Aus der Medizinischen Klinik Innenstadt
der Ludwig-Maximilians-Universität München

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Establishment of a Mouse Model with Mutagenesis Induced Hyperaldosteronism

Dissertation
Zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilian-Universität zu München

vorgelegt von
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aus Athen
2009

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Tag der mündlichen Prüfung:	18.06.2009

To my father

Contents

1. Introduction

1.1 Renin – Angiotensin – Aldosterone (RAA) System

The RAA system represents a cascade of different hormones and enzymes that regulate water and salt balance in the organism. Thus, the RAA system is one of the most important endocrine axes that impacts blood pressure.

The cascade begins with the release of the enzyme renin from the juxtaglomerular apparatus of the kidney. This consists of specialized cells of the capillary, which supply the glomerulus of the kidney with blood (vas afferens), of cells of the macula densa, and of cells of the connective tissue (mesangium). The juxtaglomerular apparatus measures the renal perfusion pressure of the vas afferens and the salt concentration of the urine in the urinary canaliculi and responds also to beta adrenergic stimulation and prostaglandins (Persson et al., 2004). Changes in perfusion pressure are sensed by myotransducers in the wall of the afferent arteriole. These signals are then transmitted to the juxtaglomerular apparatus to modify the level of renin secretion. The renin granules are produced and stored in the macula densa (Barajas, 1979). Reduced blood perfusion of the kidney units, reduced blood pressure measured by the pressure sensors of the vas afferens, reduced glomerular filtration rate, reduced sodium chloride concentration in the urine and activation of the sympathetic nervous system cause an increase in the release of renin (Guyton, 1991, Saxena, 1992).

Renin then acts as a protease and hydrolyses angiotensinogen, a circulation alpha 2 globulin produced in the liver into the inactive decapeptide angiotensin I. Angiotensin I is further cleaved in the lungs and several other tissues by endothelial bound angiotensin converting enzyme (ACE) into angiotensin II (ANGII), the final active octapeptide (Fig. 1). Angiotensin II is a potent vasoconstrictor, which leads to an increase of blood pressure. Angiotensin II also has prothrombotic potential through adhesion and aggregation of platelets and production of PAI-1 and PAI-2 (Skurk et al., 2001, Gesualdo et al., 1999). In addition, the same system can be activated in smooth muscle cells in conditions of hypertension, atherosclerosis or endothelial damage. Moreover, when cardiac cell growth is stimulated, a local (autocrine-paracrine) renin-angiotensin system is activated in the cardiac myocyte, which stimulates cardiac cell growth through Protein Kinase C dependent pathways (Dzau, 2001). Angiotensin II is the most important stimulator of the heart during hypertrophy, compared to endothelin-1 and A1 adrenoreceptors (Taugner et al., 1984). In the kidney, angiotensin II causes vasoconstriction of the vas efferens and thereby increases the resistance of the vessels and the blood pressure of the capillaries of the kidney. Angiotensin II also causes the release of aldosterone from the adrenal cortex. This induces the resorption of sodium and water from the urine and increases their concentration in the blood. Angiotensin II also exerts effects on

the pituitary, where it increases the secretion of adrenocorticotropic hormone (ACTH) (Schoenenberg et al., 1987) and of antidiuretic hormone (ADH) (Usberti et al., 1989). ADH increases the permeability of the distal convoluted tubules and collecting tubules in the nephrons of kidneys to water, thus, allows water reabsorption and excretion of a smaller volume of concentrated urine (Fig. 1).

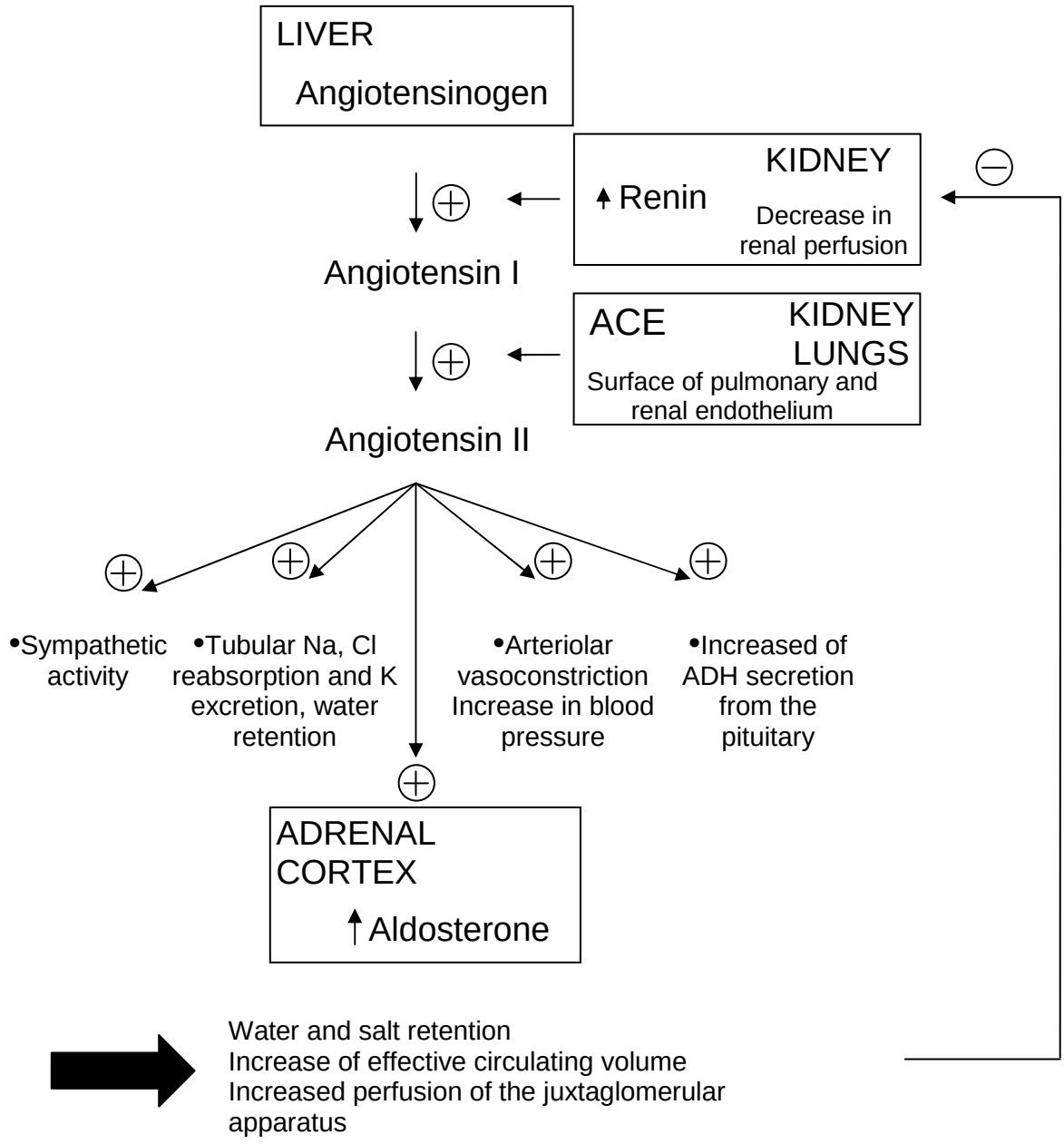


Fig. 1: Schematic presentation of the RAA system and its regulation

1.2 Aldosterone biosynthesis, metabolism and mode of action

Aldosterone is a steroid hormone produced by the outer zone (zona glomerulosa) of the adrenal cortex. Its action is to retain sodium, to increase urinary secretion of potassium and to increase blood pressure. Aldosterone is synthesized from cholesterol which after being shuttled into the mitochondria by steroidogenic acute regulatory protein (StAR) is transformed to pregnenolone by the side chain cleavage enzyme (P450scc). The enzyme 3β-hydroxysteroid dehydrogenase (3βHSD) catalyzes dehydration to progesterone, which is then further hydroxylated to form deoxycorticosterone (Otis and Gallo-Payet, 2007). A second hydroxylation by the enzyme 11β hydroxylase produces corticosterone. Aldosterone synthase is an enzyme that causes hydroxylation but can also perform an 18-oxidation which subsequently regulates the formation of aldosterone (Fig. 2) (Muller, 1998).

Aldosterone binds weakly to corticosteroid-binding protein and circulates mostly bound to albumin. Free aldosterone comprises 30-50% of its total plasma concentration. Consequently, aldosterone has a relatively short half-life, in the order of 15-20 minutes. It is rapidly inactivated in the liver, with formation of tetrahydroaldosterone. Another metabolite, aldosterone 18-glucuronide is formed by the kidney and usually represents 5-10% of secreted aldosterone (Wacker et al., 1995). A small amount of free aldosterone appears in the urine.

Aldosterone regulates the resorption of sodium and chloride ions in exchange for secretion of potassium. At the late distal tubule and collecting duct, aldosterone has two main actions: Acting on mineralocorticoid receptors on cells in the distal tubule of the kidney nephron it increases the permeability of their luminal membrane to potassium and sodium and activates their basolateral Na^+/K^+ pumps, stimulating ATP hydrolysis which ultimately results in phosphorylation of the pump (Verrey, 1999). A conformational change of the pump transports the Na^+ ions to the outside (Chen et al., 1999, Rossier et al., 2002). The phosphorylated form of the pump has a low affinity for Na^+ ions, hence reabsorbing sodium ions and water into the blood, and secreting potassium ions into the urine (Kamunina and Staub 2002). The affinity of the mineralocorticoid receptor to cortisol is similar to that of aldosterone. Nevertheless, the mineralocorticoid activity of aldosterone in comparison to cortisol is 1000 times higher because aldosterone sensitive cells transform cortisol to cortisone through 11β-HSD type II, which cannot activate the receptor. Aldosterone also stimulates H^+ secretion by intercalated cells in the collecting duct, regulating plasma bicarbonate (HCO_3^-) levels and thus acid/base balance (Winter et al., 2004).

In addition, aldosterone is likely to exert a number of effects in a variety of peripheral tissues. Aldosterone excess has been shown to result in cardiac fibrosis in animals, and studies in humans with primary aldosteronism suggest that aldosterone excess is associated with

alterations in myocardiac texture as assessed by electrocardiography. Hyperaldosteronism has also been associated with endothelial dysfunction independent of systemic blood pressure effects. There are now several studies suggesting that aldosterone is important in the pathophysiology of heart failure. Excess aldosterone has been related to extracellular matrix and collagen deposition and therefore to myocardial fibrosis (Pessina et al., 1997).

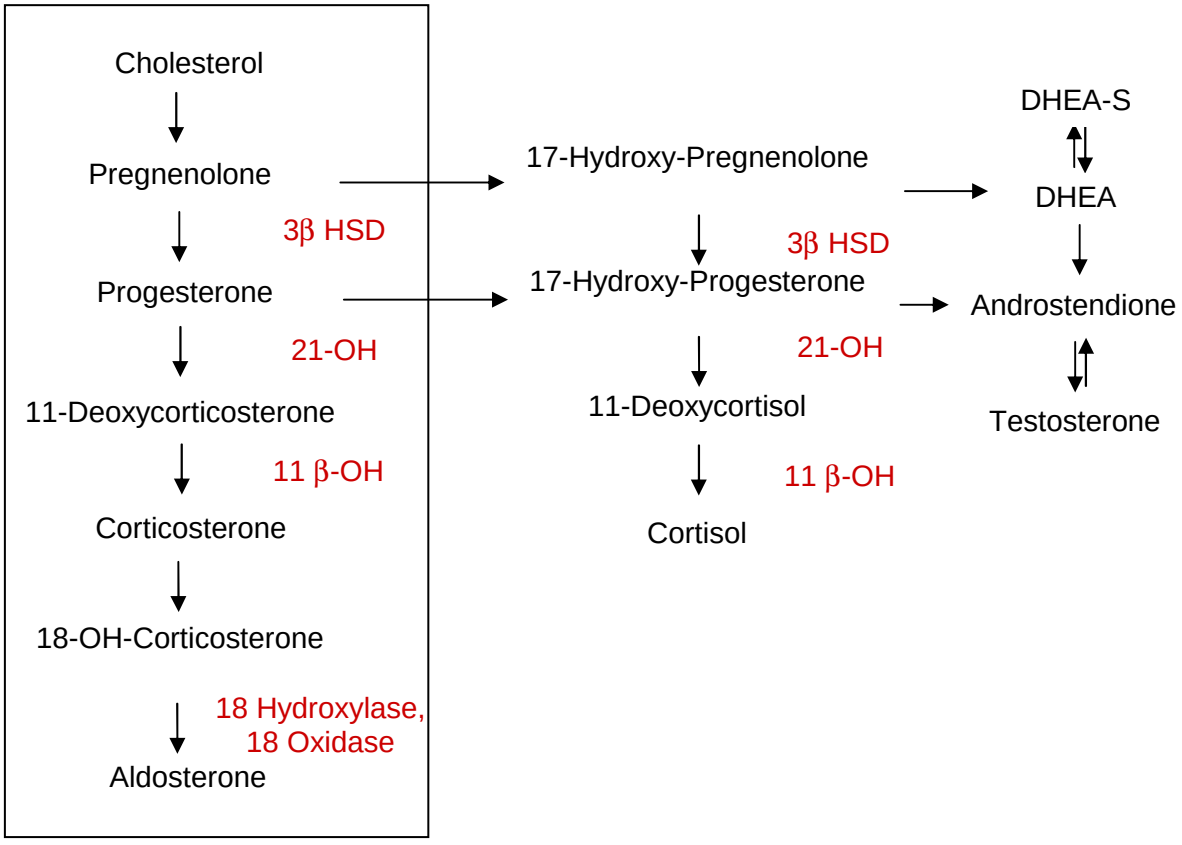


Fig. 2: Steroidogenic pathway of aldosterone biosynthesis within the adrenal zona glomerulosa

1.3 Regulation of aldosterone secretion

Aldosterone production is controlled by a complex regulatory network which adjusts plasma aldosterone concentration according to the acute and chronic changes in water and electrolytes balance. The most important regulating factors are ANG II and extracellular potassium concentration (Vinson et al., 1985; Lotshaw, 2001, Pearce et al., 2003). In contrast, ACTH, which is the main regulator of glucocorticoid production, plays only a limited role in the stimulation of aldosterone production (Lumbers, 1999). The main suppressive factors of aldosterone synthesis are sodium chloride (NaCl), and feedback mechanisms by

aldosterone which can be mimicked by the synthetic mineralocorticoid fludrocortisone (Fig. 3). Aldosterone secretion is also induced by plasma acidosis and by the stretch receptors located in the atrium of the heart. If decreased blood pressure is detected, the adrenal gland is stimulated by these stretch receptors to release aldosterone, which increases sodium reabsorption from the urine, sweat and the gut. This causes increased osmolarity in the extracellular fluid which will eventually return blood pressure toward normal. The secretion of aldosterone has a diurnal rhythm (Hurwitz et al., 2004). Many studies have shown a modulating role of other hormones such as adrenomedullin (Nussdorfer et al., 1997), vasopressin (Gallo-Payet and Guillon, 1998), and prolactin (Glasow et al., 1996) on aldosterone secretion. Pharmacological effects on the RAA system can also be observed in treatment regiments including beta-blockers, spironolactone, ACE inhibitors and diuretics.

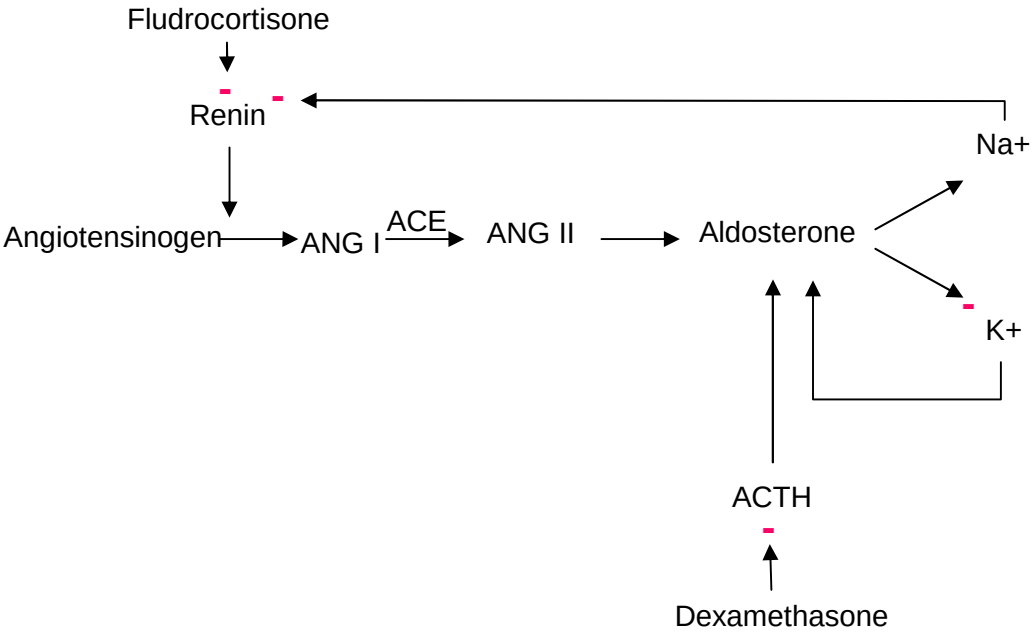


Fig. 3: Positive and negative feedback regulation on aldosterone secretion

1.3.1 Angiotensin dependent regulation (Angiotensin II, fludrocortisone)

Angiotensin II is considered to be the strongest stimulus for adrenal aldosterone synthesis. On the molecular level, angiotensin II binds to the membrane Angiotensin II receptor type 1 of the cells of the zona glomerulosa and stimulates phospholipase C, which in turn triggers InP_3-dependent Ca^{2+} release from intracellular stores, namely the endoplasmic reticulum (Spat et al., 1991). The immediate phase of aldosterone secretion is followed by sustained secretion, which is dependent on depolarisation and activation of T- and L-type Ca^{2+}

channels (Rossier et al., 1996). This sustained phase is characterised not only by increased formation of inositol phosphates but also by changes in the ratio of different inositol phosphate fractions. The final effect of this chain of events is the phosphorylation of steroid acute regulatory protein (StAR), an obligatory step in the transfer of cholesterol from the cytoplasm into the mitochondria (Christenson and Strauss, 2001).
Fludrocortisone is a synthetic corticosteroid with moderate glucocorticoid and major mineralocorticoid potency. It is primarily used to replace the missing hormone aldosterone in various forms of adrenal insufficiency such as Addison's disease and the classic salt wasting form of congenital adrenal hyperplasia (21-hydroxylase deficiency). In addition it is occasionally used to treat orthostatic hypotension. Fludrocortisone, as an aldosterone agonist reduces the endogenous secretion of aldosterone from the glomerulosa cells by negative feedback mechanism (McInnes et al., 1982).

