repress aldosterone biosynthesis.

Figures

Figure 5.3.1. Adrenocortical steroidogenic pathways for the production of mineralocorticoids and glucocorticoids. The adrenal cortex produces zone-specific steroids as a result of differential expression of steroidogenic enzymes. In the initial step of steroidogenesis, steroidogenic acute regulatory (StAR) protein is needed for the rate-limiting step of movement of cholesterol to the inner mitochondrial membrane, where cholesterol is cleaved by cholesterol side-chain cleavage (CYP11A1) to pregnenolone. Further steps of the steroidogenic pathway include the enzymes 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), 17α-hydroxylase, 17,20-lyase (CYP17), 21-hydrolylase (CYP21), 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2).

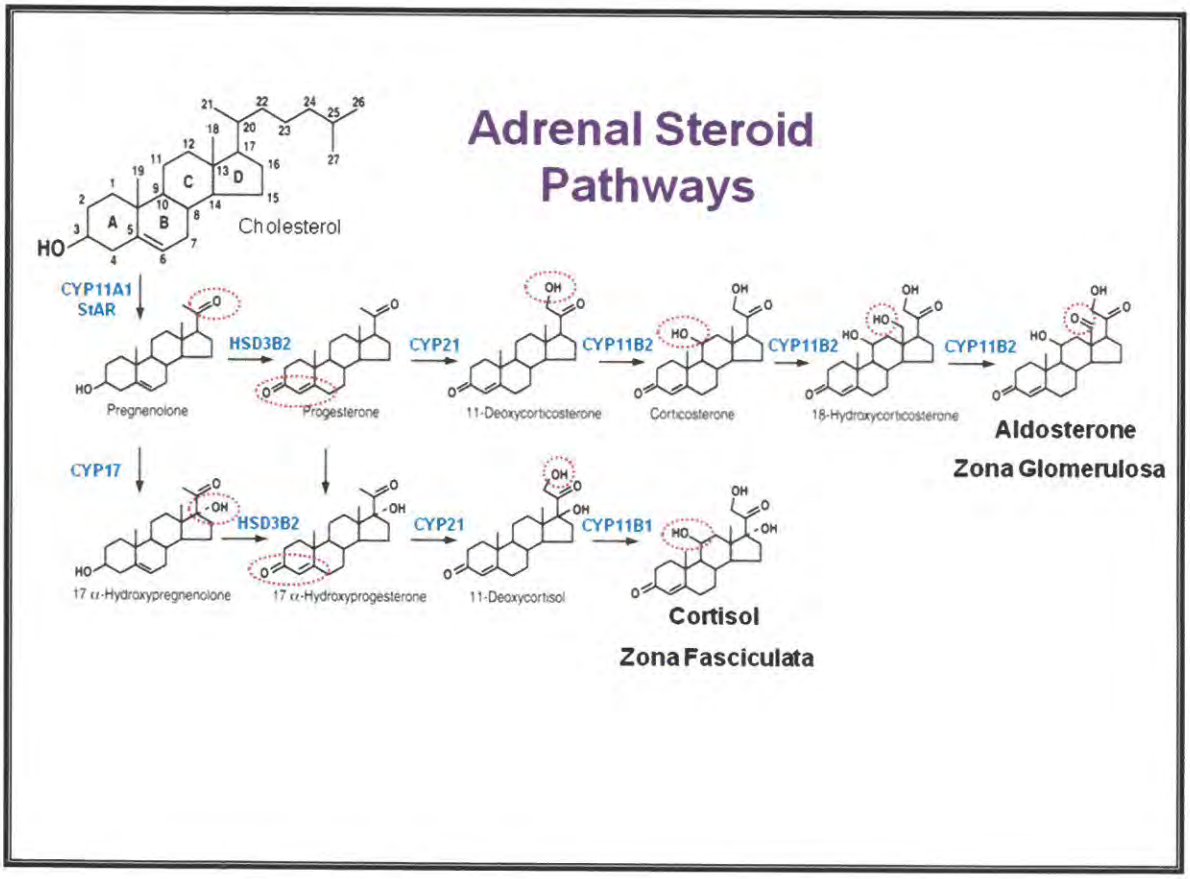


Figure 5.3.2. Acute actions of Ang II, K^+ and ACTH on adrenal glomerulosa cell aldosterone production. Ang II binds the AT_1 receptor to activate phosphoinositide-specific phospholipase C (PLC)-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds the IP_3R on the endoplasmic reticulum (ER), releasing calcium and raising cytosolic calcium concentrations. Ang II also activates, in part through protein kinase C (PKC), phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) which can be metabolized to DAG by lipid phosphate phosphatases. Small increases in extracellular K^+ depolarize the glomerulosa cell, activating the voltage-operated L-and T-type calcium channels, increasing calcium influx. Increased intracellular calcium concentration activates calcium/calmodulin-dependent protein kinases I/II (CaMK), as well as PKC isoforms. Both of these pathways can modulate not only StAR phosphorylation, but also expression, likely in part through the StAR promoter binding of cAMP response element binding protein (CREB). The DAG/PKC pathway also activates protein kinase D (PKD) which can likewise phosphorylate (and activate) CREB. DAG can be hydrolyzed by DAG lipase to release arachidonic acid, which can be further metabolized by 12-lipoxygenase to 12-hydroxyeicosatetraenoic acid (12-HETE), which also induces the phosphorylation (and activation) of CREB. ACTH can also mediate aldosterone synthesis through binding to the melanocortin type 2 *receptor* (MC2R), thus activating through a heterotrimeric G_s protein, adenylate cyclase (AC). AC converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) inducing a slow but sustained calcium influx through the L-type calcium channels. PKA also

phosphorylates CREB, thereby increasing StAR expression. Cholesterol for aldosterone production arises from cholesteryl ester hydrolase (CEH)-mediated hydrolysis of cholesteryl esters synthesized *de novo* or obtained from lipoproteins and stored in lipid droplets. Free cholesterol is shuttled to the inner mitochondrial membrane by StAR likely in complex with other cholesterol transport proteins.