1.3.2 Electrolyte dependent regulation (K^+, Na^+)

The second strongest stimulus for aldosterone secretion is potassium. Even low increases in potassium can stimulate aldosterone secretion in normal subjects. Glomerulosa cells appear to be a unique sensor of extracellular K^+ (Spat and Hunyady, 2004). Increases of the extracellular K^+ concentration by approximately 1 mmol/l are sufficient to double aldosterone output. The basis for this unique sensitivity for plasma potassium concentration is a very high background K^+ conductance, which makes the membrane voltage strictly follow the K^+ equilibrium potential. The mechanism of potassium induced aldosterone secretion is not angiotensin II dependent (Okubo et al., 1997). High plasmatic K^+ concentrations exclusively stimulate the sustained phase of aldosterone secretion via influx of Ca^{2+} through voltage dependent T- and L-type Ca^{2+} channels without release of Ca^{2+} from the endoplasmic reticulum (Lotshaw, 2001; Heitzmann et al., 2008). At pharmacological concentrations T-type channels are inactivated and continuous Ca^{2+} influx takes place through L-type channels.
In addition, the plasma concentration of sodium influences adrenal aldosterone biosynthesis. With increasing sodium load there is a decrease in renin release and angiotensin II production, reducing the organism’s ability to retain sodium. The saline load test is used in humans to investigate an autonomous and, thus, non-suppressible aldosterone production. The mechanisms underlying these effects are complex. As one of the mechanisms it has been suggested that there is a regulation of the angiotensin 1 receptors in the glomerulosa cells by sodium rendering these less angiotensin sensitive (Williams et al. 2005).

1.3.3 ACTH dependent regulation (ACTH, dexamethasone)

ACTH plays a role in the context of acute and short-term stimulation of the aldosterone secretion. The concentration of ACTH required for the stimulation of the aldosterone secretion is much higher than that required for glucocorticoid secretion and the long term effects of ACTH are inhibitory (Mitani et al., 1996; Lumbers, 1999; Sewer and Watermann, 2003). With continuous ACTH stimulation, there appears to be a conversion of glomerulosa cell function into fasciculata cell function. Thus, long term, continuous ACTH stimulation results in a paradoxical decrease in aldosterone secretion.

The molecular mechanisms of ACTH stimulation of aldosterone secretion are initiated by binding of ACTH to its G-protein coupled receptors, which then leads to activation of cAMP-dependent protein kinase A. The exact activation mechanism of StAR protein via cAMP has not been elucidated and experimental results on the effect of ACTH on mitogen activated protein kinases (MAPK) are scarce. In mouse adrenocortical carcinoma cells for example, ACTH stimulates the phosphorylation and activation of MAPK, MEK and ERK1/2, while in bovine adrenal fasciculata cells, ACTH, which stimulates steroidogenesis via cAMP, does not activate ERK1/2 and completely blocks ERK1/2 activation induced by angiotensin II (Foster et al. 2004).

Dexamethasone is a synthetic compound with glucocorticoid action. Its potency is about 20-30 times of that of hydrocortisone and 4-5 times that of prednisone. Dexamethasone is also used in a diagnostic context, namely through its potential to suppress the hypothalamo-pituitary-adrenal axis (Gross et al., 2007) in the diagnosis of autonomous glucocorticoid secretion. As ACTH has a short-term action on the renin-angiotensin-aldosterone axis, dexamethasone is likely to cause suppression of the aldosterone secretion through down-regulation of the physiological ACTH secretion from the pituitary.

1.4 Primary aldosteronism

Arterial hypertension represents one of the most common diseases in the developed countries. According to recent epidemiologic studies, about 20% of the adult population is diagnosed with any form of hypertension (Fardella et al., 2000). Most patients suffer from an idiopathic form of the disease. However, in a significant proportion of hypertensive patients a secondary cause can be identified. The most frequent cause of secondary arterial hypertension is primary aldosteronism. Primary aldosteronism is caused by autonomous hypersecretion of the mineralocorticoid aldosterone that can be associated with specific clinical symptoms.

1.4.1 Etiology

Primary aldosteronism represents a group of disorders in which aldosterone production is inappropriately high, relatively autonomous from the RAA system and non-suppressible by sodium load. The vast majority of cases is caused either by an aldosterone producing adrenal adenoma or by a bilateral adrenal hyperplasia, with two thirds of patients being diagnosed with bilateral hyperplasia and one third with aldosterone producing adenoma (Beuschlein and Reincke, 2007). Among patients with an adrenal adenoma, women are more frequently affected than men, and usually they are younger than patients with bilateral adrenal hyperplasia (Schirpenbach and Reincke, 2007). Only in rare instances primary aldosteronism is due to a unilateral adrenal hyperplasia, an adrenal carcinoma or a familiar form of hyperaldosteronism (type I and II).

1.4.2 Epidemiology

In the past, the prevalence of the classical form of primary aldosteronism had been described to range between 0.1-1% of all hypertensives. Recently accumulated evidence has changed these assumptions. After the introduction of a more sensitive screening method, with the measurement of the aldosterone to renin ratio, more patients suffering from the normokalemic form of the disease have been identified, and with them the prevalence increased to approx. 10-15% of all hypertensive subjects. Thus, primary aldosteronism is considered to be the most prevalent cause of secondary hypertension. In patient groups with refractory hypertension the proportion of primary aldosteronism increases further to 20% (Calhoun et al., 2002). Specifically, groups with a high prevalence of primary aldosteronism include patients with moderate to severe hypertension (8-13%), resistant hypertension (17-23%), patients with spontaneous or diuretic induced hypokalemia and patients with hypertension with adrenal incidentaloma (1.1-10%), (Funder et al., 2008). In recent studies, only a minority of patients with primary aldosteronism (9 to 37%) had hypokalemia and this form was present only in the more severe cases (Mulatero et al., 2004). 50% of patients with an aldosterone producing adrenal adenoma and 17% of those with idiopathic hyperaldosteronism had serum potassium concentrations <3.5 mmol/l. However, all patients with primary aldosteronism have more hypertension-related organ damage in comparison to patients with essential hypertension (Rossi et al., 2006).

1.4.3 Genetics of primary aldosteronism

There is high homology between aldosterone synthase and the P450c11b, the product of CYP11B1 gene, which is necessary for the conversion of 11-deoxycortisol to cortisol. P450c11b transcription is mainly regulated by ACTH through a cAMP and protein kinase A dependent mechanism. Both genes are localized on chromosome 8 and have 90-95% identical nucleotide sequence in introns and exons (Kawamoto et al., 1990). In vitro studies have shown that some mutations of CYP11B1 can be responsible for specific forms of hyperaldosteronism. Curnow et al. have demonstrated that substitution of Ser288Gly and Val320Ala can transform the cortisol forming CYP11B1 to an aldosterone producing enzyme (Curnow et al., 1997).

Glucocorticoid remediable hyperaldosteronism, also known as familial hyperaldosteronism type 1, is an autosomal dominant disease characterized by hyperaldosteronism and production of abnormal adrenal steroids (18-oxocortisol and 18-hydroxycortisol). The genetic background includes unequal crossing-over of the CYP11B1 and CYP11B2 genes and the formation of a hybrid gene (Hampf et al., 2001). This resulting gene is responsible for the production of aldosterone but responds to ACTH instead of being regulated by angiotensin II.

Familial hyperaldosteronism type 2 consists of a heterogeneous group of autosomal dominant diseases without well defined genetic causes. Linkage analysis in large families with familial hyperaldosteronism type 2 identified the locus 7q22 as one of the suspected regions (Jackson et al., 2002; So et al., 2005). However, the exact genetic mechanisms of the disease remain unresolved. In addition, it has yet to be investigated, if genes in this locus play a significant role in the pathogenesis of the sporadic cases of primary aldosteronism.

1.4.4 Clinical features

In its classic form, primary aldosteronism is defined by hypertension, hypokalemia and metabolic alkalosis (Conn, 1955). The disruption of electrolyte balance is responsible for most of the clinical symptoms, such as muscle weakness, cramps, constipation, polyuria, paresthesias and intermittent paralysis and electrocardiographic (ECG) alterations. The majority of the patients, however, suffer from the normokalemic form of the disease, and have hypertension as single clinical symptom. Blood pressure in patients with primary aldosteronism can range from borderline elevation to severely hypertensive levels. The mean blood pressure of patients with primary aldosteronism shows no significant difference between those suffering from an adrenal adenoma or from bilateral adrenal hyperplasia. The heart is usually mildly enlarged and electrocardiographic changes reflect modest left ventricular hypertrophy and potassium depletion (Rossi et al., 2008).

1.4.5 Diagnosis

As specific therapeutic interventions are available for patients with primary aldosteronism, it is important to identify these patients as part of a broad screening including the subjects with the normokalemic form of the disease. Most of the patients have a potassium value in the lower normal range and the only effective screening procedure is by definition of the aldosterone to renin ratio (ARR) in plasma (Funder et al., 2008). Some drugs, however, can influence this ratio and give false positive or negative results. Before proceeding to ARR testing, diuretica, beta blockers and ACE inhibitors should be stopped for at least 1 week, whereas the aldosterone antagonist spironolactone due to its very long half life should be paused to a minimum of 4 weeks before the test.

If the ARR shows pathological values, an autonomous aldosterone secretion should be considered, which needs to be substantiated by independent confirmation tests. These tests include the measurement of the aldosterone metabolites in 24h urine samples and the normal saline load test, where 2l of isotonic NaCl 0.9% are infused over 4 hours followed by blood sampling for the measurement of aldosterone concentration (Mulatero et al., 2006). An additional confirmation test, not often used in routine testing, is the fludrocortisone suppression test. For this test, the patient receives 4 times a day for 4 days fludrocortisone per os (p.o.) and normal saline substitution simultaneously. If aldosterone secretion is insufficiently suppressed, the diagnosis of primary aldosteronism is confirmed (Stowasser et al., 2001). The next step is the differential diagnosis between a unilateral adrenal adenoma and the bilateral adrenal hyperplasia. Therefore abdominal imaging by computer tomography can be useful, as well as orthostatic testing. In most instances, however, the invasive adrenal vein sampling with determination of the aldosterone to cortisol ratio has to be performed to proof a unilateral or bilateral source of aldosterone secretion (Young et al., 2004).

1.4.6 Therapy

Treatment strategies depend on the cause of primary aldosteronism. For unilateral adrenal adenoma, first line therapy is unilateral laparoscopic adrenalectomy. Before surgery it is advisable to treat the patients for one month with spironolactone, in order to avoid postoperative hypoaldosteronism. In patients with the correct diagnosis after surgery hypertension and serum potassium are improved in nearly 100% of the patients and blood pressure is normalized in 33-66% of patients (Young, 2003). If a patient is unable or unwilling to undergo surgery, then a medical treatment with a mineralocorticoid receptor antagonist, like spironolactone or epleronone is another therapeutic option.

Patients suffering from bilateral adrenal hyperplasia are treated with a mineralocorticoid receptor antagonist. Spironolactone is the first choice and has proven effective in blood pressure reduction but can cause side effects such as breast tenderness, muscle cramps and decreased libido mainly through its anti-androgenic mode of action (Jeunemaitre et al., 1987). Epleronone can be used as an alternative, as a newer, selective mineralocorticoid receptor antagonist, without anti-androgen and progesterone antagonist effects (McManus et al., 2008, de Gasparo et al., 1987). Epleronone, however, is not officially approved for the treatment of patients with primary aldosteronism in Germany.
For cases of glucocorticoid remediable hyperaldosteronism, the use of the lowest dose of glucocorticoids that can normalize blood pressure and potassium levels is the treatment of choice. Alternatively, treatment with a mineralocorticoid receptor agonist should be considered (Stowasser and Gordon, 2001).

1.5 Strategies to explore genetic mechanisms of diseases

Mouse models play an important role for the investigation of the genetic background of human diseases. There are two different methods in approaching these diseases. The first being the phenotypical characterisation of mice after a targeted genetic modification in knock-out or transgenic models, the second is the phenotype driven mutagenesis screen. In the second case, mice are treated with a highly mutagenic substance that can cause random mutations, the phenotype of the offspring then guides the investigation of the unknown genotype.

1.5.1 Genomewide association studies

In the case of phenotype driven genetics the genomic association is accomplished utilizing common single nucleotide polymorphisms (SNPs). Genetic mapping turns hypothesis driven research on its head. It is based on the theory that systematic genomewide study of DNA variation in relation to disease can lead to the localization of the causative gene (Hirschhorn and Daly, 2005, Wang et al., 2005, Laird and Lange 2006). Like linkage mapping, such studies can identify only a region of interest within the genome. To conclusively pinpoint causal genes and mutations, genes within each of these regions must be sequenced in cases and controls, and supplemented with functional studies. In diseases that follow mendelian patterns of inheritance, this process typically reveals many different causal mutations in disease related genes. Therefore large cohorts are required, since many different genetic polymorphisms may influence the disease, and since common identified SNPs are often not themselves the causative variants in each such gene (Neale and Sham

2004). The opportunities for genomewide association studies to identify new genomic risk loci, which may harbor rare mutations of larger effect, will not be exhausted, until large samples are assembled for each disease and trait of clinical importance. The underlying genetic causes for most diseases probably include dozens of risk alleles, some common and of small effect and others of rare and larger effects. Whereas genomewide association studies offer a method for finding the former, the latter require sequencing of DNA from large numbers of individuals, in genes implicated by genomewide studies, in biologic candidate genes, and ultimately throughout the genome. In some cases the identified genes have known functions but were not known to be involved in the disease. However, in most cases the genes identified had not previously been identified by functional systems or mendelian genetics, proving that genomewide association studies can help to fill critical gaps in the current knowledge of physiology (Hunter et al., 2008).

1.5.2 ENU mutagenesis screen

Ethylnitrosourea is an alkylating agent that causes ethylation of nucleic acids that ultimately result in point mutations. ENU exerts mutagenic action on DNA of premeiotic spermatogonial stem cells (Russel et al., 1979; Rinchik, 1991) i.e. A-T base pair substitutions and/or small intragenic lesions (Popp et al., 1983; Harbach et al., 1992). Many of the mutant offsprings produced by ENU treated mice will therefore be hypomorph (partial loss-of-function), although gain-of-function as well as complete loss of function mutants can also be expected (Grunwald and Streisinger, 1992; Justice et al., 2000). This allows the production of a large number of F1 founder animals from a single treated male, minimizing the number of animals required and the handling of ENU.

The aim of ENU treatment is to saturate the genome with mutations. The exact ENU dose required for a genetic screen has been defined by different methods. Quite a few studies (Russel et al., 1979; Hitosumachi et al., 1985; Shedlovsky et al., 1986; Davis and Justice, 1998) have calculated the optimal dose and the rate of recessive mutations in 1 mutation per locus, per 700 gametes. The frequency of mutations is 0.5 – 1.0 mutation per Mbp and 20 point mutations are expected per G1 gamete. The biggest problem is the infertility and mortality of treated animals. While some strains can tolerate higher ENU dosage, for others toxicity is a limiting factor. With a dosage of 3 times 100mg/kg the strains C57BL/6J, C57BL6Ros and C3HeB/FeJ can survive, but only the C3HeB/FeJ can remain fertile (Justice et al., 2000).

In the Munich ENU project which takes place at the Institute of Experimental Genetics of the Helmholtz Center Munich, C3HeB/FeJ male mice are treated with 3 injections of ENU at weekly intervals. After a sterility period, injected males are mated to wild type untreated

females. Only F1 litters deriving from ENU treated males which underwent a minimal sterility period of 100 days are chosen for phenotyping to ensure that they were generated with mutagenic treatment derived sperms. These F1 mice can either be analyzed directly for dominant mutations or bred further to subsequently study recessive phenotypes. Very large numbers of mice can be analyzed in a dominant F1 screen (Fig. 4).

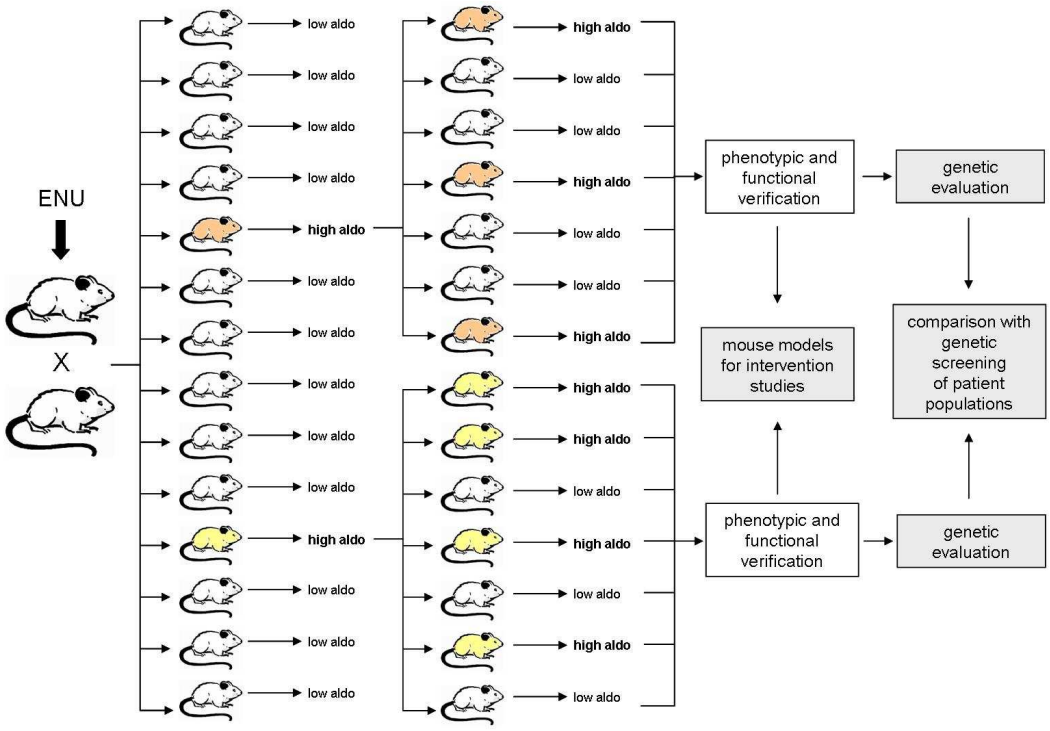


Fig. 4: Schematic presentation of an ENU Mutagenesis Screen for the phenotype ‘’hyperaldosteronism’’.

1.6 Objectives of the study

The physiological mechanisms of the regulation of aldosterone secretion have not been elucidated in detail until now. In addition, the genetic cause of primary aldosteronism has only been defined in familial hyperaldosteronism type I, whereas for the vast majority of sporadic cases of the disease it still remains unclear.

Our study had two major objectives: First, to further investigate the regulation of aldosterone secretion *in vivo* and to define normal aldosterone values in a mouse model. So far in mice only few experiments have been performed on the regulation of adrenal aldosterone secretion. Therefore, normal aldosterone values of different mouse strains and of both genders under baseline conditions and after functional tests had to be defined. In addition, a series of stimulation and suppression experiments have been designed. These groundwork experiments would identify the aldosterone response after the initiation of different functional tests and would be used as a guide for time dependent experiments. These time dependent experiments would allow definition of the pattern of aldosterone secretion upon specific modification of the RAA system. As far as the molecular basis is concerned, these experiments could also clarify the pattern of expression of aldosterone synthase, the final enzyme in the chain and the rate limiting step, which leads to aldosterone production in a time dependent manner.

The second objective of our study was to screen for affected mice with hyperaldosteronism in an ENU mutagenesis project. These mice would not only be a suitable mouse model for intervention studies for the diagnosis and treatment of primary aldosteronism, but would also potentially reveal genetic alterations responsible for this phenotype. Therefore, the affected animals had to be identified and characterized according to their hormonal and biochemical profile and their cardiovascular phenotype.

2. Materials and methods

2.1.1 Materials

Reagents:

Acetic acid (Merck)

ACTH (Synacthen Novartis)

Agarose (PeqLAB)

Aldosterone (Fluka)

Angiotensin II (Fluka)

Boric acid (Merck)

Bovine Serum Albumine (Sigma)

Bovine Gamma Globuline (Sigma)

Dexamethasone (Sigma)

Dichloromethane (Merck)

Diethylenetriaminepentaaceticacid (DTPA Sigma)

Di-sodium hydrogen phosphate (Na_2HPO_4 x 2 H_2O Fluka)

Ethanol absolute (Merck)

Ethidium Bromide (Roth)

Ethylene diamine tetraacetic acid (EDTA-2Na-2H_2O Sigma)

Europium labelled streptavidin (Perkin-Elmer, Wallac)

Fludrocortisone (Sigma)

Isoflurane (Forene Abbot)

Methanol (Merck)

Mounting medium (MICROM)

NaCl 0.9% (BRAUN)

Neomycin sulphate (AppliChem GmbH)

Paraffin (Merck)

Paraformaldehyde (PFA, Sigma)

PBS (Phosphate buffered Saline Sigma)

Phenylmethanesulfonylfluoride (PMSF, DiaSorin RENCTK Renin Activity kit)

Picric acid solution, saturated (Fluka)

Polyethylenglycol 10000 (Merck)

Potassium carbonate (K_2CO_3 Merck)

Potassium chloride (KCl Merck)

Potassium-hydrogen-phthalate (Merck)

Rat renin substrate – Plasma from 5/6 nephrectomized rats (CharlesRiver, France)

Sirius red (Direct Red 80, Fluka)
Sodium azide (NaN_3 Merck)
Sodium chlorid (NaCl Merck)
Sodium hydrogen carbonate ($NaHCO_3$ Merck)
Sodium maleate (Sigma)
Thenoyltrifluoroacetone (Sigma)
Tri-N-octylphosphinoxide (Merck)
Tris-hydroxymethylaminomethane (Merck)
Triton X-100 (Sigma)
Tween 20 (Sigma)
Tween 40 (Sigma)
Xylol (Merck)

Antibodies:
Monoclonal anti-aldosterone antibody (Prof. Dr. Gomez-Sanchez, University of Mississippi)
Polyclonal rabbit anti-mouse immunoglobulin (DAKO)

Molecular biology reagents:
Loading Dye (6x Fermentas)
DNA Ladder Bench Top 100 bp Promega)
dNTP's (Promega)
MML-V (Promega)
Oligo (dT) primer (Promega)
Reaction buffer (Promega)
RNAsin plus (Promega)
RNA extraction kit (Promega SV Total RNA Isolation System)
LightCycler FastStart DNA Master SYBR Green I (Roche)

Instruments-devices:
Automatic microtiter plate washer (TECAN, SLT)
Horizontal shaker (WALLAC DELFIA PLATESHAKE)
Fluorometer (VICTOR, Perkin-Elmer)
Centrifuge (BIOFUGE fresco Heraeus)
Electrophoresis system (BIORAD Powerpac)
PCR machine (PeqLAB primus 25 advanced)
LightCycler Real time PCR machine (Roche)
UV Camera (INTAS UV-System)

Automatic Gamma-Counter (Wallac 1470 Wizard)
Cobas Integra 800 (Roche)
Rotary microtome (MICROM)
Microtiter plates (Maxisorp NUNC)
Microscope slides (Superfrost Plus, Menzel Gläser)

Commercially available kits:
RENCTK Renin Activity RIA kit (DiaSorin)

2.1.2 Buffers

Wash buffer for mictotiter plates
20 Tablets PBS (Phosphate buffered Saline Sigma)
2 ml Tween 20 (Sigma)
4 L ddH_2O

DIN Spuke (10x)
4.2 g sodium hydrogen carbonate ($NaHCO_3$ Merck)
0.5 g sodium chlorid (NaCl Merck)
0.2 g potassium carbonate (K_2CO_3 Merck)
Usage solution: Dilution of stock solution 1: 10 with ddH_2O

Coating buffer:
8.9 g di-sodium hydrogen phosphate (Na_2HPO_4 x 2 H_2O Fluka)
800 ml ddH_2O
Phosphoric acid in drops until pH=7.4
ddH_2O up to 1 L

LKC Assay buffer:
45 g sodium chlorid (NaCl Merck)
30 g trishydroxymethylaminomethane (Sigma)
2.5 g sodium azide (NaN_3 Merck)
0.5 g Tween 40 (Sigma)
3 L ddH_2O
With conc. HCl reach pH 7.75
25 g BSA (Bovine Serum Albumine Sigma)
2.5 g Bovines Gamma Globuline (Sigma)

39.35 mg Diethylenetriaminepentaaceticacid (DTPA Sigma)

dd H_2O up to 5 L

Let stay overnight and then filter with 8µ Filter and finally with 0.8µ filter

Enhancement solution:

Stock solution A

5.55 g thenoyltrifluoroacetone (=100mM Sigma)

966.5 mg tri-N-octylphosphinoxide (=10mM Merck)

50 ml triton X-100 (Sigma)

DdH2O up to 250 ml

Stock solution B

3.46 g KH-phthalate (=68mM)

15 ml glacial acetic acid (Merck)

ddH_2O up to 250 ml (pH 3.15)

final solution: Mix 2.5 ml *stock solution A* and 25 ml *stock solution B* and fill up with ddH_2O up to 250 ml

DCM-PEG:

50 mg Polyethylenglycol 10000 (Merck)

500 ml Dichloromethane (Merck)

TBE 10x:

Tris 108 g

Boric acid 55 g

EDTA-2Na-2H_2O 7.4 g

ddH_2O 1 l

HCl in drops until pH=8.2

1% Agarose Gel:

1 mg agarose

100 ml TBE 1x

In the microwave until melting of the agarose

Cool down and add:

5 µl Ethidium Bromide

Maleate buffer:

0.2 M sodium maleate pH=6.0

5mM phenylmethanesulfonylfluoride (PMSF) provided by the manufacturer (DiaSorin RENCTK Renin Activity kit)
10mM EDTA
0.1% neomycin sulphate

Picro-sirius red solution:
0.5 g Sirius red
500 ml Saturated aqueous solution of picric acid

4% Paraformaldehyde solution:
40 g paraformaldehyde (PFA)
1 litre of 1x PBS solution warmed to 65^0C under the hood
The solution has finally to be placed on ice and stored in -20^0C

2.2 Animals

The experiments were performed on 12 weeks old male and female C3HeB/FeJ wild type animals from the group of Prof. Hrabé de Angelis (Helmholtz Centre München). All animal studies were performed according to protocols examined and approved by the Regierung von Oberbayern (Az. 55.2-1-54-2531-36-07, 55.2-1-54-2531-134-07) and according to the German Animal Protection Law.
The animals were kept in a non-specific pathogen free animal facility at an ambient temperature of 22+/-2℃, (relative humidity 60+/-5%) and on a 12h-12h light-dark circle. The standard mouse cage size was 15 cm x 27 cm x 42 cm and the bottom was covered with wood fibres (Lignocel Fa. J.Rettenmaier & Söhne). The animals were fed standard breeding chow ad libitum (Ssniff R/M-H) with free access to tap water, if not mentioned otherwise. They were maintained in groups of 6-8 individuals of the same sex. The composition of the chow diet is as follows: crude protein 19%, crude fat 3.3%, crude fibre 4.9%, crude ash 6.4%, metabolizable energy 12.8 MJ/kg, sodium 0.24% and potassium 0.91%.
At least one week before starting the experiments all mice were transported to the animal house of the Medizinische Klinik Innenstadt. They could relax completely from the transport, so that the results could not be influenced by stress.

2.3 Experiments

All experiments for the establishment of stimulation and suppression tests on mice were performed on 20 animals per experiment and per sex. All the experiments for the investigation of the time dependent aldosterone secretion were performed on 5 female animals per time point and per experiment. The female animals were chosen for the second group of experiments because of their better responsiveness on the substances used.
The working scheme for the first group of experiments is shown in Fig. 5.

2.3.1 Evaluation of stimulation and suppression tests

For each of the functional tests a group of 20 male and 20 female 12 weeks old wild type mice was established. The first day, after weighting the animals, blood samples were taken after retro-orbital blood collection, under isoflurane anesthesia and the aldosterone values of these mice under baseline conditions were defined.

ACTH stimulation – dexamethasone suppression

The second day at 9:00 40 ng/g ACTH (Synacthen Novartis) were injected without anesthesia intraperitoneally in every mouse, and an hour later blood samples were taken after retro-orbital blood collection, under isoflurane anesthesia. The same day, at 20:00 all animals received an intraperitoneal injection of 50ng/g dexamethasone (Sigma). On the third day at 9:00 all mice were euthanized by isoflurane inhalation for final blood collection.

Angiotensin II stimulation

The second day at 9:00 200 ng/g Angiotensin II (Fluka) were injected without anesthesia intraperitoneally in every mouse, and an hour later all mice were euthanized by isoflurane inhalation for final blood collection.

Potassium supplementation

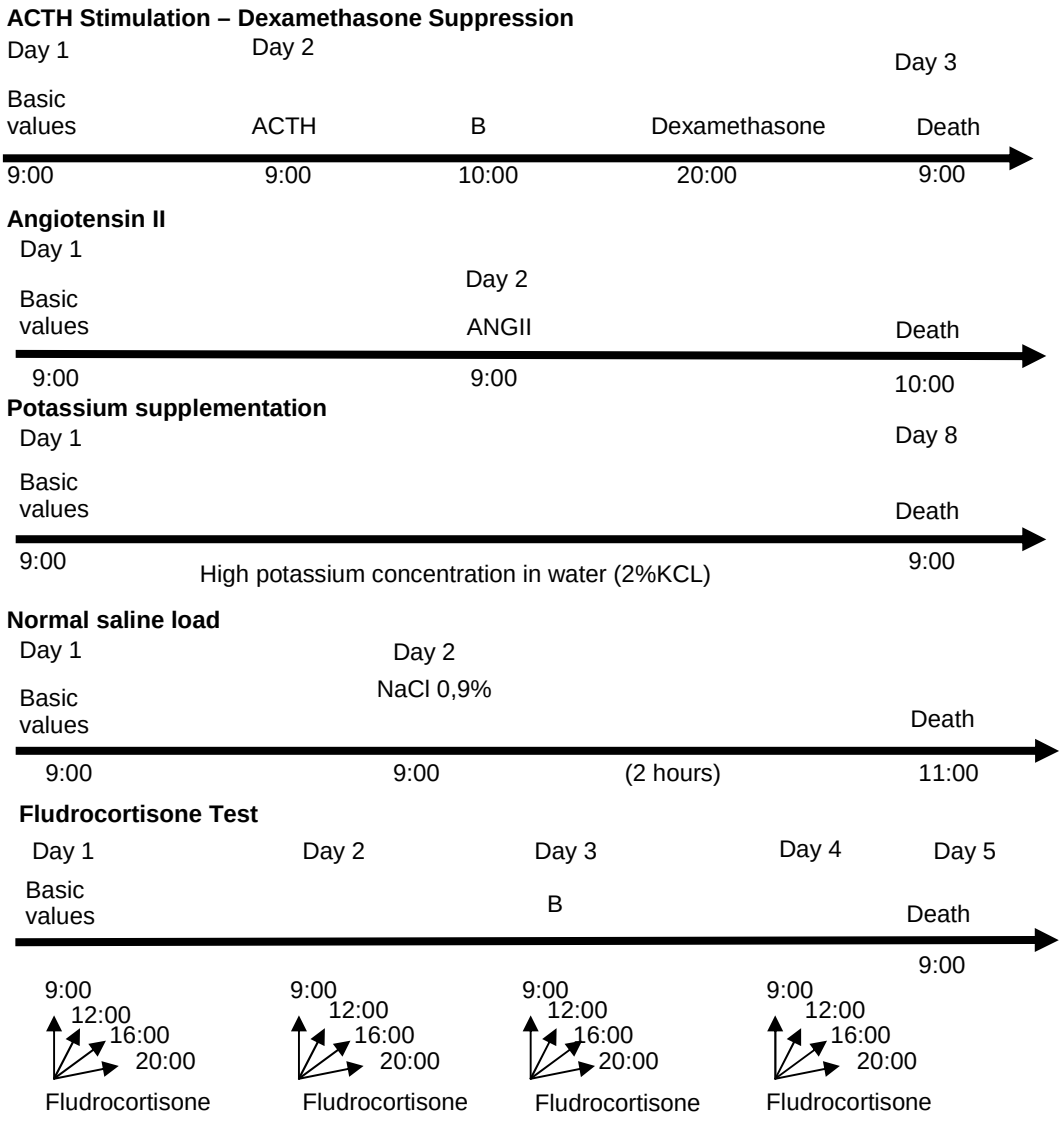
From day 2 and for 8 days animals could drink instead of tap water a water solution with 2% KCl ad libitum. On the 8^{th} day at 9:00 all animals were euthanized by isoflurane inhalation for final blood collection.

Normal saline load

The second day at 9:00 50 µl/g NaCl 0.9% were injected without anesthesia intraperitoneally in every mouse, and two hours later all mice were euthanized by isoflurane inhalation for final blood collection.

Fludrocortisone suppression test

For the next 4 days all animals received 4 times a day, with 4 hours intervals 1.5 ng/g fludrocortisone by intraperitoneal injection, without anesthesia. The 5th day at 9:00 all animals were euthanized by isoflurane inhalation for final blood collection.