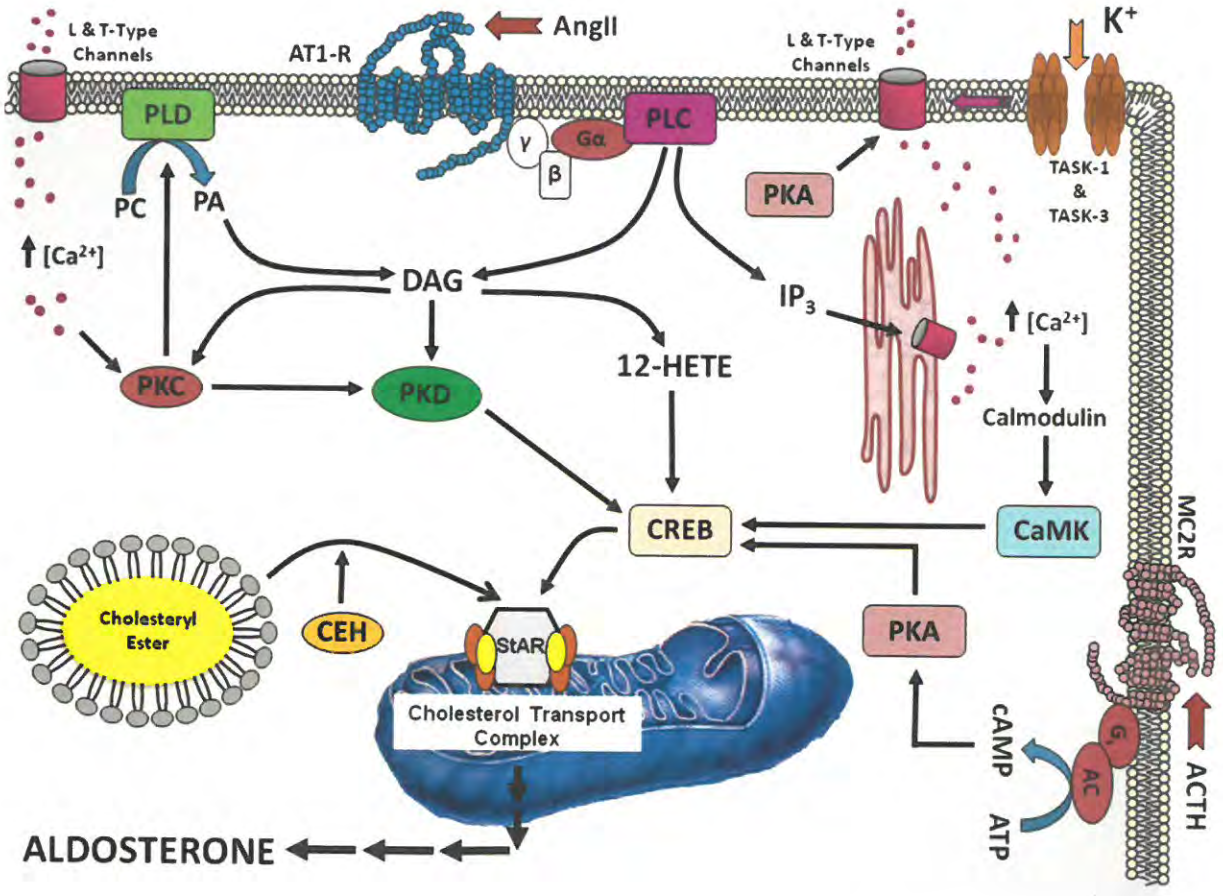
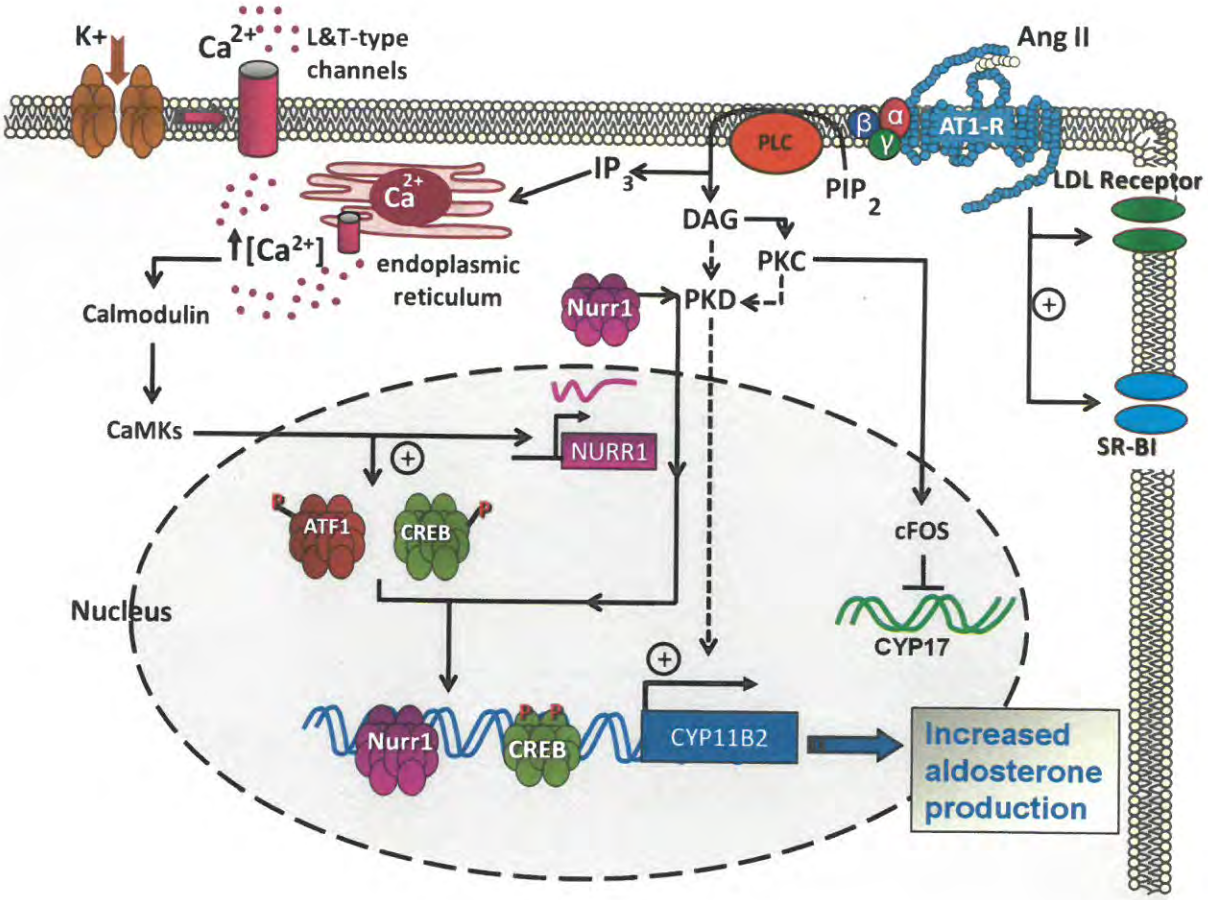


Figure 5.3.3. The chronic production of aldosterone is regulated by Ang II and potassium (K^+). Ang II binds type 1 Ang II receptors (AT_1-R) and activates phospholipase C (PLC) which causes hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG

activates protein kinase C (PKC) which inhibits transcription of 17α-hydrolyase (CYP17) through transcription factors such as cFOS. DAG may also increase the activity of protein kinase D (PKD), which has been shown to increase CYP11B2 transcription. IP_3 causes the release of intracellular calcium and the activation of calcium-calmodulin kinases (CaMKs). Small increases in extracellular K^+ also depolarize the glomerulosa cell, increasing calcium influx and activating CaMKs. CaMKs increase expression and/or phosphorylation and activation of transcription factors that increase CYP11B2 transcription. Further, binding of Ang II to the AT_1-R also increases the expression of LDL and HDL receptors, which increases cholesterol availability for steroidogenesis.



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SECTION IV

EFFECT OF KCNJ5 MUTATIONS ON GENE EXPRESSION IN ALDOSTERONE-PRODUCING ADENOMAS AND ADRENOCORTICAL CELLS

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ABSTRACT

Context: Primary aldosteronism is a heterogeneous disease that includes both sporadic and familial forms. A point mutation in the *KCNJ5* gene is responsible for familial hyperaldosteronism type III. Somatic mutations in KCNJ5 also occur in sporadic aldosterone producing adenomas (APA). **Objective:** Define the effect of *KCNJ5* mutations on gene expression and aldosterone production using APA tissue and human adrenocortical cells. **Methods:** Microarray analysis was used to compare transcriptome profiles of female derived APA samples with and without *KCNJ5* mutations and of HAC15 adrenal cells overexpressing either mutated or wildtype KCNJ5. Real-time PCR validated a set of differentially expressed genes. Immunohistochemical staining localized KCNJ5 expression in normal adrenals (NA) and APA. **Results:** We report a 38 % (18/47) prevalence of *KCNJ5* mutations in APA. KCNJ5 immunostaining was highest in the zona glomerulosa of NA and heterogeneous in APA tissue, and KCNJ5 mRNA was 4-fold higher in APA compared to NA ($p<0.05$). APA with and without *KCNJ5* mutations displayed slightly different gene expression patterns, notably the aldosterone synthase gene (*CYP11B2*) was more highly expressed in APA with *KCNJ5* mutations. Overexpression of KCNJ5 mutations in HAC15 increased aldosterone production and altered expression of 36 genes by >2.5-fold ($p<0.05$). Real-time PCR confirmed increases in *CYP11B2* and its transcriptional regulator, *NR4A2*. **Conclusions:** *KCNJ5* mutations are prevalent in APA, and our data suggest that these mutations increase expression of *CYP11B2* and *NR4A2*, thus increasing aldosterone production.

Key Words: KCNJ5 mutations, primary aldosteronism, aldosterone producing adenoma, CYP11B2, aldosterone

Forty-seven human adrenals were collected from APA patients from different centers. PA patients were studied following procedures that have been described previously [204, 409, 410]. All samples were used under Institutional Review Board (IRB) approval with written informed consent obtained from each patient.

Immunohistochemistry

Sections from formalin-fixed paraffin-embedded specimens were incubated with anti-KCNJ5 antibody. EnVision reagent (Dako) coupled with peroxidase-labeled polymer was incubated as secondary antibody. The slides were visualized with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2, counterstained with hematoxylin, and mounted.

Sequencing of KCNJ5

KCNJ5 cDNA was PCR amplified using intron-spanning primers [5] and sequenced using the following primers: Forward (5'-CGACCAAGAGTGGATTCCTT-3') and Reverse (5'-AGGGTCTCCGCTCTCTTCTT-3') [5].

RNA extraction and Gene expression assays.

RNA extraction and gene expression assays were performed as described previously [411].

Microarray analysis

RNA from 24 female APA samples were hybridized to an Illumina bead chip (Illumina, San Diego, CA). The arrays were scanned at high resolution on the iScan system

(Illumina). Results were analyzed using GeneSpring GX (version 11.5) software (Silicon Genetics, Redwood City, CA).

Molecular cloning of KCNJ5

The cDNAs encoding human $KCNJ5^{WT}$, $KCNJ5^{G151R}$, $KCNJ5^{L168R}$ were purchased from Invitrogen/Geneart and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA).

Cell culture and experimentation

HAC15 human adrenocortical carcinoma cells were cultured as described previously [411, 412] and electroporated using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany). Culture medium was assayed for aldosterone by radioimmunoassay.

RESULTS

Tissue expression of KCNJ5

KCNJ5 mRNA levels were quantified by real-time PCR in human placenta (n=4), testes (n=3), ovarian follicles (n=4), brain (n=4), fetal adrenals (n=4), adult adrenals (n=30) and APA (n=30). *KCNJ5* transcript levels were significantly higher in adrenocortical tissue compared to placenta, gonads and brain ($p<0.05$). Within the adrenal tissues, *KCNJ5* was 4-fold higher in APA compared to normal adrenals ($p<0.05$) (Supplemental Figure 1A). No significant difference in *KCNJ5* expression was observed between APA with or without *KCNJ5* mutations (data not shown). Immunohistochemical analysis revealed that KCNJ5 expression localizes in both the adrenal zona glomerulosa and outer part of

the fasciculata (Supplemental figure 1B); in APA, KCNJ5 expression was higher in the adenoma compared to the surrounding adrenal cortex (Supplemental Figure 1C).

Prevalence of KCNJ5 mutations in aldosterone producing adenomas

Of the 47 APA tissues, the overall prevalence of *KCNJ5* mutations in APA was 38% (Supplemental Table S1). Amongst the 18 APA with *KCNJ5* mutations, 8 APA (17%) had p.G151R and 10 APA (21%) had p.L168R mutations. The remaining samples contained only wildtype *KCNJ5* sequences. Of the 8 p.G151R mutations, 2 derived from the substitution c.451G>C and 6 from the substitution c.451G>A. Of note, *KCNJ5* mutations were more frequent in APA from female patients than males (71% vs 29%, p=0.05). Further, patients with mutated *KCNJ5* displayed lower serum potassium levels compared with wildtype APA (Supplemental Table S2).

Transcriptome analysis of APA with and without KCNJ5 mutations

Oligonucleotide microarrays were used to perform a transcriptome analysis of 24 APA from female patients, 15 with mutations in *KCNJ5* (8 p.L168R and 7 p.G151R) and 9 without mutations. APA with mutations in *KCNJ5* exhibited 24 differentially expressed genes compared to APA with wildtype *KCNJ5* (defined as 2.5 fold increase or decrease in mRNA levels: Figure 1 and Supplemental Table S3 and S4). Transcripts with the greatest differences in expression are shown in a heatmap presentation in Figure 1A. Interestingly, *CYP11B2* was one of the genes displaying differential expression. Our microarray analysis was validated by real-time PCR on a larger subset of samples, which showed 3-fold higher *CYP11B2* transcripts in tumors with the *KCNJ5* mutation compared to tumors without the mutations ($p<0.05$, Figure 1B).

Expression of KCNJ5 mutations in HAC15 cells

To better define the effects of the *KCNJ5* mutations on adrenal cell function, we overexpressed KCNJ5 in HAC15 adrenal cell model by transfection with pcDNA3.1/KCNJ5^{WT}, pcDNA3.1/KCNJ5^{G151R}, pcDNA3.1/KCNJ5^{L168R} or empty vector, Gene expression was analyzed using oligonucleotide microarrays. A total of 36 up-regulated genes (including *CYP11B2) and* 3 down-regulated genes were identified with significantly altered expression ($p<0.05$) in HAC15 cells expressing KCNJ5 mutations compared to wild type KCNJ5 (Figure 2A and Supplemental Table S5 and S6). *HSPA6* (Heat shock 70 KDa protein) and *NR4A2* (nuclear receptor subfamily 4, group A, member 2) were the two most up-regulated genes, with fold changes of 40.5 and 20.9, respectively. Also NR4A3 (nuclear receptor subfamily 4, group A, member 3) was upregulated (11.7-fold). Real-time PCR validated results on a broader sample set (Figure 2B). Moreover, the overexpression of KCNJ5 mutations resulted in increased aldosterone production (1.9 ± 0.2 fold in 48 h), when compared to KCNJ5^{WT} transfected cells.

DISCUSSION

Although great strides have been made in our understanding of the pathophysiology of PA, the molecular mechanisms causing the deregulated adrenal cell growth and aldosterone production remain poorly defined. Recently, mutations in *KCNJ5* gene were implicated in the pathogenesis of both FH-III and sporadic APA [5, 413-415]. The functional relevance of these mutations in the pathophysiology of APA and the regulation of aldosterone production are still unknown. Herein, we confirm recently published

prevalence findings and demonstrate that mutations in the *KCNJ5* gene cause augmented aldosterone production and *CYP11B2* expression in adrenal cells.

Recent studies have reported the prevalence of mutated *KCNJ5* in sporadic APA from different centers to range between 14% and 65.2% [414, 415]. We observed an overall 38% prevalence of *KCNJ5* mutations in APA, with a higher percentage in females. Further, all 5 of our Japanese APA had *KCNJ5* mutations. This result might relate to the patient population or center differences in referral patterns, yet a recent study also showed a high (65.2%) prevalence of *KCNJ5* mutations in Japanese APA. Further studies on a larger cohort of samples is warranted to determine if mutations APA are related to ethnicity. Serum K^+ levels were lower in patients with *KCNJ5* mutations, whereas plasma aldosterone, PRA and blood pressure did not differ between groups. Serum K^+, which reflects the chronic effect of aldosterone on the kidney, is a more reliable indicator of the severity of PA than serum aldosterone, which fluctuates with use of antihypertensive medications, time of day, dietary sodium, conditions of sampling and assay method.