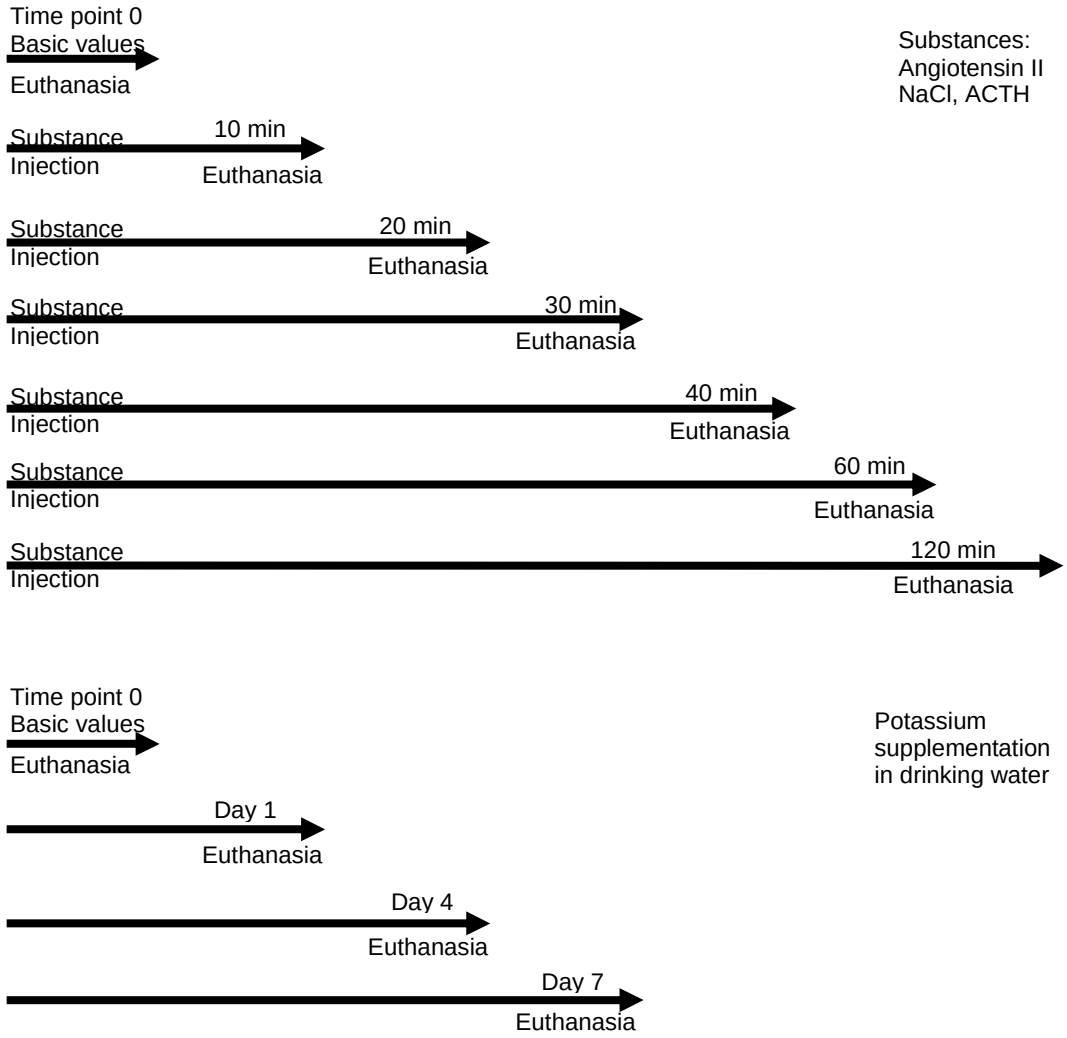


B: Blood sampling

Fig. 5: Working scheme for the first group of stimulation and suppression experiments

2.3.2 Time dependent stimulation and suppression experiments

The working schemes of the time dependent experiments are shown in Fig. 6.



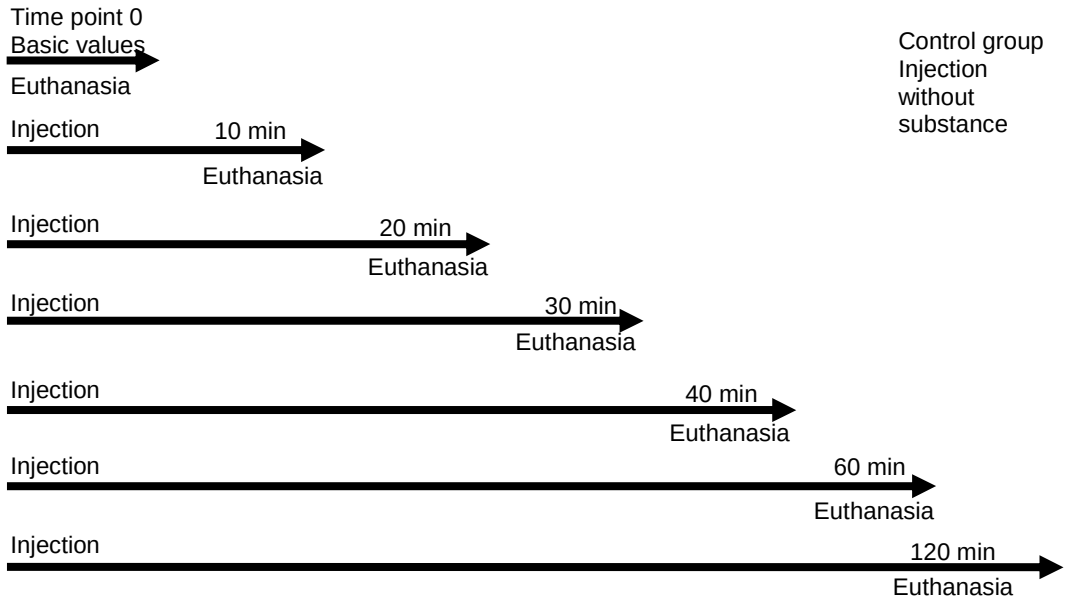


Fig. 6: The working schemes of the time dependent experiments

Angiotensin II Stimulation

Seven groups of five 12 weeks old female mice were established. The mice of the first group were euthanized by isoflurane inhalation for collection of blood and adrenals under baseline conditions. The mice from the 6 other groups received an intraperitoneal injection of 200 ng/g Angiotensin II without anesthesia. After 10, 20, 30, 40, 60 and 120 minutes 5 mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection.

ACTH stimulation

Four groups of five 12 weeks old female mice were established. The mice of the first group were euthanized by isoflurane inhalation for collection of blood and adrenals under baseline conditions. The mice from the 3 other groups received an intraperitoneal injection of 1µg/g ACTH (Synacthen Novartis) without anesthesia. After 10, 30 and 60 minutes, 5 mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection.

Normal saline load

Seven groups of five 12 weeks old female mice were established. The mice of the first group were euthanized by isoflurane inhalation for collection of blood and adrenals under baseline conditions. The mice from the 6 other groups received an intraperitoneal injection of 50 µl/g

NaCl 0.9% without anesthesia. After 10, 20, 30, 40, 60 and 120 minutes 5 mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection.

Potassium supplementation in drinking water

Four groups of five 12 weeks old female mice were established. The mice of the first group were euthanized by isoflurane inhalation for collection of blood and adrenals under baseline conditions. The mice from the 3 other groups could drink instead of tap water a water solution with 2% KCl ad libitum. After 1, 4 and 7 days 5 mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection.

Control group

Six groups of five 12 weeks old female mice were established. They all received an intraperitoneal injection without any substance without anesthesia. After 10, 20, 30, 40, 60 and 120 minutes 5 mice per time point were euthanized by isoflurane inhalation for final blood and adrenal collection.

2.3.3 Organ sampling

After isoflurane anesthesia, mice were decapitated and the abdominal cavity was opened horizontically. Both adrenal glands of each mouse were identified and removed. Directly after the collection, the surrounding fat tissue was removed under stereoscope and the adrenals were weighted, snap frozen and stored in liquid nitrogen (-80℃). Subsequently the thoracic cavity was opened vertically; the heart was identified, removed and stored in 4% PFA.

2.4 Blood tests

2.4.1 Blood sampling

The collection of blood samples had to take place under stressless conditions. To avoid an influence of the examined parameters through HPA axis activation, handling of the animals until blood sampling was kept to an interval of less than one minute. Blood sampling took place between 8:00 and 11:00 am for all animals. For mice that would participate in further experiments, a retro-orbital blood collection under isoflurane anesthesia was required. For all other animals trunk blood was taken by decapitation after isoflurane anesthesia. For both

methods animals were anesthetised individually in a glass jar containing isoflurane vapour (Forene, Abbot).
For the retro-orbital blood sampling, after effective anesthesia, the retro-orbital vein plexus of the mouse was punctuated with a glass capillary and from each animal 0.3 ml of blood could be taken. After isoflurane inhalation, the animals could be decapitated. This procedure allowed the collection of 0.7 ml of mixed arterial-venous blood. The blood was collected in EDTA covered 1.5 ml eppendorf tubes to avoid coagulation and after centrifugation (10000g x 10min) the plasma was separated and kept at -20℃ until the measurement.

Definition of biochemical profile

Potassium values of the plasma samples were obtained in the Zentrallabor der Medizinischen Klinik Innenstadt by an ion selective electrode method (ISE direct), on an automated Cobas Integra 800 analyzer. Urea, creatinine, uric acid and total protein values were measured in the German Mouse Clinic of the Helmholtz Institute in Munich.

2.4.2 Aldosterone measurement

Due to the low plasma aldosterone concentration in the picomole range, the measurement of aldosterone in small plasma volumes constitutes a difficult part of the hormonal analysis. The amount of plasma that can be collected from a mouse is often less than 50 µl, which represents a limiting factor for endocrine hormone analysis.
Commercially available assays for the measurement of aldosterone use polyclonal antibodies with varying affinity and specificity with respect to recognizing the aldosterone molecule. In addition, the available assays are usually radioimmunoassays that need bigger plasma volumes and include the risk of exposure to radioactivity.
In our laboratory (Endokrinologisches Labor Medizinische Klinik Innenstadt) a new time resolved competitive aldosterone immunoassay was recently developed (Manolopoulou et al., 2008). This allows measurement of aldosterone from very small plasma volumes (Fig. 7). The specificity and reproducibility of the method was confirmed in 50 µl sample volume. The assay incorporates a monoclonal anti-aldosterone antibody, which is characterised by very limited cross reactivity to cortisol and corticosterone (Gomez-Sanchez et al., 1987). Biotinylated aldosterone used as a tracer in the immunoassay was prepared by coupling aldosterone 3-O-carboxymethyloxime with a commercially available biotin hydrazide derivate according to previously described procedure (Dressendorfer et al., 1992). The principle of the assay is the competitive binding of the biotinylated aldosterone and the aldosterone of the

sample on the anti-aldosterone antibody. The detection of the tracer bound is then performed by the addition of Europium – labelled streptavidin as second marker.

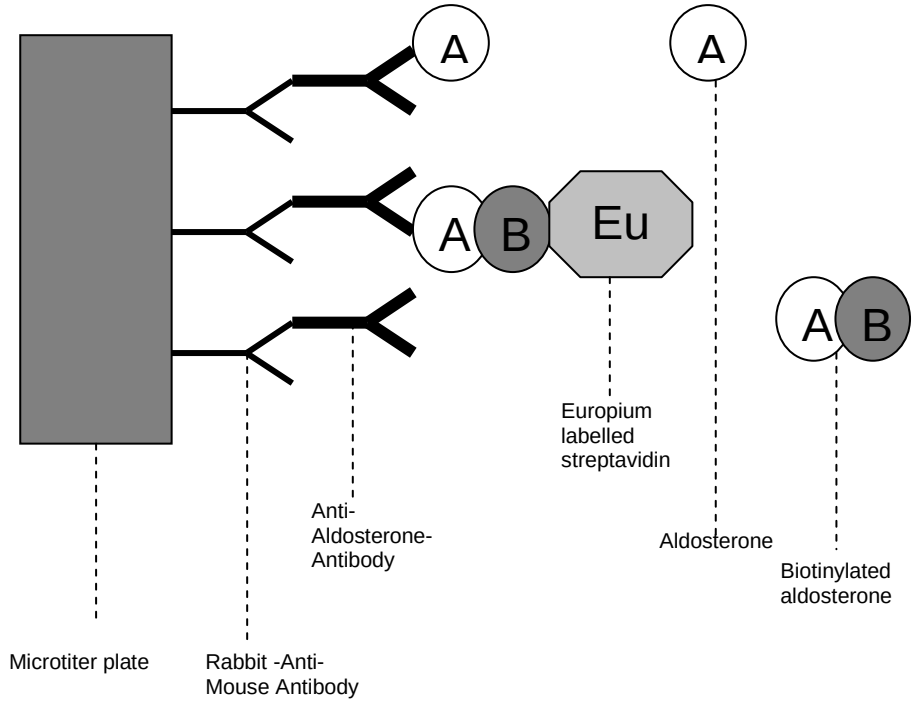


Fig. 7: Schematic presentation of the utilized aldosterone assay (A: Aldosterone, B: biotine, EU: Europium)

2.4.2.1 Coating of microtiter plates

The microtiter plates used for the assay were coated with antibodies following a two-step procedure. The first step involves the coating of the plate with an unspecific polyclonal rabbit-anti mouse antibody to ensure the reproducibility of the method. In the second step the specific monoclonal anti-aldosterone antibody is coated (Gomez-Sanchez et al., 1987).
First, the anti-mouse antibody (9 g/l – DAKO) was diluted in coating buffer to reach a concentration of 300 ng/well. The microtiter plate was then incubated overnight at 4℃ and on the next day the incubation was terminated by three times washing on an automatic microtiter plate washer (TECAN, SLT; Crailsheim Germany) using an in house prepared wash buffer. The initial concentration of the secondary anti-aldosterone antibody was unknown, but a dilution of 1:15000 in LKC buffer was required before coating the plate. After an overnight incubation at 4℃ the plate was ready to be used.

2.4.2.2 Standard curves and controls

A salt buffer (DIN) was used as the matrix for preparation of the standards. To create the single points of the standard curve 10 mg of aldosterone powder (Fluka) were weighted and dissolved in 1 ml EtOH. This solution was further diluted in DIN Spuke to receive the different concentration points from 5 pg/ml to 2000 pg/ml. The controls were created by pooling plasma samples from healthy mice with low (10 pg/ml), medium (50 pg/ml) and high (200 pg/ml) plasma aldosterone concentration. Both the standard curves and the controls were stored in aliquots at -20℃ and were thawed only im mediately before use.

2.4.2.3 Aldosterone extraction from plasma samples

The plasma samples (50 µl) collected from each animal were thawed and pipetted in a glass tube. 2 ml of dichloromethane (DCM)/polyethylene glycol 10000 (PEG 50mg/l) were added and then vortexed at low speed for 30 min. After the two phases separated, the organic phase (1.7 ml) was transferred to a new clean tube and left to evaporate in a water bath at 50℃. The sample then was reconstituted with 160 µl of DIN Spuke and 10 µl of pure methanol. Accordingly, values obtained by measurement were multiplied by four to compensate for the dilution factor.

2.4.2.4 Aldosterone Assay

After reconstitution, 50 µl of each sample, each point of the standard curve and of the controls were pipetted in duplicate onto the previously coated microtiter plate. Subsequently, 100 µl biotinylated aldosterone tracer (Manolopoulou et al., 2008) was pipetted into each well (5pg/well). The plate was sealed with self adherent foil to avoid evaporation and incubated overnight at 4℃. The following day the incubation was terminated by washing three times on an automatic microtiter plate washer. 200 µl Europium labelled streptavidin (Perkin-Elmer, Wallac) were added in each well and incubated for 30 min at room temperature on a horizontal shaker. After washing the plate six times, 200 µl of the enhancement solution were added per well, followed by incubation at room temperature for 15 min, used to transfer the europium conjugate into a fluorescent complex. The amount of biotinylated aldosterone bound was then measured using a fluorometer (VICTOR, Perkin-Elmer) (Fig. 8).

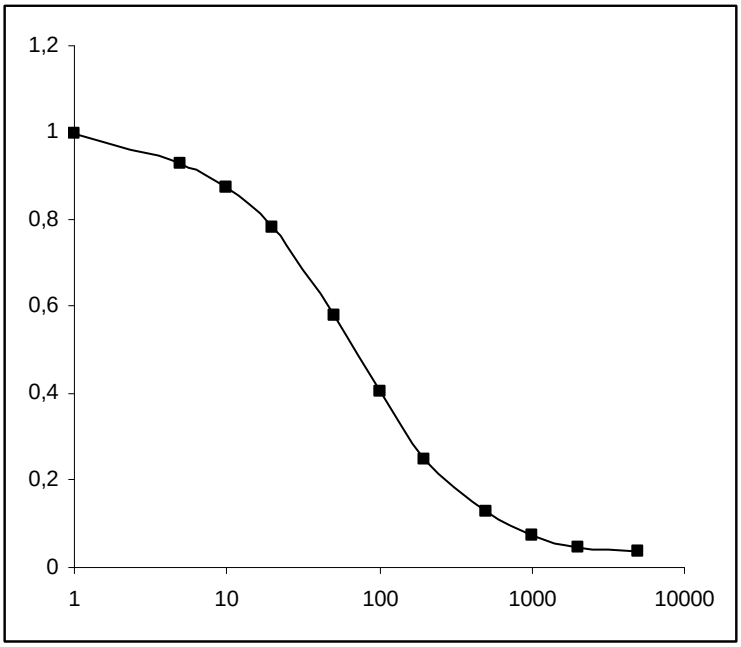


Fig. 8: Example of standard curve of a time – resolved fluorescent immunoassay for aldosterone

2.5 Plasma Renin Activity (PRA) Assay

The plasma renin activity was measured by a commercially available Angiotensin I Radioimmunoassay Kit from DiaSorin. The method was modified according to Heitzmann et al., 2008. Each plasma sample was diluted 1:20 with maleate buffer. The rat renin substrate was diluted 1:3 with maleate buffer. 50 µl of the diluted probe, 22.2 µl of the rat renin substrate, 27.7 µl of the generation buffer included in the RENCTK kit and 2 µl PMSF (RENCTK kit) were mixed. 51 µl of this mixture were incubated for 90 min in a water bath at 37°C and the remaining 51 µl were incubated for 90 min on ice (blank sample). 45 µl of both 37°C sample and blank sample were dispensed in RIA coated tubes and incubated for 23 hours at room temperature. The same procedure was performed also for the calibrators. After the incubation, the content of the tubes was aspirated and the radioactivity of the 37°C and blank tubes was measured by a Gamma-Counter.

For the calculation of the results the corresponding blank value was subtracted from each 37°C sample value. The obtained value was multiplied by 40.8 (the dilution factor of the sample) and this was divided by the incubation time in hours.

$$PRA = \frac{(ng\ 37℃ - ng\ 4℃) \times 40.8}{hours\ of\ incubation} = ng/ml/hour$$

2.6 Histology procedures

2.6.1 Paraffin embedding of tissues

Organs sampled from sacrificed mice for histological analysis were immediately placed in a 4% PFA solution and kept overnight at 4℃. The next day the organs were washed twice with PBS and incubated in a 30%, a 70%, a 96% and a 100 % ethanol solution, a xylol solution and a 1:1 xylol:liquid paraffin solution for two hours. After incubation overnight in liquid paraffin at 60℃ and the samples were embedded in p araffin blocks.
The paraffin blocks were cut in 5 µm thick paraffin sections by microtome and left on the microscope slides at room temperature overnight to dry.

2.6.2 Picro-sirius red staining

The paraffin sections were de-waxed by incubation for 3 min in each one of the following solutions: 2 x Xylol, 2 x 100% ethanol, 2 x 96% ethanol, 1 x ddH_2O. Then the sections were incubated for one hour in the picro-sirius red solution and washed in two changes of acidified water (0.5% glacial acetic acid). After removing the water from the slides, these were dehydrated in three changes of 100% ethanol, cleared in xylol and mounted in a resinous medium.

2.7 Evaluation of RNA expression levels

2.7.1 RNA extraction

The mouse adrenals kept at -80℃ were used for RNA extraction. To obtain sufficient amounts of mRNA, both adrenals of each individual animal were pooled before extraction. The adrenals were homogenized while still frozen and the extraction procedure was followed using the Promega SV Total RNA Isolation System according to the manufacturer's instructions. The concentration of the RNA obtained, diluted in nuclease-free water, was then defined by photometric analysis and integrity of extracted RNA was confirmed by electrophoresis on 1% agarose gel. 2 µl RNA from each sample, diluted in 8 µl ddH_2O with

the addition of 3 µl loading Dye were pipetted in each well of the agarose gel together with 3 µl of the DNA Ladder. The specific RNA bands were visualised after 40 min (110 V) under a UV Camera (254 nm).

2.7.2 Reverse transcription polymerase chain reaction

The isolated single stranded RNA was reverse transcribed into complementary DNA (cDNA). 1 µg of total RNA was incubated for 5 min at 70℃ with 1 µl of an oligo (dT) primer (Promega) to denature RNA secondary structure and then was quickly chilled on ice (10 min) to let the primer anneal to the RNA. Subsequently 1.25 µl dNTP's (Promega), 5 µl RNAsin plus (Promega), 5 µl reaction buffer (Promega), 1 µl MML-V (Promega) and 3.75 µl water were added per sample. The mixture was then heated at 40℃ for 10 min, at 48℃ for 50 min and finally, for the inactivation of the enzyme, at 70°C for 15 min.

2.7.3 Real-time PCR

Quantification of mRNA levels was accomplished by Real-time PCR with the LightCycler (Roche) Real Time PCR machine. The kit used was the LightCycler FastStart DNA Master SYBR Green I and the procedure was completed according to the manufacturer's instructions.
The amplified genes were:
Murine ß-actin (as housekeeping gene)
Primers: Fwd: TCATGAAGTGTACGTGGACATCC
Rev: CCTAGAAGCATTTGCGGTGGACGATG
Murine CYP11ß2 (aldosterone synthase)
Primers: Fwd: CAGGGCCAAGAAAACCTACA
Rev: ACGAGCATTTTGAAGCACCT

The PCR conditions for the amplification of the ß-actin gene were as follows:
Denaturation: 95℃ for 10 min
Amplification: 95℃ for 10 sec (denaturation)
56℃ for 5 sec (annealing)
72℃ for 13 sec (extension)
40 Cycles
Melting curve: 69℃ for 60 sec (annealing)
98℃ with ramp rate 0.1℃/sec (melting)

The PCR conditions for the amplification of the CYP11ß2 gene were as follows:

Denaturation: 95℃ for 10 min

Amplification: 95℃ for 10 sec (denaturation)
63℃ for 5 sec (annealing)
72℃ for 16 sec (extension)
40 Cycles

Melting curve: 69℃ for 60 sec (annealing)
98℃ with ramp rate 0.1℃/sec (melting)

2.8 Statistics

All values in this study represent the mean values ± standard error of the mean (SEM). Statistical significance was determined using the Mann-Whitney unpaired t-test with a p-value <0.05 considered as statistically significant.

3. Results

3.1 Definition of normal aldosterone values in C3HeB/FeJ mice

The first goal of this study was to define the normal aldosterone values of the C3HeB/FeJ strain which was planned to be used for the ENU screen as well as for the stimulation and suppression experiments. Measurement of baseline aldosterone levels in 12 weeks old male (n=55) and female (n=69) mice revealed the baseline aldosterone values of 173 ± 16 pg/ml (mean ± SEM) and 92 ± 6 pg/ml, respectively, which resulted in statistically significant difference of aldosterone levels between the genders (P<0.001) (Fig. 9).

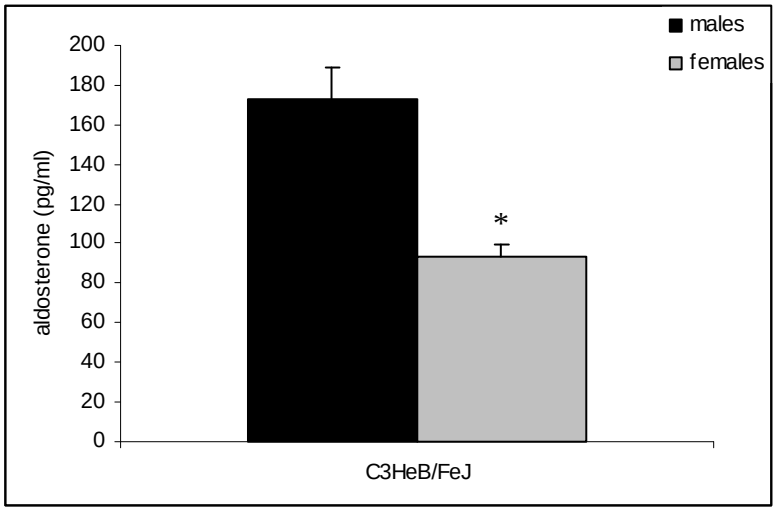


Fig. 9: Aldosterone values of 12 weeks old C3HeB/FeJ male and female mice

For the aldosterone screening process it was necessary to retest the animals with initially high aldosterone values to confirm sustainability of the observed phenotype. The second test was set when the mice were 16 weeks old. Therefore, the aldosterone values under baseline conditions were also defined in 16 weeks old C3HeB/FeJ animals. In comparison to the 12 weeks old mice baseline aldosterone levels tended to be lower for the male mice (157 ± 10 pg/ml vs. 173 ± 16 pg/ml, P=0.45) and significantly higher for the females (140 ± 11 pg/ml vs. 92 ± 6 pg/ml, P<0.001) (Fig. 10).

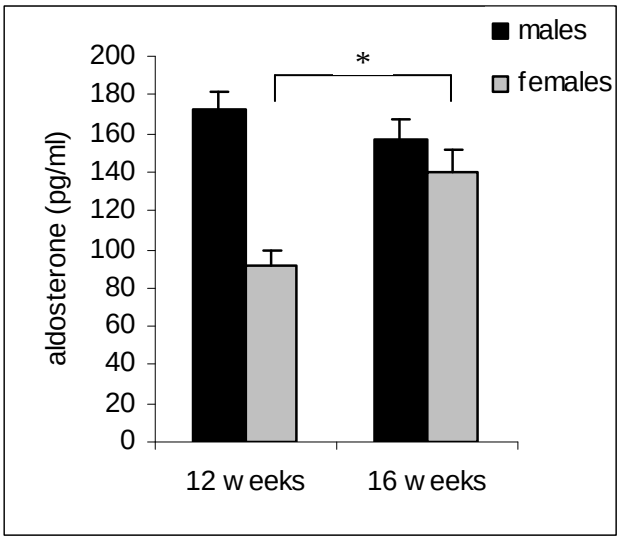


Fig. 10: Comparison of aldosterone values between 12 and 16 weeks old C3HeB/FeJ male and female mice

3.2 Strain dependent variation in baseline aldosterone values

In addition to C3HeB/FeJ mice, the baseline aldosterone values of two other common strains were measured under baseline conditions in 12 weeks old adult animals. The strain which would show similar aldosterone values to the C3HeB/FeJ could then be chosen for future backcross breeding necessary for linkage analysis. In addition, for these association studies significant genetic differences have to be present in comparison to the C3HeB/FeJ strain. Thus, the strains chosen to be tested were the Balb/c and the C57BL/6J strain of mice. The mean aldosterone values for the Balb/c mice were 150 ± 13 pg/ml (mean ± SEM) for the males (n=20) and 136 ± 16 pg/ml for the female (n=20) mice, with no statistically significant difference between both strains (P=0.40). In contrast, the aldosterone values for the C57BL/6J were 237 ± 13 pg/ml for the males (n=40) and 210 ± 15 pg/ml for the females (n=30), which were significantly higher for both genders in comparison to the C3HeB/FeJ mice (P<0.001). No gender dependent differences were present in both Balb/c and C57BL/6J animals respectively (Fig. 11).

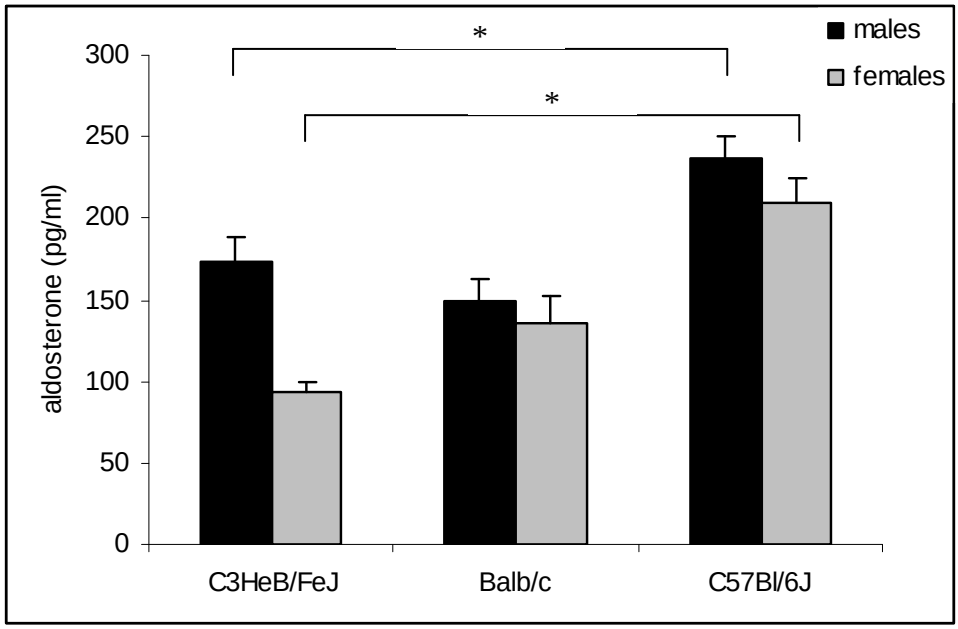


Fig. 11: Comparison of aldosterone values between male and female animals of three common strains.

3.3 In vivo stimulation and suppression tests for aldosterone secretion

The substances chosen to modulate the RAA system were among those used in clinical practice for the evaluation of aldosterone secretion: The ACTH stimulation test, the dexamethasone suppression test, the normal saline load test and the fludrocortisone suppression test were chosen, for the definition of the normal aldosterone values in mice under these conditions. Two of the major stimuli of the aldosterone secretion, angiotensin II and potassium were also included into the experiments.

3.3.1 Angiotensin II stimulation and fludrocortisone suppression tests

Sixty minutes after injection of 200 ng/g angiotensin II, aldosterone levels were significantly stimulated in 12 weeks old male (n= 20, 284 ± 5 pg/ml, vs. baseline n=20, 145 ± 8 pg/ml, P<0,001) and female (n=20, 286 ± 7 pg/ml vs. baseline n= 20, 91 ± 6 pg/ml, P<0.001) animals. In contrast to baseline evaluation no significant differences between the genders were observed (P=0.81).

For the fludrocortisone suppression test 20 male and 20 female C3HeB/FeJ mice were treated with 4 injections of 1.5 ng/g fludrocortisone daily for four days. The aldosterone response was then evaluated by measurement of the plasma aldosterone values, which

were significantly lower in comparison to those under baseline conditions (P<0.001, males: 77 ± 7 pg/ml, females: 37 ± 7 pg/ml) (Fig. 12).

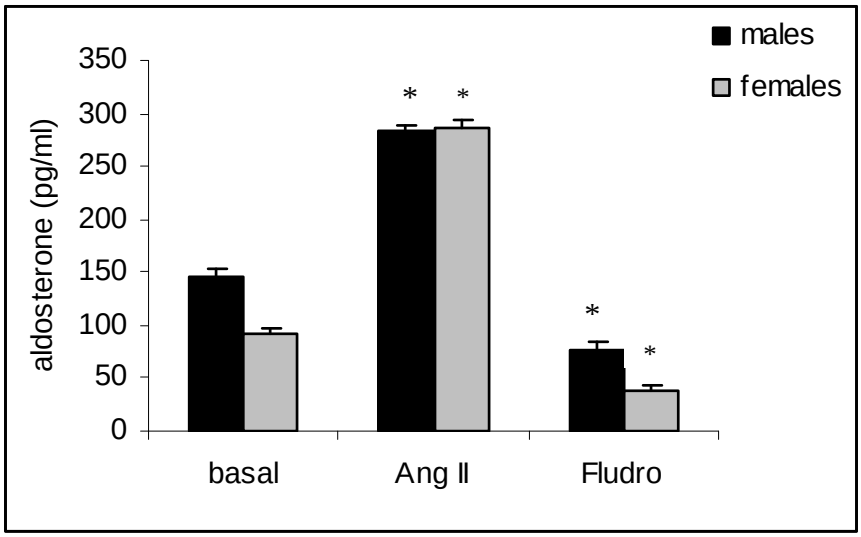


Fig. 12: Aldosterone response in C3HeB/FeJ mice after angiotensin II and fludrocortisone treatment

3.3.2 Potassium and sodium dependent effects on aldosterone secretion

For evaluation of potassium dependent aldosterone secretion, 12 weeks old C3HeB/FeJ mice (n=20 males, n=20 females) were treated for 8 days with drinking water supplemented with high levels of potassium. The plasma aldosterone values varied between males and females, but were in both cases significantly higher in comparison to the untreated population (P<0.001, males: 312 ± 34 pg/ml, females: 192 ± 17 pg/ml).

The normal saline load test was performed in order to imitate the test performed in human subjects for the differential diagnosis of primary aldosteronism. 20 male and 20 female 12 weeks old C3HeB/FeJ mice received an intraperitoneal injection of 1 ml NaCl 0.9%. Two hours later plasma aldosterone measured revealed significantly lower levels in comparison to the untreated groups of both sexes (P<0.001, males: 22.3 ± 2.2 pg/ml, females: 22.6 ± 2 pg/ml, vs. baseline males: n=20, 145 ± 8 pg/ml, females: n= 20, 91 ± 6 pg/ml) (Fig. 13).

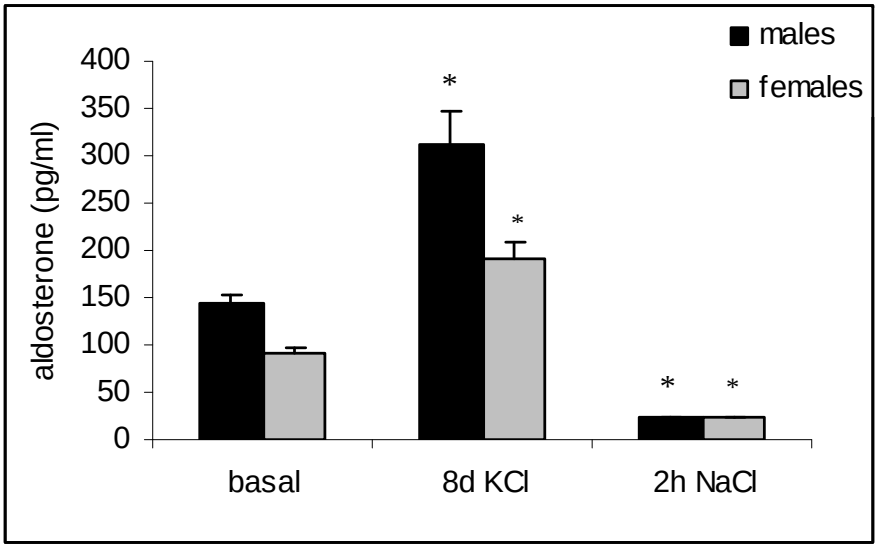


Fig. 13: Aldosterone response in C3HeB/FeJ mice after treatment with potassium and sodium chloride

3.3.3 ACTH stimulation and dexamethasone suppression

The ACTH stimulation test was performed in 12 weeks old mice (n=20 males, n=20 females) in order to evaluate the aldosterone response in wild type animals. The aldosterone values measured after ACTH stimulation were significantly higher in comparison to those under baseline conditions ($P<0.001$, males: 199 ± 11 pg/ml, females: 170 ± 9 pg/ml).
Conversely, the aldosterone values obtained after the dexamethasone suppression test in 12 weeks old mice (n=20 males, n=20 females), 13 hours after the injection of 50 ng/g dexamethasone, resulted in significant suppression of the aldosterone secretion in both sexes ($P<0.001$, males: 20.1 ± 15.3 pg/ml, females: 17.2 ± 6.1 pg/ml) (Fig. 14).