In order to better define molecular differences between tumors with and without *KCNJ5* mutations, we performed a transcriptome analysis on APA tumors. Array comparison was done using only female samples to avoid the presence of gender bias in gene discovery. APA with and without *KCNJ5* mutations had a slightly different gene expression patterns, with reelin (*RELN*) and 5-hydroxytryptamine (serotonin) receptor 2B (*HTR2B*) as the two most up-regulated genes. Real-time PCR on a larger subset of APA samples also validated these results. The expression of the transcript encoding the late rate-limiting enzyme involved in aldosterone synthesis, *CYP11B2*, was also

significantly higher in APA with *KCNJ5* mutations than in tumors without these mutations. We have previously established the pathways that regulate *CYP11B2* transcription, and increased intracellular calcium is a key step in the signaling mechanisms shared by both AngII and potassium [83, 116, 148, 239, 316]. Both KCNJ5 amino acid substitutions, p.G151R and p.L168R, seem to increase sodium influx, cell depolarization, and subsequent over production of aldosterone [5].

To better define the influence of *KCNJ5* mutations on *CYP11B2* expression, we overexpressed wild type or mutant KCNJ5 in the HAC15 cells. Over-expression of *KCNJ5* mutations increased aldosterone secretion, whereas wildtype *KCNJ5* did not. In addition, both p.G151R and p.L168R mutations increased *CYP11B2* transcription compared to wild type KCNJ5, suggesting a direct link between KCNJ5 mutations and activation of aldosterone production through increased *CYP11B2* transcript levels. Importantly, we also observed an increase in key regulators of *CYP11B2* transcription, NR4A2 and NR4A3, the final effectors of the multiple signaling pathways activated by AngII and potassium in adrenal cells [239, 316]. However, no difference in transcript levels for *NR4A2* or *NR4A3* was observed between APA with or without *KCNJ5* mutations, because these transcription factors are likely a common final event needed for increased transcription of *CYP11B2* regardless of the primary molecular mechanism. In this respect, a cell model might be a better way to define genes that are regulated in the short term by mutated *KCNJ5*. . Exposure of cells to various stresses, as probably occurs with the sodium and calcium influx in cells transfected with KCNJ5 mutants could explain the overexpression of the heat shock proteins *HSPA6, HSPA7* and *HSPA1B* in

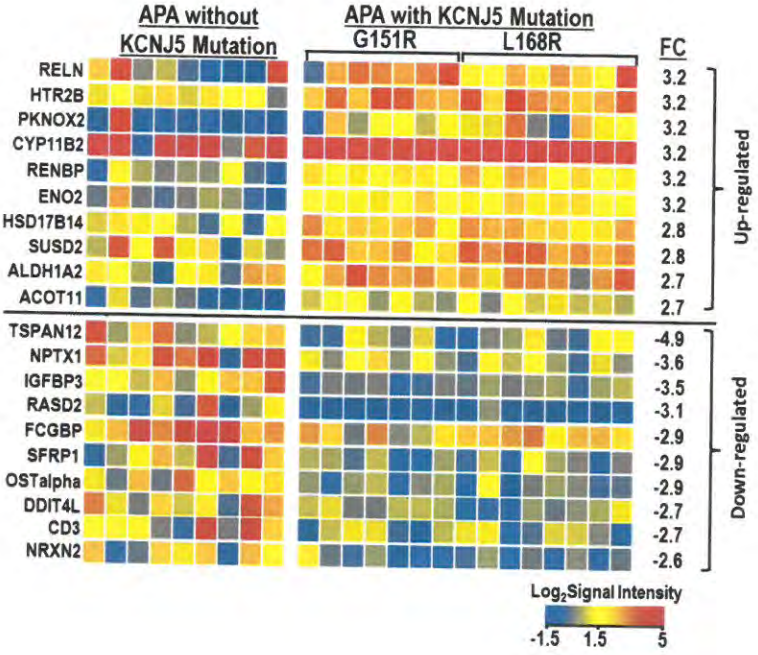
HAC15 cells with mutant KCNJ5, especially, Hsp70, which is involved in protection from stress-induced apoptosis [416].

In conclusion, we propose that KCNJ5 is primarily an adrenal glomerulosa-expressed protein, found at high levels in APA. While the role of the wildtype KCNJ5 protein in the regulation of aldosterone biosynthesis remains unclear, our findings confirm that two recurring mutations in the *KCNJ5* gene are commonly found in APA tumors. Transcriptome and real-time PCR analyses demonstrate that APA with KCNJ5 mutations exhibit enhanced *CYP11B2* expression. Finally, we found that overexpressing the KCNJ5 mutations but not wildtype KCNJ5 in adrenal cells increased aldosterone production by augmenting the transcription of *CYP11B2* and of its regulatory transcription factors. Together, our findings support a model in which the recurring KCNJ5 mutations p.G151R and p.L168R cause PA by activating the transcription of genes required for aldosterone production in adrenal cells.

FIGURE LEGENDS

Figure 5.4.1. Comparison of gene expression in aldosterone producing adenoma with./without KCNJ5 mutations. Panel A. Heatmap representation of the ten genes with the highest differential expression in APA with or without *KCNJ5* mutations. Genes were selected based on a significance of $p<0.05$ and a differential expression of at least 2.5 fold. Heatmap data are presented as Log_2 of the signal intensity value. Absolute fold change (FC) is also provided. Panel B-E. Validation of microarray using real-time PCR. Four genes were selected to confirm microarray analysis by using real-time PCR on a larger subset of RNA samples from women with APA (13 $KCNJ5^{WT}$ and 20 KCNJ5 mutant APA). Comparison of APA with and without KCNJ5 mutations demonstrated a significant up-regulation of *CYP11B2* (3.08-fold change), *RELN* (2.47-fold change) *and HTR2B (2.21 fold,)* but a significant down regulation of *TSPAN12* (-5.08-fold change. Data are presented as normalized (cyclophylin) transcript fold change with each bar representing the mean ± SE. * $p< 0.05$ versus APA with wild type KCNJ5.

(A)