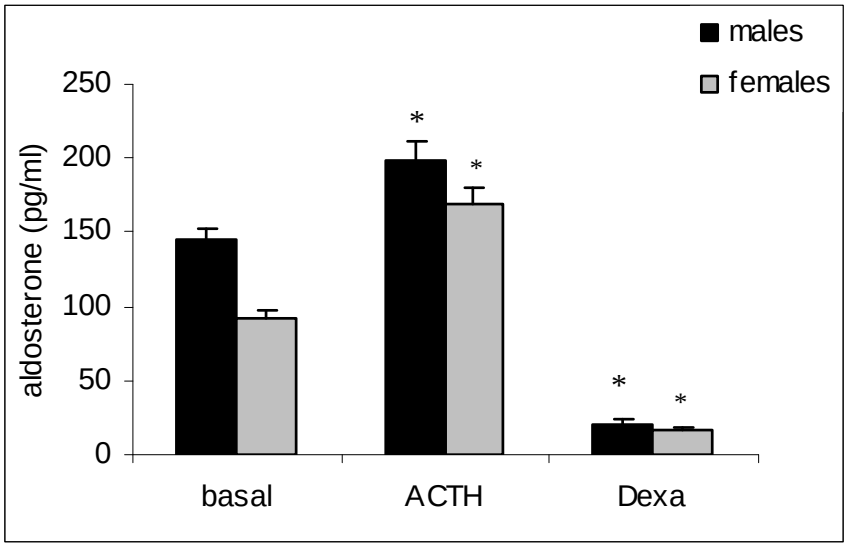


Fig. 14: Aldosterone secretion in C3HeB/FeJ mice after ACTH and dexamethasone treatment

3.4 Time dependant stimulation and suppression experiments

Based on the result of the presented stimulation and suppression experiments angiotensin II, normal saline (NaCl 0.9%), ACTH and potassium were chosen for further evaluation of the time dependent adrenal aldosterone release. Female animals were chosen to take part in this series of experiments, because of their more distinguished response to the above presented functional tests.

3.4.1 Control Group

The control group was established in order to elucidate any kind of interactions caused by the treatment of the animals and especially by the injection procedure. The mice of this group received sham intraperitoneal injection without application of substance, so that potential stress related effects of the injection itself could be investigated. Animals were then euthanized at the same time points as chosen for all experiments.

Interestingly, aldosterone secretion in mice 10 minutes after sham injection was significantly higher than under baseline conditions (baseline: 88 ± 10 pg/ml, 10 minutes: 228 ± 23 pg/ml, $P<0.005$). This phenomenon was still present but less pronounced in the 20 minutes sampling (154 ± 18 pg/ml, $P<0.05$) while the aldosterone values of treated mice reached the baseline values 30 minutes after injection (Fig. 15).

The aldosterone synthase expression was evaluated as one of the rate limiting steps in aldosterone synthesis. Real time analysis revealed a statistically significant increase 10

minutes after the injection (153% ± 5%, P<0.05) which came back to baseline expression levels in the 20 minutes group (98% ± 7%, P=0.93) and thereafter (Fig.16).

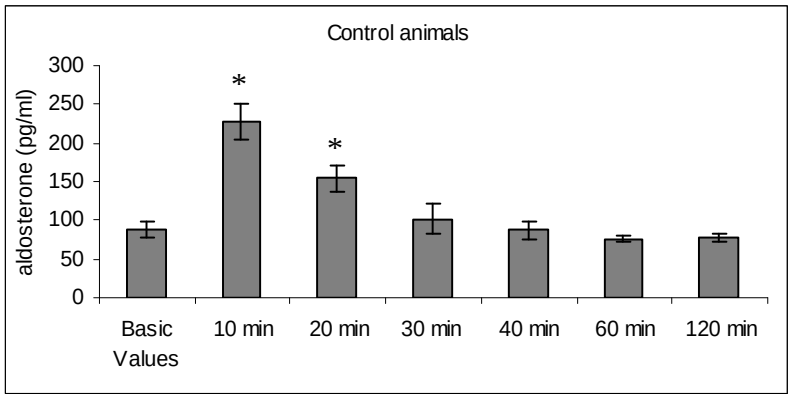


Fig. 15: Aldosterone secretion in C3HeB/FeJ female mice after a sham intraperitoneal injection

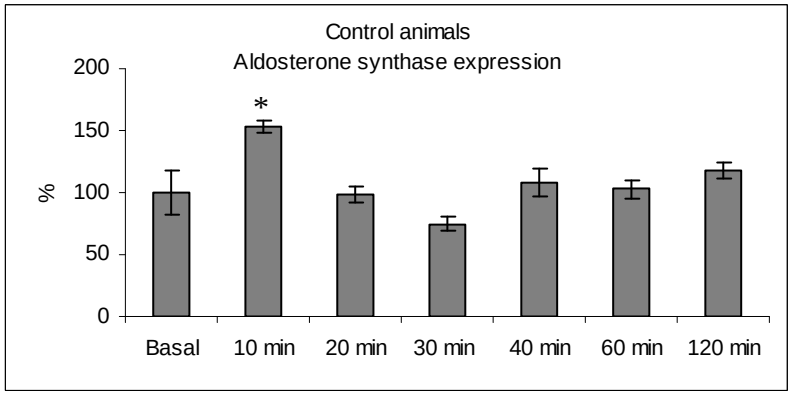


Fig. 16: Aldosterone synthase expression levels in C3HeB/FeJ female mice after a sham intraperitoneal injection

3.4.2 Angiotensin II stimulation

For evaluation of the time dependency of angiotensin II induced aldosterone secretion 200 ng/g of angiotensin II were injected intraperitoneally in each mouse. Groups of mice were euthanized 10, 20, 30, 40, 60 and 120 minutes later and blood and adrenals were collected. Plasma aldosterone was measured in all time groups and was compared to the baseline values. 10 minutes after the initial injection, the aldosterone values were significantly elevated (baseline: 88 ± 10 pg/ml, 10 minutes: 858 ± 126 pg/ml, P<0.001). The following measurements at 20, 30 and 40 minutes showed a stepwise reduction but the aldosterone secretion still remained significantly higher in comparison to the baseline values (P<0.01, 20 min: 585 ± 133 pg/ml, 30 min: 485 ± 69 pg/ml, 40 min: 297 ± 48 pg/ml) (Fig. 17).

The corresponding aldosterone synthase expression in the adrenal gland showed a 3.3 – fold increase 10 minutes after the angiotensin II injection (P<0.005) which was sustained up to 60 minutes later (P<0.01). 120 minutes after the stimulus the aldosterone synthase expression remained still increased but not significantly in comparison to the baseline expression levels (P=0.16) (Fig. 18).

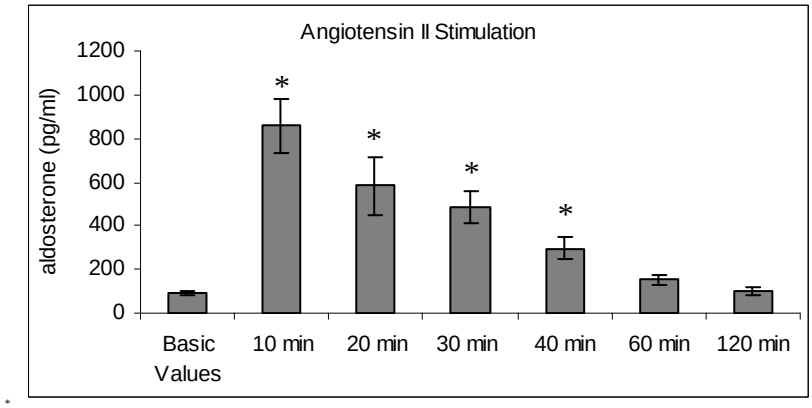


Fig. 17: Aldosterone secretion in C3HeB/FeJ female mice after an intraperitoneal angiotensin II injection

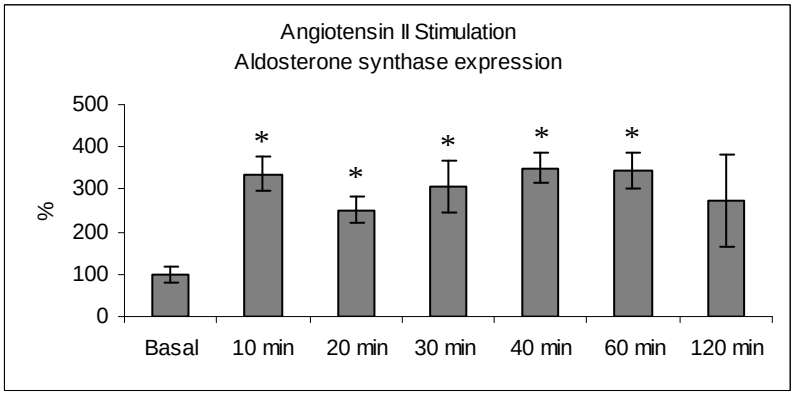


Fig. 18: Aldosterone synthase expression levels in C3HeB/FeJ female mice after an intraperitoneal injection of angiotensin II

3.4.3 Normal saline load

After an intraperitoneal injection of 1 ml NaCl 0.9% the aldosterone secretion displayed a short term increase (10 minutes: 192 ± 20 pg/ml, P<0.005) comparable to that seen after sham injection. This was followed by a slow decrease with a nadir 60 minutes after the injection, which was significantly lower than the baseline values (38 ± 3.7 pg/ml, P<0.005) (Fig. 19).

Sodium load had significant effects on aldosterone synthase expression with a significant decrease over baseline values between 20 (37% ± 6%, P<0.05) and 60 minutes (42% ± 6.2%, P<0.01) (Fig. 20).

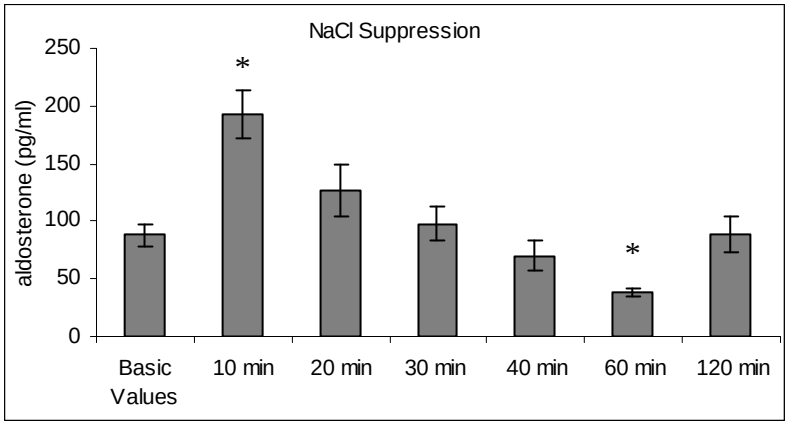


Fig. 19: Aldosterone secretion in C3HeB/FeJ female mice after sodium load test

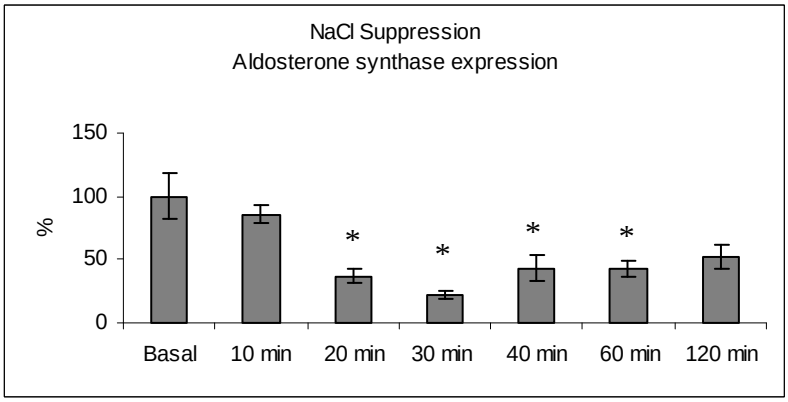


Fig. 20: Aldosterone synthase expression in C3HeB/FeJ female mice after sodium load test

3.4.4 Potassium supplementation in drinking water

In this group animals could drink ad libitum water which was supplemented with 2% potassium for one week. One, four and seven days after the start of this treatment, mice were euthanized. The plasma aldosterone measured after the first day of treatment was significantly higher than in the untreated group (298 ± 53 pg/ml, P<0.01). Aldosterone values on day 4 and 7 remained also significantly higher than under baseline conditions (day 4: 226 ± 10 pg/ml, P<0.001, day 7: 170 ± 23 pg/ml, P<0.05) (Fig. 21).

Similarly, the aldosterone synthase expression showed a 2-fold increase after the first day of treatment, which was sustained until the last day, however, without reaching statistical

significance (day 1: 186% ± 23%, P<0.05, day 4: 193% ± 53.5%, P=0.18, day 7: 215% ± 43%, P=0.06) (Fig. 22).

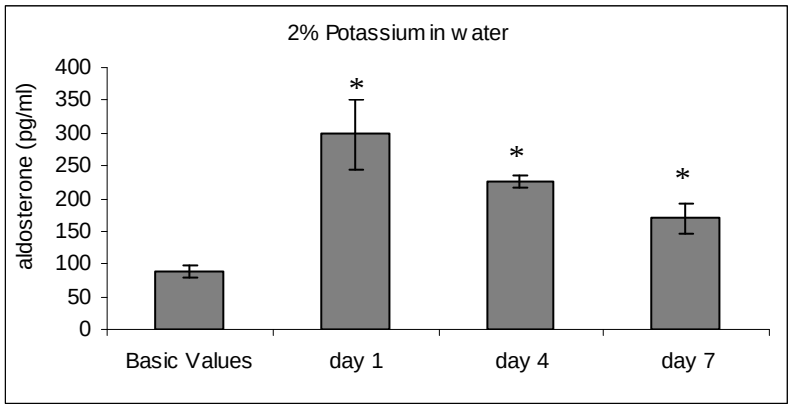


Fig. 21: Aldosterone levels in C3HeB/FeJ female mice after treatment with high potassium concentration in drinking water

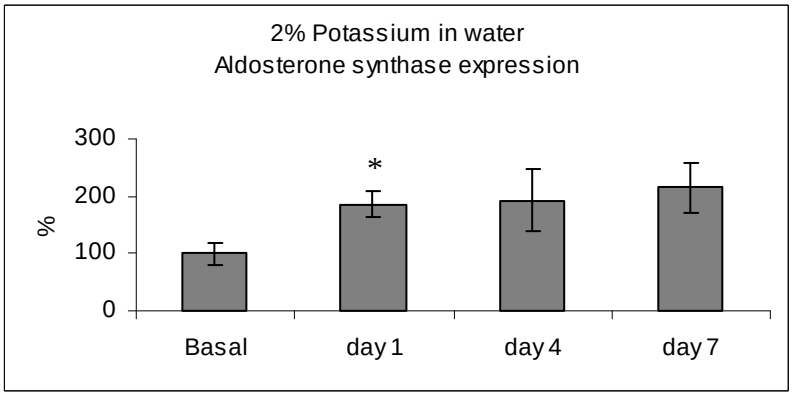


Fig. 22: Aldosterone synthase expression in C3HeB/FeJ female mice after treatment with potassium supplemented in drinking water

3.4.5 ACTH Stimulation

10 minutes after an intraperitoneal injection of ACTH the aldosterone secretion in mice was significantly higher in comparison to baseline levels (1216 ± 180 pg/ml vs. 76 ± 14 pg/ml, P<0.001), with a stepwise decrease after 30 (876 ± 218 pg/ml, P<0.01) and 60 minutes (555 ± 164 pg/ml, P<0.05), which remained significantly higher in comparison to baseline levels (Fig. 23).

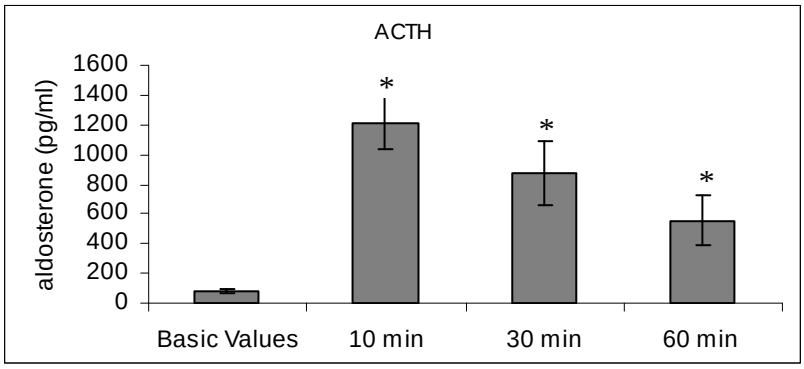


Fig. 23: Aldosterone secretion after ACTH stimulation test in C3HeB/FeJ female mice

3.5 ENU Screen

3.5.1 Initial Screen and distribution of cohorts

For the mutagenesis screen for the phenotype hyperaldosteronism, all F1 offspring from ENU-treated males and wild type females had to be checked for their aldosterone levels at the age of 12 weeks. The aldosterone values received were then compared to the aldosterone values of the wild type population of the same age and gender. The upper cut-off value of the normal range was defined as the gender dependent mean plus 3 standard deviations of the aldosterone values of the wild type population. According to this definition, for 12 weeks old male mice the upper normal limit was set at 515 pg/ml, and for female mice at 251 pg/ml (displayed in figure 24 as dashed lines).