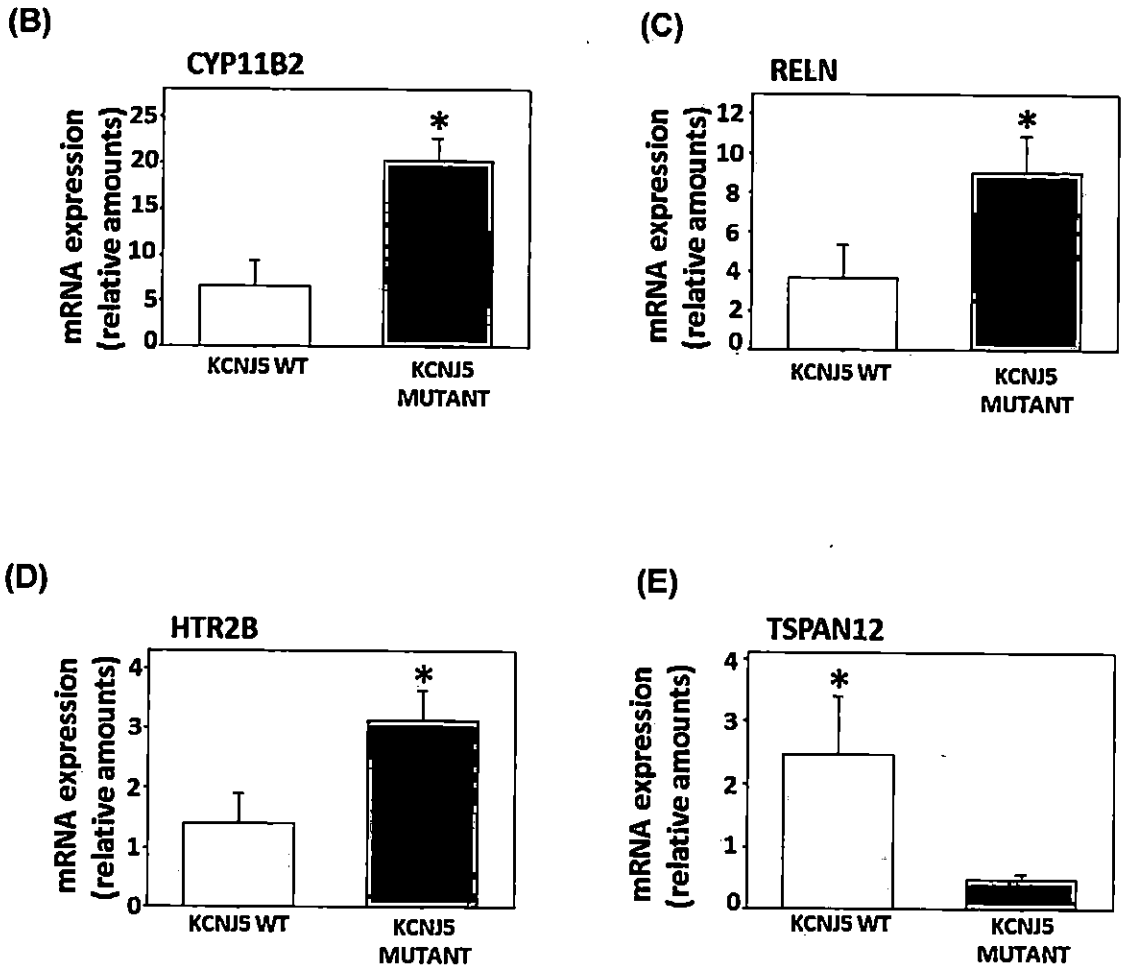
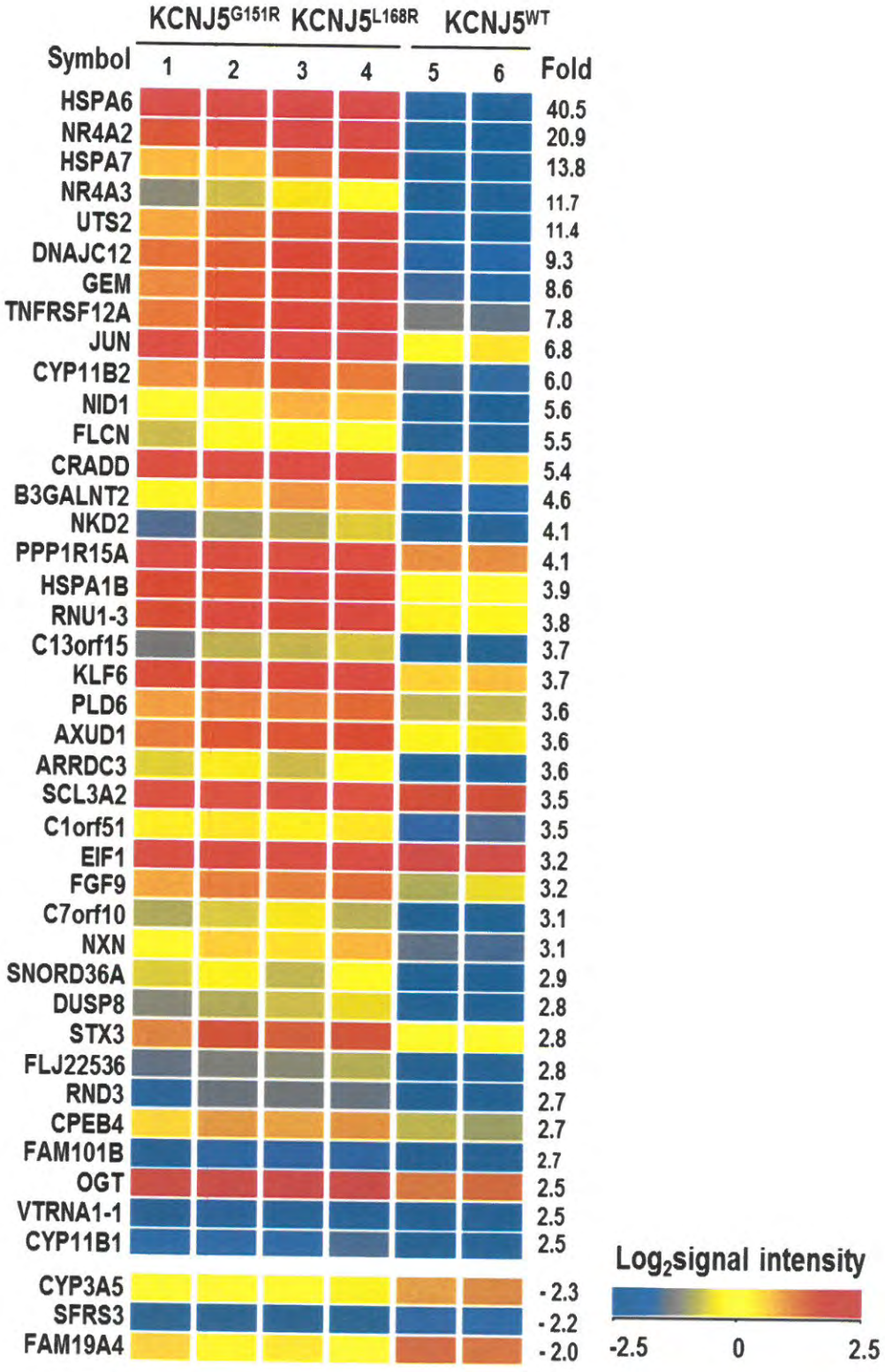


Figure 5.4.2. Effects of expression of mutant KCNJ5 (G151R and L168R) on gene expression in vitro. Panel A. Heatmap representation of differentially expressed genes in HAC15 adrenal cells overexpressing either KCNJ5 mutations (p.G151R and p.L168R) or $KCNJ5^{WT}$. Genes were selected based on a significance of $p<0.05$ and a differential expression of at least 2.0-fold. Heatmap data are presented as Log_2 of intensity value. Absolute fold change (FC) is also provided. Samples are HAC15 cells transfected with (1 and 2) $pcDNA3.1/KCNJ5^{G151R}$; (3 and 4) $pcDNA3.1/KCNJ5^{L168R}$; (5

and 6) pcDNA3.1/KCNJ5^{WT}. Panels B-E. Validation of up-regulated genes using real-time PCR. *CYP11B2* was up-regulated 6.3±0.6-fold and 7.2±1.4-fold in HAC15 cells overexpressing KCNJ5^{G151R} or KCNJ5^{L168R}, respectively, compared to HAC15 cells overexpressing KCNJ5^{WT}. *NR4A2* was markedly up-regulated 98 ± 28-fold and 38 ±6-fold in HAC15 cells overexpressing KCNJ5 ^{L168R} or KCNJ5 ^{G151R}, respectively, versus KCNJ5^{WT}, with a relative fold change L168R/G151R of 2.6 ($p<0.05$). No statistically significant differences were observed between the effects of the G151R and L168R mutations on the gene expression levels of *CYP11B1*, *CYP11B2* and *NR4A3* in HAC15 cells. No statistically significant difference was observed between HAC15 cells overexpressing KCNJ5^{WT} and pcDNA3.1 mock-transfected cells for any of the four selected genes. Each bar represents the mean ±SD of relative fold-change of gene expression in 5 independent experiments. Each assay was performed in triplicate, and *PPAI* (cyclophylin) was used as endogenous control. *$p<0.05$ compared to WT; ** $p<0.05$ compared to WT and G151R.