The offspring tested presented with a wide range of aldosterone values. Much more female mice were characterized by pathological values in comparison to the male animals. All animals that showed once pathological aldosterone levels were retested at the age of 16 weeks. In the initial screen 2864 mice took part, with 1450 male and 1414 female animals. Out of these 83 animals initially showed high aldosterone levels (18 males and 65 females). After the confirmation measurement only 11 mice had sustained high aldosterone values (1 male and 10 female). Two of the pathological female mice died before further breeding for unrelated reasons. All remaining animals were included into the further breeding process, by being mated to wild type mice of the opposite gender. From these breeding processes four lines with affected offspring could be generated so far. As these lines were derived from four different ENU treated males, the presence of different mutations as the modifying genetic cause of the observed phenotype is likely.

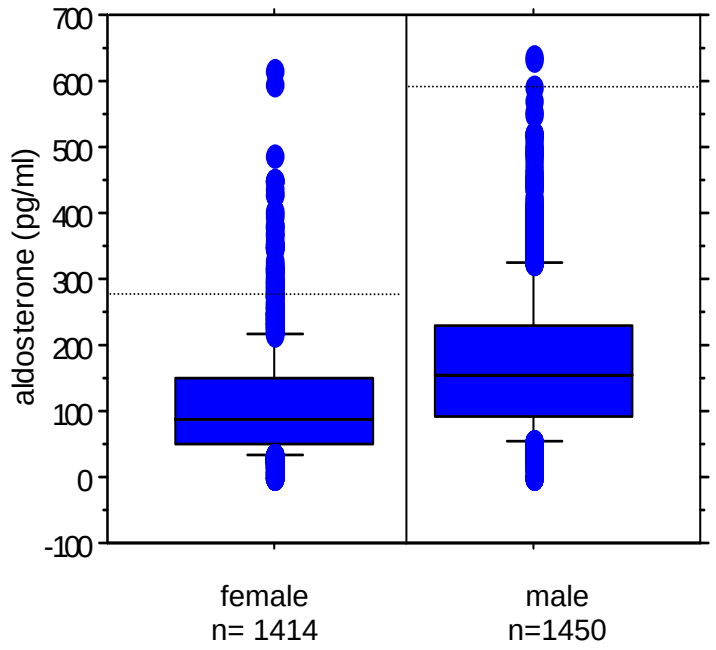


Fig. 24: Range of aldosterone values of F1 animals from the ENU Screen

Taken together, out of all offspring tested for the phenotype hyperaldosteronism during the ENU mutagenesis screen, 2.9% showed initially high aldosterone values and 13.2% of these had sustained hyperaldosteronism. Out of these 90.9% were females and only 9.1% were males. In total, 0.38% of all mice screened had confirmed hyperaldosteronism (Fig. 25).

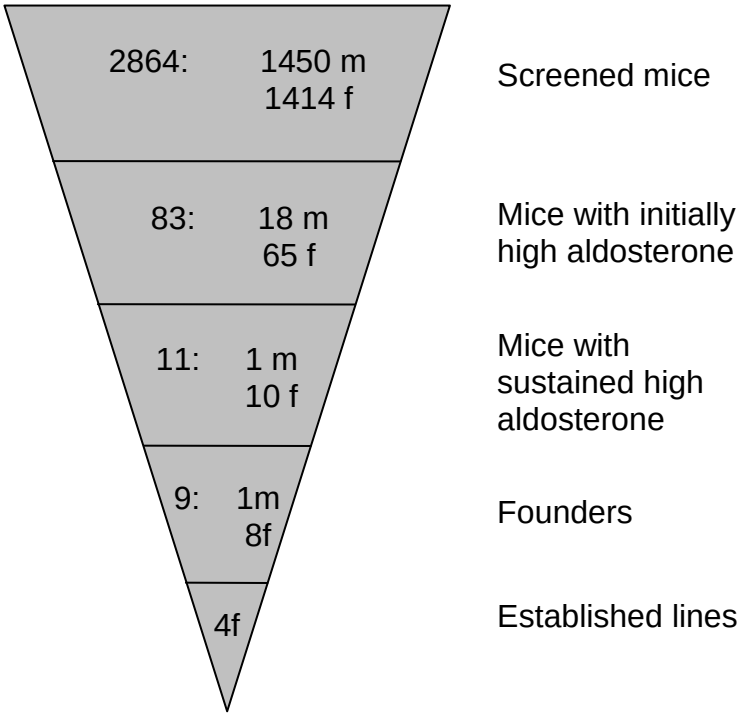
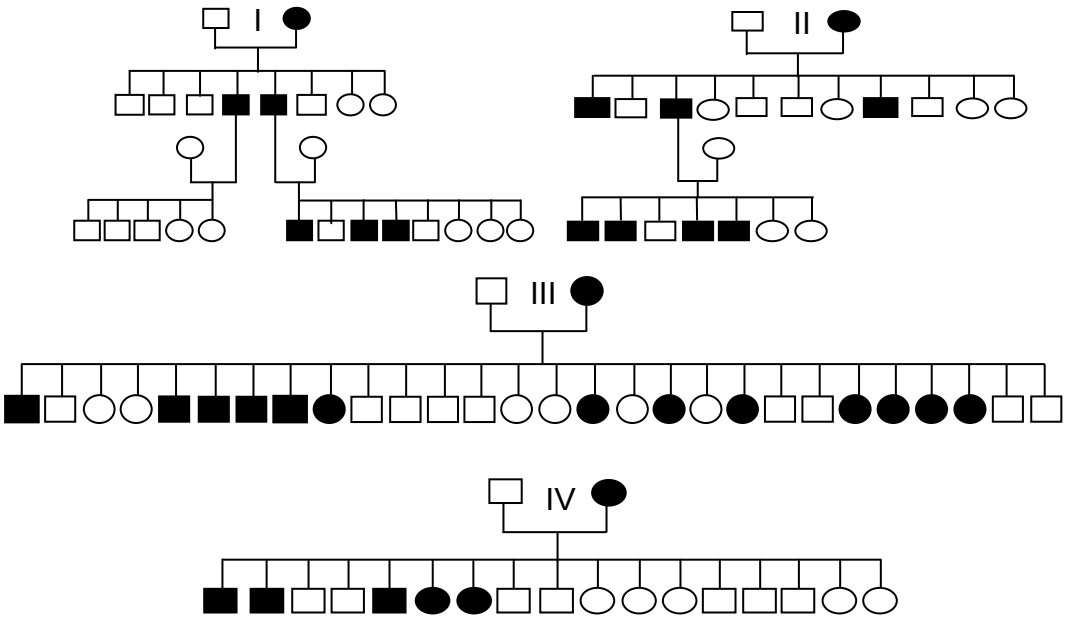


Fig. 25: Statistics of the ENU Screen for the phenotype hyperaldosteronism

3.5.2 Genetic traits of established lines

Further breeding provided us with the opportunity to gather first evidence for possible pattern of inheritance. In all the established four pedigrees (I-IV) the founder was a female animal, which originated in each case from a different ENU treated male parent (Fig. 26). This might explain the different inheritance pattern of the trait in each pedigree, as a different genetic background is likely to be present. In line I and II, only male animals displayed the pathological phenotype by a rate of 36% and 58%, respectively. In the line III the phenotype was expressed in both male and female mice by 36% and 57%, respectively. In the line IV males and females were almost equally affected with 30% and 29%, respectively.



	Male	Female
I	36% (5/14)	0% (0/7)
II	58% (7/12)	0% (0/6)
III	36% (5/14)	57% (8/14)
IV	30% (3/10)	29% (2/7)

Fig. 26: Pedigrees of established mutant lines and percentages of affected animals in each affected mouse line.

3.5.3 Phenotypical characterization

The animals were separated into affected and unaffected individuals according to their plasma aldosterone, with the cut-off value as defined above. Accordingly, affected mice had significantly higher aldosterone values than their littermates (P<0.001). The affected animals were submitted to further tests for the definition of their hormonal and biochemical profile, in order to characterize in details their hyperaldosteronism. The first parameter checked was renin, which tended to be lower in the affected animals in comparison to their normal littermates (unaffected: 120 ± 40 ng/ml/hr, affected: 75 ± 9 ng/ml/hr, P=0.43). The aldosterone to renin ratio was calculated in both groups and was significantly increased in the affected animals (unaffected: 1.83 ± 0.54 pg/ml/ng/ml/hr, affected: 5.9 ± 1.63 pg/ml/ng/ml/hr, P<0.01). Moreover, potassium levels also were significantly lower in the affected animals (unaffected: 5.44 ± 0.05 mmol/l, affected: 4.78 ± 0.23 mmol/l, P<0.05) as would be expected for primary aldosteronism (Fig. 27).

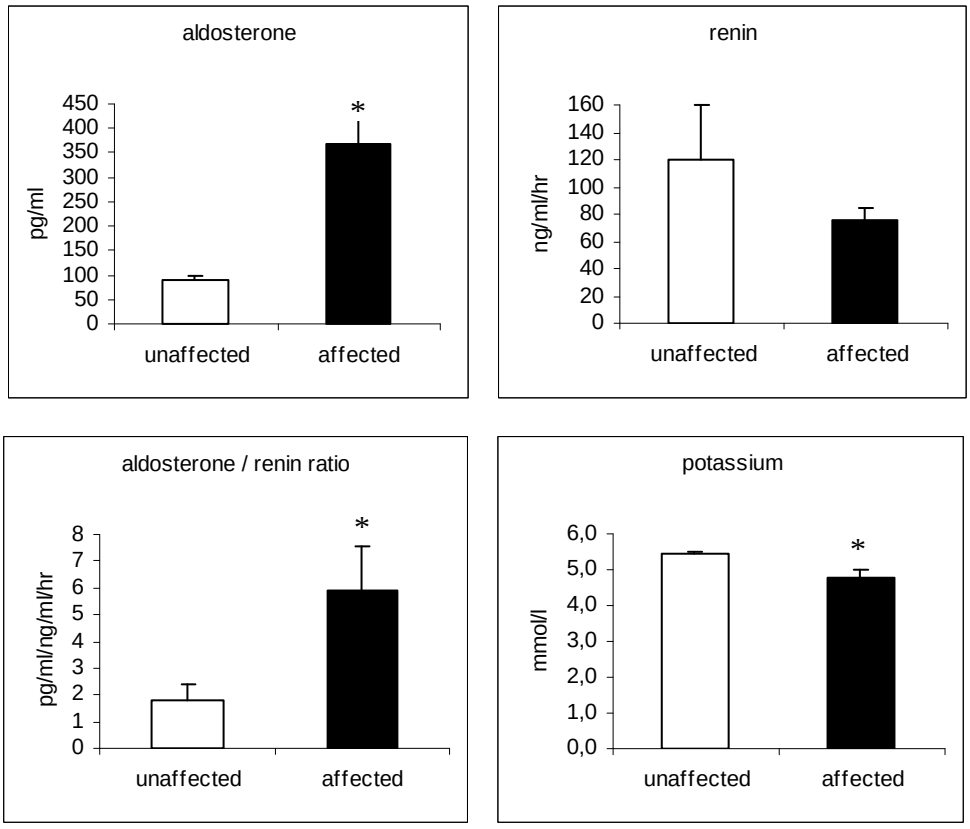


Fig. 27: Aldosterone and renin levels, aldosterone to renin ratio and potassium levels in affected animals in comparison to their unaffected littermates.

The adrenal expression of aldosterone synthase, the responsible enzyme for the last step of aldosterone production, was also investigated and proofed to be 6-7-folds higher in affected

animals in comparison to their unaffected littermates (unaffected: 100% ± 8%, affected: 649 ± 76%, P<0.001) (Fig. 28).

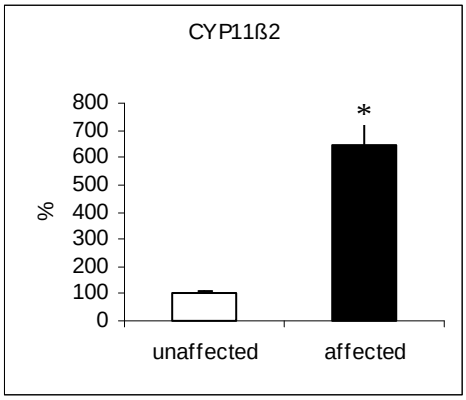


Fig. 28: Aldosterone synthase expression in affected animals in comparison to their unaffected littermates

Other biochemical parameters tested so far in these lines showed no significant difference between affected and non-affected animals: The levels of total protein between normal animals and their affected littermates were within the normal range, without any significant difference (unaffected: 5.4 ± 0.09 g/dl, affected: 5.25 ± 0.08 g/dl, P=0.36). Creatinine, as a parameter of renal function showed a trend towards lower levels in the affected animals, without any significant differences in comparison to the healthy mice (unaffected: 0.348 ± 0.007 mg/dl, affected: 0.340 ± 0.007 mg/dl, P=0.53). Urea was slightly increased in the affected animals (unaffected: 49.1 ± 2.4 mg/dl, affected: 56.5 ± 1.8 mg/dl, P=0.087), but remained still within the normal range. Uric acid finally showed similar values in both groups (unaffected: 3.19 ± 0.11 mg/dl, affected: 3.93 ± 0.14 mg/dl, P=0.22) (Fig. 29).

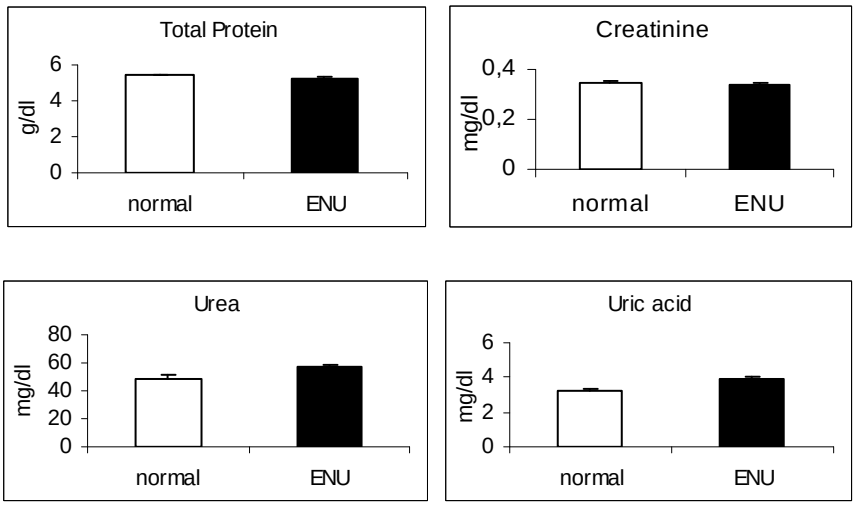


Fig. 29: Biochemical parameters in affected and non affected animals

For the investigation of the cardiac phenotype of affected animals their hearts were embedded in paraffin and horizontally sectioned. The sections were then stained with picro-sirius red, a staining for collagen, where the collagen fibers are coloured red. Representative sections of hearts from affected animals were compared with wild type littermates (Fig. 30). The hearts from affected animals displayed higher staining intensity than those of the unaffected animals. Thus, an increase in collagen fibers in the hearts of affected animals is to be expected, similar to the clinical picture of primary aldosteronism with cardiac fibrosis.

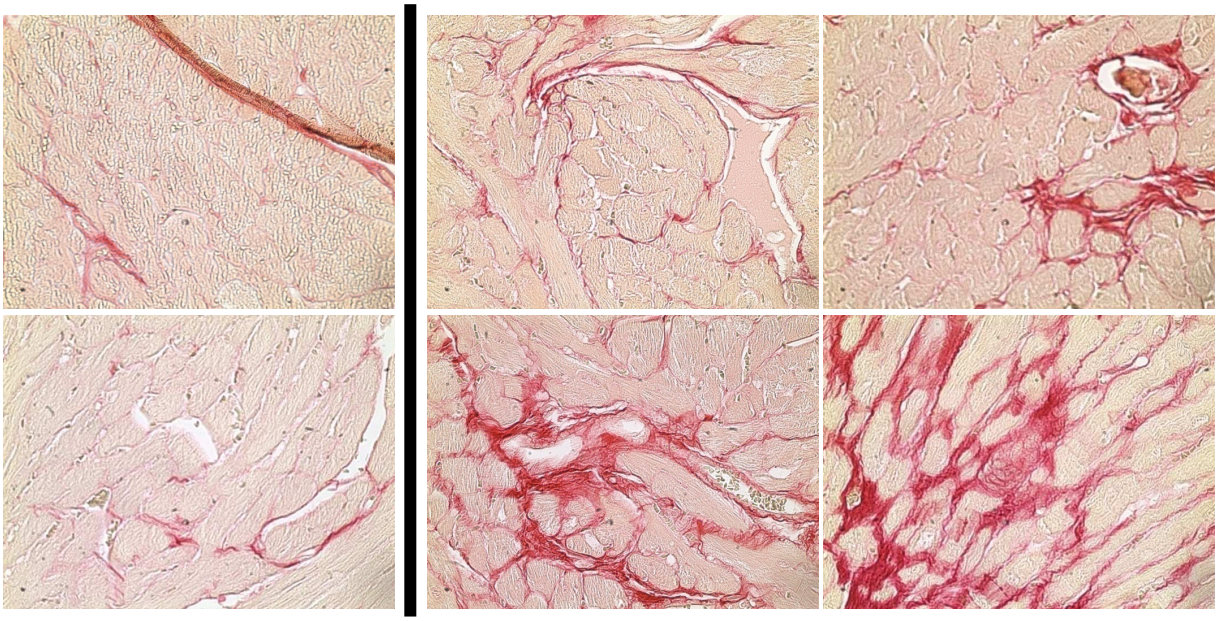


Fig. 30: Picro-sirius red staining of horizontal sections of left ventricles of wild type (left) and of affected animals (right). Increased collagen deposits (red colour) in the left ventricles of affected animals suggest cardiac fibrosis.