(A)


KCNJ5G151R
KCNJ5L168R
KCNJ5WT
Symbol
1
2
3
4
5
6
Fold
HSPA6 40.5
NR4A2 20.9
HSPA7 13.8
NR4A3 11.7
UTS2 11.4
DNAJC12 9.3
GEM 8.6
TNFRSF12A 7.8
JUN 6.8
CYP11B2 6.0
NID1 5.6
FLCN 5.5
CRADD 5.4
B3GALNT2 4.6
NKD2 4.1
PPP1R15A 4.1
HSPA1B 3.9
RNU1-3 3.8
C13orf15 3.7
KLF6 3.7
PLD6 3.6
AXUD1 3.6
ARRDC3 3.6
SCL3A2 3.5
C1orf51 3.5
EIF1 3.2
FGF9 3.2
C7orf10 3.1
NXN 3.1
SNORD36A 2.9
DUSP8 2.8
STX3 2.8
FLJ22536 2.8
RND3 2.7
CPEB4 2.7
FAM101B 2.7
OGT 2.5
VTRNA1-1 2.5
CYP11B1 2.5
CYP3A5 -2.3
SFRS3 -2.2
FAM19A4 -2.0
Log2signal intensity
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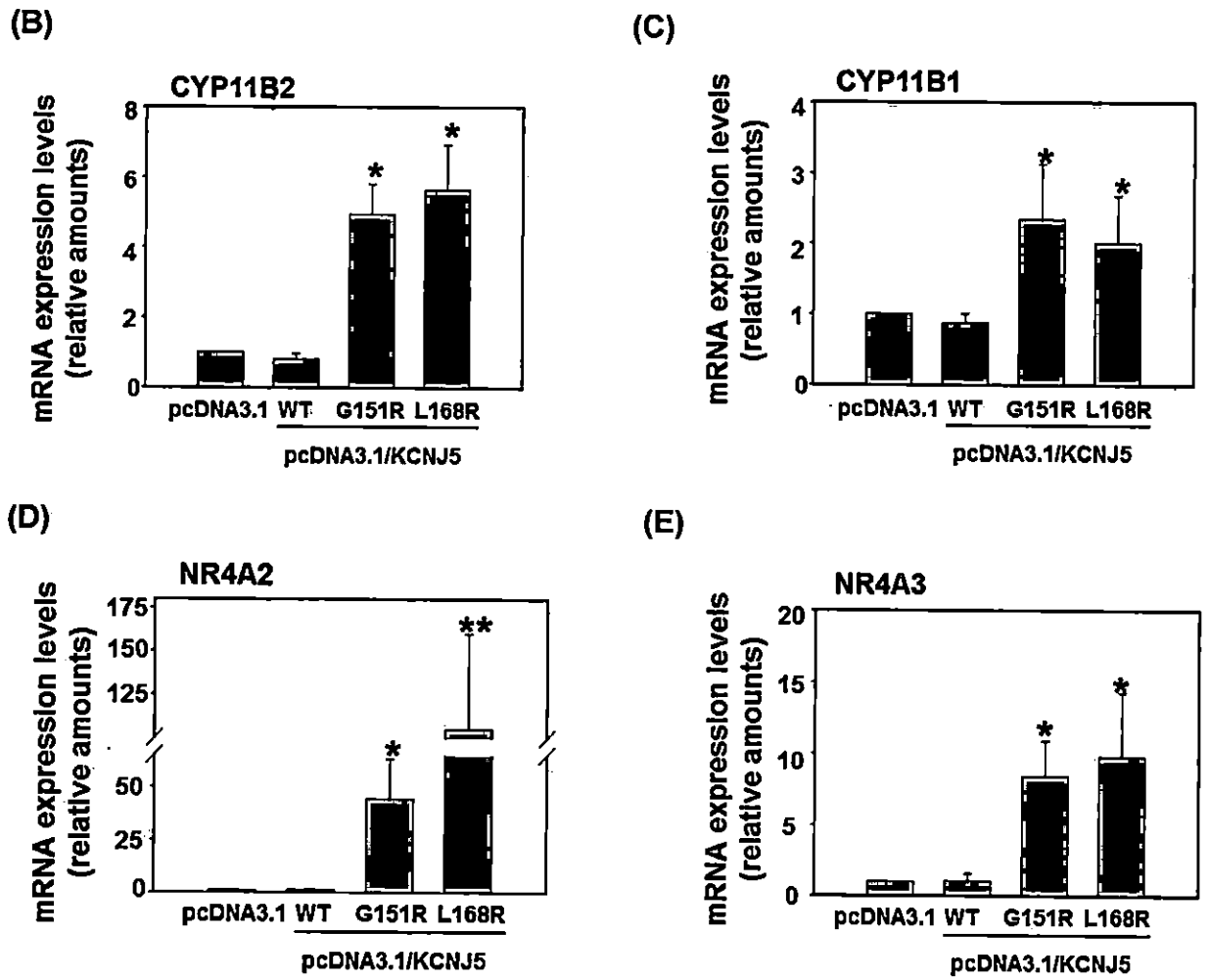
0
2.5

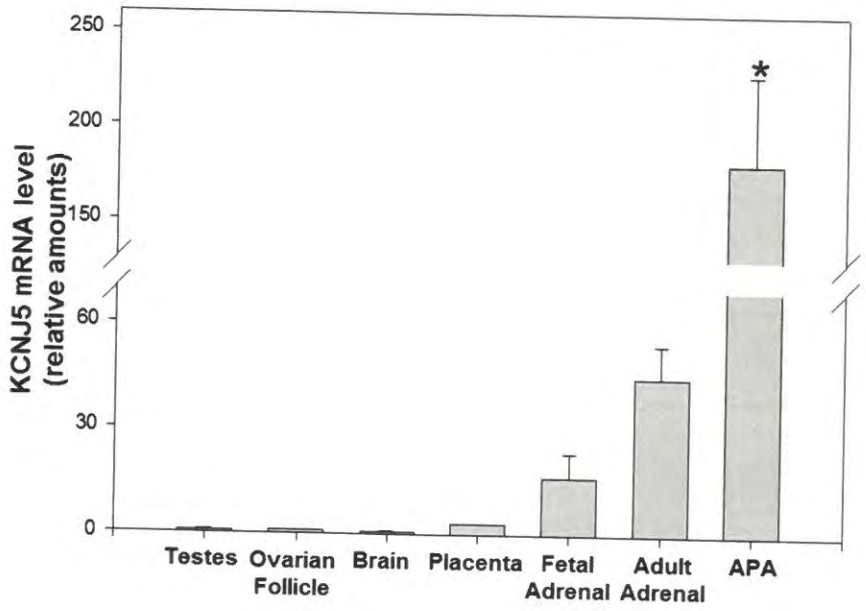


Supplemental Figures

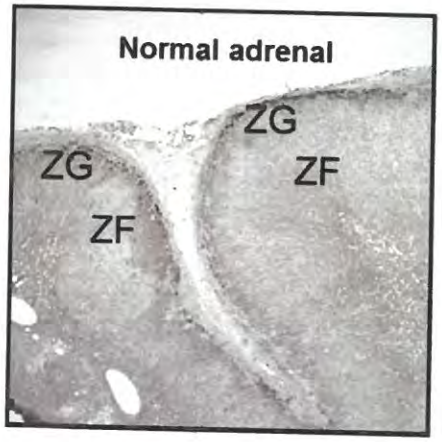
Supplemental Figure 5.4.S1. Expression of KCNJ5 across various steroidogenic tissues, and across the adrenal zones. Panel A. KCNJ5 transcript levels in human steroidogenic tissues and brain. As detailed in the Supplemental Methods section, KCNJ5 mRNA expression was studied by quantitative PCR in testes (n=4), ovarian follicle (n=4), brain (n=3), placenta (n=4), fetal adrenal (n=4), adult adrenal (n=30) and APA (n=30). Data were normalized to 18S rRNA and expressed as fold change over brain expression. Each bar represents the mean ±SE of each group of samples (*,

p<0.05). Panel B. Immunohistochemical staining of KCNJ5 in normal adrenal using the protocol detailed in the Supplemental Methods. Panel C. Immunohistochemical staining of KCNJ5 in a representative APA using the protocol detailed in the Supplemental Methods.

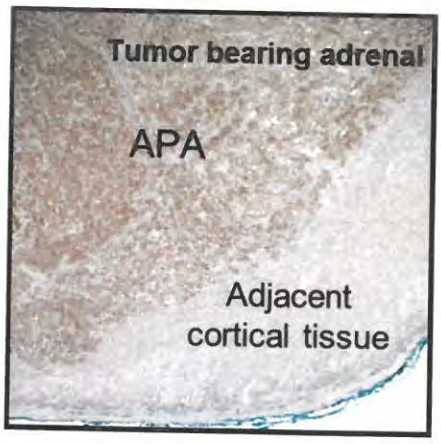
(A)



(B)



(C)



Supplemental table 5.4.S1. Prevalence of KCNJ5 mutations in aldosterone producing adenomas.

	Padova (Italy)	Dallas (TX)	Augusta (GA)	Tokyo (Japan)	Total
No.of samples	26	10	6	5	47
No.of wild type	18	7	4	0	29
No.of mutated(%)					
p.G151R (%)	5(19%)	0	0	3(60%)	8 (17%)
p.L168R (%)	3(12%)	3(30%)	2(33%)	2(40%)	10(21%)
p.T158A (%)	0	0	0	0	0 18(38%)
Total (%)	8(31%)	3(30%)	2(33%)	5(100%)	

Supplemental table 5.4.S2. Clinical and biochemical data of APA patients according to KCNJ5 status. Data are expressed as mean ± standard deviation or median [25th-75th percentile]. SBP= systolic blood pressure; DBP= diastolic blood pressure; NS= not significant

	KCNJ5 WT	KCNJ5 mutated	p-value
Age (years)	48±12	49±8	N.S.
Females (%)	50%	71%	0.05
SBP (mmHg)	168±32	161±17	N.S.
DBP (mmHg)	99±7	101±11	N.S.
Serum Aldosterone (ng/dL)	37[31-45]	33[23-39]	N.S.
Plasma Renin Activity (ng/mL/h)	0.3 [0.2-0.5]	0.2[0.1-0.4]	N.S.
Serum K^+ (mEq/L)	3.5±0.38	3.0±0.4	0.036

Supplemental table 5.4.S3. Statistically significant up and down regulated genes in KCNJ5 mutated APAs compared to KCNJ5-WT APA.

Gene Symbol	Gene Name	Chromosome	Probe ID	Regulation	Average fold change
RELN	Reelin (RELN), transcript variant 2	chr7	4490221	up	3.2
HTR2B	5-hydroxytryptamine (serotonin) receptor 2B	chr12	6740037	up	3.2
PKNOX2	PBX/knotted 1 homeobox 2	chr11	870575	up	3.2
CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2	chr8	2060114	up	3.2
RENBP	Renin binding protein	chrX	5810678	up	3.2
ENO2	Enolase 2 (gamma, neuronal)	chr12	50402	up	3.2
HSD17B14	Hydroxysteroid (17-beta) dehydrogenase 14	chr19	2230133	up	2.8
SUSD2	Sushi domain containing 2	chr22	110592	up	2.8
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2	chr15	510687	up	2.7
ACOT11	Acyl-CoA thioesterase 11	chr1	1050253	up	2.7
RAB3C	RAB3C, member RAS oncogene family	chr5	4570474	up	2.6
APOD	Apolipoprotein D	chr3	7150634	up	2.5
TSPAN12	Tetraspanin 12	chr7	3830193	down	4.9
NPTX1	Neuronal pentraxin I	chr17	6100468	down	3.6
IGFBP3	Insulin-like growth factor binding protein 3	chr7	6840372	down	3.5
RASD2	RASD family, member 2	chr22	10397	down	3.1
FCGBP	Fc fragment of IgG binding protein	chr19	130609	down	2.9
SFRP1	Secreted frizzled-related protein 1	chr8	6960379	down	2.9
OSTalpha	Organic solute transporter alpha	chr3	5560364	down	2.9
DDIT4L	DNA-damage-inducible transcript 4-like	chr7	380019	down	2.7
CD36	CD36 molecule (thrombospondin receptor)	chr7	3310538	down	2.7
NRXN2	neurexin 2 (NRXN2), transcript variant beta	chr11	3360678	down	2.6
BCAT1	Branched chain aminotransferase 1, cytosolic	chr1	7650524	down	2.5
FAM43A	Family with sequence similarity 43, member A	chr3	1690630	down	2.5

Supplemental table 5.4.S4 Microarray comparison of transcript levels in HAC15 cells transfected with KCNJ5-WT, KCNJ5-L168R and KCNJ5-G151R. Date provided are the statistically significant up and down regulated genes in KCNJ5-G151R and KCNJ5L168R transfected cells compared to KCNJ5WT transfected cells. As described in the Supplemental Methods, HAC15 cells were electroporated with 3 µg KCNJ5 expression plasmid in 100 µl of Nucleofector solution R using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany). After electroporation, cells were plated in 6-well plates with 5 mL of growth medium/well and recovered for 24 h, then starved overnight (18 h) in 0.1% low serum media (0.1% Cosmic calf serum and antibiotics).RNA was then isolated and used for microarray analysis.

Gene Symbol	Gene Name	Chromosome	Probe ID	Regulation	Average fold change
HSPA6	Heat shock 70kDa protein 6	chr1	160092	up	41
NR4A2	Nuclear receptor subfamily 4, groUp A, member 2	chr2	6400364	up	21
HSPA7	Heat shock 70kDa protein 7 (HSP70B)	chr1	7200079	up	14
NR4A3	Nuclear receptor subfamily 4, groUp A, member 3	chr9	6200026	up	12
UTS2	Urotensin 2 (UTS2), transcript variant 2	chr1	6290228	up	11
DNAJC12	DnaJ (Hsp40) homolog, subfamily C	chr10	6900538	up	9.3
GEM	GTP binding protein overexpressed in skeletal muscle	chr8	1170246	up	8.6
TNFRSF-12A	Tumor necrosis factor receptor superfamily, member 12A	chr16	6510377	up	7.8
JUN	Jun oncogene	chr1	6510367	up	6.8
CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2	chr8	2060114	up	6.0
NID1	Nidogen 1	chr1	4780239	up	5.6
FLCN	Folliculin	chr17	6980148	up	5.5

Gene Symbol	Gene Name	Chromosome	Probe ID	Regulation	Average fold change
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	chr12	160376	up	5.4
B3GALNT2	Beta-1,3-N-acetylgalactosaminyltransferase 2	chr1	7210647	up	4.6
NKD2	Naked cuticle homolog 2 (Drosophila)	chr5	1340600	up	4.1
PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	chr19	3850433	up	4.1
HSPA1B	Heat shock 70kDa protein 1B	chr6	5670152	up	3.9
RNU1-3	RNA, U1 small nuclear 3	chr1	160242	up	3.8
C13orf15	Chromosome 13 open reading frame 15	chr13	360110	up	3.7
KLF6	Kruppel-like factor 6 (KLF6)	chr10	160376	up	3.7
PLD6	Phospholipase D family, member 6	chr17	1340707	up	3.6
AXUD1	AXIN1 Up-regulated 1	chr3	7560041	up	3.6
ARRDC3	Arrestin domain containing 3	chr5	4060672	up	3.6
SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	chr11	5420575	up	3.5
C1orf51	Chromosome 1 open reading frame 51	chr1	2510068	up	3.5
EIF1	Eukaryotic translation initiation factor 1	chr17	3710544	up	3.2
FGF9	Fibroblast growth factor 9	chr13	6420746	up	3.2
C7orf10	Chromosome 7 open reading frame 10	chr7	4040097	up	3.1
NXN	Nucleoredoxin	chr17	2140431	up	3.1
SNORD36A	Small nucleolar RNA, C/D box 36A	chr9	2750446	up	2.9
DUSP8	Dual specificity phosphatase 8	chr11	4890041	up	2.8
STX3	Syntaxin 3 (STX3)	chr11	2350703	up	2.8
FLJ22536	Hypothetical locus LOC401237	chr6	20168	up	2.8
RND3	Rho family GTPase 3	chr2	4210524	up	2.7
CPEB4	Cytoplasmic polyadenylation element binding protein 4	chr5	1500131	up	2.7
FAM101B	Family with sequence similarity 101	chr17	580767	up	2.7
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	chrX	1070300	up	2.5
VTRNA1-1	Vault RNA 1-1	chr5	4220255	up	2.5
CYP11B1	Cytochrome P450, family 11,	chr8	4220398	up	2.5

Gene Symbol	Gene Name	Chromosome	Probe ID	Regulation	Average fold change
	subfamily B, polypeptide 1				
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	chr7	6250059	down	2.3
SFRS3	Splicing factor, arginine/serine-rich 3	chr6	1090300	down	2.2
FAM19A4	Family with sequence similarity 19	chr3	5050100	down	2.0

SUPPLEMENTAL METHODS

Patients selection and tissues

Forty-seven human adrenal glands were collected from APA patients. All samples were used under Institutional Review Board (IRB) approval with written informed consent obtained from each patient. Serum aldosterone/plasma renin activity ratio (ARR) (normal values: PRA [1.5-6 ng/ml/h] and aldosterone 5-30 ng/dL] was used as screening test, and the diagnosis of PA was confirmed by captopril test, oral salt loading, or intravenous saline loading tests. All patients underwent CT scanning and AVS using strict criteria for both cannulation and lateralization as described elsewhere [210, 409]. All patients with APA were adrenalectomized by laparoscopy. Diagnosis of APA was confirmed by histology and post-surgery evaluation (correction of hypokalemia, normalization of ARR, cure or significant improvement of hypertension, normal suppression of aldosterone).

Normal human adult adrenal, ovarian follicles, ovarian corpora luteum, brain, placenta and testis tissues were obtained through the Cooperative Human Tissue Network (Philadelphia, PA) and Clontech (Palo Alto, CA). Fetal adrenals (14–19 wk gestation) were obtained from pathological examination following elective pregnancy terminations from Advanced Bioscience Resources with informed consent.

Immunohistochemistry

Sections (4 μm) from formalin-fixed paraffin-embedded specimens were incubated with anti-KCNJ5 antibody (Sigma, St. Louis, MO) at a dilution of 1:100 for 30 minutes. Envision reagent coupled with peroxidase-labelled polymer (DAKO, Carpinteria, CA,) was incubated as secondary antibody for 15 minutes. The slides were visualized with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 (DAKO), counterstained with hematoxylin, and mounted.

RNA extraction and gene expression assays

Adrenal tissue was homogenized in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) with 700 μL of RLT buffer (Qiagen, Hilden, Germany) with 10% β-mercaptoethanol. RNeasy Mini Kits (Qiagen) were used for total RNA was isolation according to the manufacturer's instructions. The purity and quantity were assessed by a ND-1000 NanoDrop spectrophotometer NanoDrop Technologies, Wilmington, DE).

For cDNA generation a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to reverse transcript 2 μg total RNA. Real time PCR was performed for KCNJ5, Reelin (RELN), 5-hydroxytryptamine (serotonin) receptor 2B (*HTR2B*), tetraspanin 12 (TSPAN12), nuclear receptor subfamily 4, group A, member 2 (NR4A2) and nuclear receptor subfamily 4, group A, member 3 (NR4A3) using TaqMan gene expression assays (Applied Biosystems) and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The primer and probe set for human aldosterone synthase (CYP11B2) and *11β-hydroxylase (CYP11B1)* were designed using Primer Express 3.0 (Applied Biosystems) and purchased from Integrated DNA Technologies, Inc., (Coralville, IA) as described previously [417]. Real-time PCR was performed with 5 μl cDNA, 10 μl TaqMan Fast Universal PCR Master Mix (2X; Applied Biosystems), 900 nM of each primer, and 400 nM probe in a total volume of 20 μl per well. Gene

expression levels were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method, using 18S RNA or cyclophillin (PPAI) (for elctroporation studies) as endogenous controls.

Sequencing of KCNJ5

KCNJ5 cDNA was PCR amplified using intron spanning primers [5]. PCR was performed on 100 ng of cDNA (4 µL of reverse transcription product) in a final volume of 50 µL containing 1.5 mM $MgCl_2$, 200 nM of each primer, 200 µM deoxynucleotide triphosphate and 1.25 U GoTaq DNA Polymerase (Promega, Fitchburg, WI,) using primer sets described by Choi et al [5]. The PCR product was sequenced (MacrogenUSA, Rockville, MD) using the following primers: Forward (5'-CGACCAAGAGTGGATTCCTT-3') and Reverse (5'-AGGGTCTCCGCTCTCTTCTT-3') [5].

Microarray analysis

RNA samples used for microarray experiments were assayed for purity and quality using an Experion Automated Electrophoresis System (Bio Rad, Life Sciences Group, Hercules, CA). RNA from 24 APAs was hybridized to an Illumina bead chip containing more than 48,000 probes representing over 25,000 human genes (Illumina, San Diego, CA). The arrays were scanned at high resolution on the iScan system (Illumina) at the GHSU core facility. Results were analyzed using GeneSpring GX (version 11.5) software (Silicon Genetics, Redwood City, CA).

Briefly the microarray data were filtered on flags (present, marginal) and were analyzed using unpaired t-test with Benjamini and Hochberg false discovery rate (FDR) correction. P value < 0.05 was considered to be significant.

Cell culture and experimentation.

HAC15 human adrenocortical carcinoma cells were cultured in growth media consisting of Dulbecco's Modified Eagle/F12 (Invitrogen) supplemented with 10% Cosmic calf serum

(HyClone, Logan, UT), 1% insulin/transferrin/selenium Premix (BD Biosciences, Sparks, MD) and antibiotics, including 1% penicillin/streptomycin (Invitrogen) and 0.1% gentamicin (Sigma-Aldrich).

HAC15 cells (3 x 10^6 cells) were electroporated with 3 µg plasmid in 100 µl of Nucleofector solution R using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany). After electroporation, cells were plated in 6-well plates with 5 mL of growth medium/well and recovered for 24 h, then starved overnight (18 h) in 0.1% low serum media (0.1% Cosmic calf serum and antibiotics).

Culture medium from transfected cells was assayed for aldosterone using a radioimmunoassay kit from Siemens Healthcare Diagnostics (Los Angeles, CA), and radioactivity was read by a multicrystal γ-counter (Berthold Technologies, Bad Wildbad, Germany).

Statistical analyses

IBM SPSS Statistics 19 (SPSS INC, Chicago, IL) and Sigma Stat 3.1.1 were used for statistical analyses. Data are expressed as mean ± S.D unless specified differently and individual experiments were repeated at least three times. Differences between variables were evaluated using one-way ANOVA (including one-way ANOVA on rank), unpaired *t* tests or Mann-Whitney test. A probability of less than 0.05 was considered statistically significant.

CHAPTER 4

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