4. Discussion

4.1 Definition of normal baseline aldosterone values

In the present study we defined the normal aldosterone values in male and female mice of three inbred strains, commonly used as murine *in vivo* models. We were able to demonstrate that aldosterone values of wild type mice not only showed significant differences between strains but also between genders. Aldosterone values in wild type male mice of the strain C3HeB/FeJ had consistently higher values than female mice of the same strain and age. Gender differences in aldosterone values could also be shown in another common strain, C57BL/6J and Balb/c. More detailed investigation showed that male C57BL/6J mice had higher aldosterone than females. Contrastingly, Balb/c mice of both genders had similar aldosterone values without any significant differences. Other studies have demonstrated lower aldosterone values in female mice but without significant difference in comparison to males (Manolopoulou et al., 2008), or no difference between both genders (Sausbier et al., 2005). In another study (Bielohuby et al., 2007), the analysis of the zonal composition of the adrenal gland showed an increased total volume of the zona glomerulosa cells in female mice in comparison to males. As this finding does not correspond to our observation of higher aldosterone levels in male animals, crude differences in zonation seem not to account for the gender related differences in aldosterone output. Another possible explanation for differences in aldosterone levels could be influences by other gender specific hormones with modulating effects on the RAA system. Progesterone presents one putative candidate, which through its weak mineralocorticoid activity could suppress aldosterone secretion. So far, however, only controversial data are available on the role of progesterone for negative feedback action on aldosterone output *in vivo* (Rafestin-Oblin et al., 2002). A gender-dependent response of the HPA axis has also been reported, with sexual dimorphism on the corticosterone levels of animals, but aldosterone levels had not been examined in this study (Rivier, 1994). In this case, inflammatory mechanisms played a role in the sex-dependent phenotype. Taken together, it remains unclear to date whether gender dependent differences in secretion of steroids such as progesterone and corticosterone are causative for the observed lower aldosterone levels in female animals.

We also observed a variation between the values of mice of different age. As shown before (Manolopoulou et al., 2008) there are differences in the mean aldosterone values of 3, 5, 7, 9 and 11 weeks old mice. Evaluation of adrenal zonation also displays an evolving pattern during puberty (Bielohuby et al., 2007). So far, variation of aldosterone values in adult mice has not been reported. Here we demonstrate that aldosterone values in 12 weeks old male mice are overall higher than in 16 weeks old animals. In female mice the opposite

phenomenon could be demonstrated, with 16 week old mice having significantly higher aldosterone values than 12 week old animals. Potentially, postpubertal changes in the adrenal function in both genders could explain this phenomenon. This is further in agreement with a study where aldosterone values in adult rats showed an age-related decline, in part as consequence of the reduced activities of biosynthetic enzymes, adenyl cyclase and L-type calcium channels, as well as the expression of P450scc enzyme in zona glomerulosa cells (Kau et al., 1999). As such, age related differences in aldosterone output have to be taken into account for interpretation of aldosterone levels in experimental animals.

Aldosterone values of 12 week old animals of the strain C3HeB/FeJ were also compared to the aldosterone values of age matched animals of two other common inbred strains, C57BL/6J and Balb/c mice. In all cases male mice presented higher aldosterone values than females. Moreover, the C57BL/6J strain overall had higher aldosterone values than animals from the strain C3HeB/FeJ. Specifically, male C57BL/6J mice had the highest aldosterone values of all strains while Balb/c animals had intermediate values, which are quite similar to the C3HeB/FeJ strain. Differences between common mouse strains have only been reported for corticosterone (Cabib et al., 1990, Freund et al., 1988) and insulin levels (Kayo et al., 2000). In contrast, so far there have been no in-depth studies describing strain related differences in aldosterone plasma levels. Differences in modifying genes, e.g. at the level of feedback mechanisms to the RAA system or the HPA axis regulating the aldosterone secretion (Cabib et al., 1990) could explain these findings. Overall, this strain-dependent hormonal response could be an interesting target for future research to define potential modifier genes of aldosterone output. For the ENU screen a backcross between affected C3HeB/FeJ animals and a strain with similar aldosterone values will be necessary for further association studies. The Balb/c strain is appropriate for this purpose since it has similar aldosterone values in comparison to the C3HeB/FeJ mice, and pathological values are, thus, readily recognized. In addition, this strain has significant differences in its genome in comparison to the C3HeB/FeJ, so that it will be possible to define loci involved in the pathological phenotype based on linkage analysis (Reinhard et al., 2005).

4.2 Stimulation and suppression experiments

The stimulation and suppression experiments for the aldosterone secretion were designed according to the stimulation and suppression tests used for the diagnosis of primary aldosteronism or other functional disorders of the hypothalamus-pituitary-adrenal axis.

Notably, sham injection had measurable effects on aldosterone release most likely through stress dependent activation of the HPA axis. There was a significant increase in the secretion of aldosterone 10 minutes after the stressful event of the injection, which was

however normalized 20 minutes later. This finding could be interpreted as an acute ACTH dependent effect on the adrenal gland, since stress stimulates the hypothalamus-pituitary-adrenal axis and potentially the zona glomerulosa cells (Stier et al., 2004). The possibility that anesthesia or blood sampling might have influenced the steroid levels could be minimized, since rapid sampling carried out in <3 min does not trigger a stress response (Vahl et al., 2005). On the contrary, to our knowledge, a correlation between the injection as a stressful event and an increase in the aldosterone secretion has not yet been described.

Research on the actions of ACTH on the adrenal cortex has focused so far mainly on its maintenance of glucocorticoids in plasma and its role in steroidogenesis via transfer of cholesterol across the mitochondria in conjunction with steroidogenic acute regulatory protein (StAR). Although the ACTH stimulation test is carried out for the assessment of adrenal function, there is controversial evidence for the actions of ACTH on the production of aldosterone in rodents. One study showed aldosterone levels below limits of detection in mice with a complete lack of ACTH through targeted deletion of the pituitary pre-hormone POMC (Yaswen et al., 1999) while in another study aldosterone levels were significantly lower at 1 month of age in Pomc-null mutants (Karpac et al., 2005). Contrastingly, Linhart and Majzoub, 2008 showed an increase in both aldosterone and renin values in Pomc knockout mice, consistent with the presence of secondary hyperaldosteronism. ACTH is considered to act as an acute regulator of aldosterone synthesis in stress situations (Muller, 1995). In our experiment, aldosterone secretion remained increased even one hour after the Synacthen injection, which would implicate a very high aldosterone response within the first minutes after the stimulation. As expected, dexamethasone injection caused a significant decrease in plasma aldosterone levels, confirming the ACTH effect on the aldosterone production.

One of the two most important stimuli of aldosterone secretion is potassium. Elevated extracellular potassium concentrations directly stimulate aldosterone production from glomerulosa cells (Williams, 2005). Accordingly, as was demonstrated, treatment with increased potassium concentration in the drinking water for 8 days caused a significant increase in the aldosterone values in mice, which is in agreement with findings from a previous study (Arrighi et al., 2001). Potassium dependent stimulation of chronic aldosterone secretion relies on a relatively straightforward pathway, from an increased intracellular calcium signal, leading to depolarization-induced activation of voltage-gated Ca^{2+} channels, resulting in activation of the calcium binding protein calmodulin (CaM) by CaM kinase (CaMK) I and/ or IV. This mechanism induces the expression of CYP11B2 through upregulation of transcription factors including Nurr1 and NGF1B (Bassett et al., 2004). The aldosterone increase in our experiment was already obvious after the first day of treatment and was sustained up to the last day of the experiment. Of note, the difference in the

aldosterone values between both genders was sustained even during this stimulation experiment. As expected, the normal saline load test, used in patients for the confirmation of primary aldosteronism showed a significant reduction of aldosterone secretion one hour after the intraperitoneal injection of NaCl 0.9%. In other studies a similar pattern of adrenal response to sodium load has been shown, either after injection or induced by a high sodium diet (Okubo et al., 1997). The aldosterone values between male and female animals showed no difference after this test and were significantly lower than in the baseline population only 60 minutes after the initial injection. The initial increase in aldosterone secretion, 10 minutes after the injection was comparable to the increase in aldosterone secretion observed in the sham injection group. The normal saline load effect disappeared 2 hours after the injection.

Angiotensin II is the physiological stimulus of aldosterone secretion within the RAA system. Accordingly, injection of recombinant angiotensin II in the animals caused the expected increase in the aldosterone secretion. As also shown in other studies, there is a dose-dependent aldosterone response after angiotensin II injection (Cao et al., 2006). In comparison to the ACTH dependent stimulation of aldosterone secretion, angiotensin II induced aldosterone levels were lower, which could be related to dosage dependent effects, or could be explained by the acute action of ACTH in aldosterone biosynthesis. However, the fast increase of aldosterone secretion 10 minutes after the injection cannot be explained solely on the basis of injection caused stress, since the aldosterone values were significantly higher in comparison to those of the sham injection control group.

Fludrocortisone suppression testing is performed in humans, as a diagnostic tool for the assessment of the adrenal function, and in particular for the diagnosis of primary aldosteronism (Mattsson and Young, 2006). This aldosterone agonist has similar effects on the adrenal gland in rodents and this experiment performed in mice could demonstrate a significant decrease in the aldosterone production in male and female mice, most likely through suppression of renin activity and angiotensin II secretion. Taken together, the observed changes in aldosterone secretion upon specific stimulation and suppression tests are in accordance with expected modulation of the RAA system and the HPA axis and provide the groundwork for functional tests in genetically altered animals such as these from the ENU screen.

4.3 Aldosterone synthase expression after stimulation and suppression experiments

Steroids, as very hydrophobic molecules, are not stored in steroidogenic tissues such as the adrenal cortex but are acutely synthesized from cholesterol on demand. As such, regulation of steroidogenic enzymes on the mRNA level is a major regulatory mechanism for acute and chronic increase in steroid production and release.

The intraperitoneal injection itself in a mouse causes some alterations in aldosterone secretion and simultaneously in the aldosterone synthase expression. In our experiments, the aldosterone synthase expression measured 10 minutes after a sham injection showed a 1.5 fold increase in comparison to the baseline expression. The increase in aldosterone synthase mRNA after the injection of angiotensin II was expected, since the aldosterone synthase dependent enzymatic activity is the limiting step for the production of aldosterone from the adrenal gland. A detectable and significant increase in aldosterone synthase mRNA was, however, not expected as soon as 10 minutes after the initial injection. This early regulation was also observed in the normal saline load test, where the aldosterone synthase shows a temporary increase in the first minutes after injection, as well as in the control group, in both cases probably because of the stress induced ACTH stimulation. To our knowledge, regulation of the expression of aldosterone synthase within such a short time frame has not been reported to date. In other studies it has been shown that acutely - within minutes to hours after a stimulus - aldosterone production is controlled by the transport of cholesterol into the mitochondria, mediated by increased expression and function of StAR (Cherradi et al., 1998). Only chronically - within hours to days - aldosterone production has been reported to be regulated at the level of aldosterone synthase (Basset et al., 2004). Therefore, further molecular mechanisms of regulation of aldosterone synthase on the mRNA level have to be investigated in the future. Furthermore, in this study the aldosterone synthase expression remained increased for at least 60 minutes after the angiotensin II injection but went back to baseline within a short period of time upon less specific activation of the HPA axis (e.g. sham injection, sodium load). In addition, 20 minutes after the normal saline load test a decrease in the aldosterone synthase expression could be observed, which was sustained up to 60 minutes later, whereas the aldosterone secretion showed a corresponding significant reduction only 60 minutes later. In the potassium supplementation group, the aldosterone synthase revealed an increased expression 1 day after the start of the experiment, but the later expression tended to vary, with concomitant reduction of the aldosterone secretion during the following days. Taken together, expression levels of aldosterone synthase display fast as well as sustained changes that are mostly parallel with aldosterone output. As such, regulation of aldosterone synthase on the mRNA level is likely to contribute to aldosterone output from the adrenal cortex.

4.4 ENU Screen

Random mutagenesis screens such as the one performed at the Helmholtz Center Munich have increased our knowledge of functional significance of randomly induced genetic mutations. In addition, it has provided a significant increase in the number of mouse models available to the scientific community. All F1 littermates derived from ENU treated male animals are subject to analysis for dominant mutations. At the Munich Mutagenesis Screen, in a screening of over 14000 mice for a large number of clinically relevant parameters, 182 mouse mutants for a variety of phenotypes have been recovered (Hrabé de Angelis et al., 2000). In one study, over one hundred F1 mice were tested and out of these 3 mutant lines were established (~3%), (Mohan et al., 2008). In another study though, out of the 6400 F1 mice tested, only 3 mutated lines (0,04%) could be established (Reijmers et al., 2006). In our study so far, we have established 4 mutated lines from ~2800 F1 mice screened (0,14%) whilst another 5 mouse lines are still in the breeding process. Thus, relatively low proportions of retrieved lines might reflect the very strict criteria applied to select affected animals: All animals tested had to present with aldosterone values above 3 standard deviations of the mean of the wild type population. To further decrease the possibility of false positive cases through randomly elevated aldosterone the animals selected out of this process had to undergo a second aldosterone measurement. For this second screening, age dependent normal values from wild type controls had to be applied. After the second test, the number of mice adhering to all criteria was low, but the heritability test performed in those mice has so far proved that offspring from all candidate mutants preserved the pathological phenotype.
So far, in the F1 generation we could identify only female animals displaying the pathological phenotype. Although this observation could be due to chance, specific molecular reasons for this finding could be considered: It has recently been demonstrated that potassium channels from the TASK family are of particular importance for the regulation of aldosterone secretion. Specifically, targeted deletion of TASK1 (Heitzmann et al., 2008) or TASK1 and TASK3 (Davies et al., 2008) resulted in a phenotype of primary aldosteronism. Interestingly, only female animals displayed this phenotype with concomitant zonation defects in the adrenal cortex. This phenotype was reversible upon testosterone treatment. Taken together, these data provide an example of specific gender related mechanisms that could also play a role in the gender distribution observed in our initial ENU screen. Another explanation for this fact could be that male animals with the same altered genetic background express a more severe clinical phenotype and die before reaching the age required for the screening process. One possible mechanism for this could be the gender dependent physiological response to aldosterone which has been reported to be greater in males than in females (Morris et al.,

1973). In addition, females showed a faster clearance rate of aldosterone and its metabolites from the plasma (Morris et al., 1976).

Interestingly, the four established lines displayed a different pattern of inheritance of the pathological trait. In line I and II, all affected animals were male, while in line III the affected male to female ratio was ~ 1:2, and in line IV the ratio was 1:1. As all established lines were derived from different ENU treated founder males, distinct genetic mutations are also likely to contribute to the observed gender ratio. Although the limited number of screened animals still includes the possibility of skewed gender ratio by chance, distribution in line I and II is consistent with an X linked trait, while for line III and IV an autosomal locus is likely to cause this phenotype. Future linkage analysis will be necessary to further determine the causative mutations.

Overall, persistent elevation of aldosterone levels in affected animals could have been caused by autonomous aldosterone secretion by the adrenal cortex (primary aldosteronism) or through renin dependent mechanisms (secondary aldosteronism). Thus, to further define the cause of persistent hyperaldosteronism in the affected animals, measurement of renin activity had to be performed. During the initial phenotypical characterization of the affected animals, primary aldosteronism as the cause of the phenotype could be confirmed: The affected animals showed not only high aldosterone levels but also reduced renin activity in comparison to their wild type littermates. The aldosterone to renin ratio is considered the gold standard method in differential diagnosis between primary aldosteronism and secondary forms of hyperaldosteronism (Funder et al., 2008). As the aldosterone to renin ratio was significantly higher in affected mice, this finding strongly suggests the presence of primary aldosteronism. Moreover, as expected in the setting of primary aldosteronism, significantly lower potassium levels were detectable in affected animals. These findings together with normal kidney values excluded a primary kidney defect as the cause of high aldosterone values. Consistent with the adrenal being the source of high aldosterone output, in all four established lines high expression levels of aldosterone synthase in the adrenals of affected animals were evident with a 6-7 fold increase in comparison to unaffected littermates. Theoretically, according to the criteria applied in the screening process, it would have been expected to identify also animals with hyperaldosteronism due to secondary causes. One potential explanation for the screening result whereby only animals with primary aldosteronism were defined could be that diseases causing secondary hyperaldosteronism are combined with other co-morbidities such as renal failure and subsequently resulting in a lethal phenotype. Moreover, the strict screening parameters with sustained high aldosterone levels above 3 standard deviations of gender and age matched normal controls might have lead to identification of animals with primary aldosteronism which is usually characterized by higher aldosterone values in comparison to secondary causes.

The cardiac phenotype of affected animals also displayed increased collagen deposits in the heart, as it is also observed in human subjects suffering from primary aldosteronism (Pessina et al., 1997). However, it has been shown that cardiac fibrosis cannot be caused by a chronic increase in plasma aldosterone levels alone, and that a synergistic effect of salt, aldosterone and other confounding factors is responsible for this phenotype (Wang et al., 2004; Köhler et al., 1996). Future phenotypic characterization including measurement and monitoring blood pressure in affected vs. unaffected animals will be necessary to further define the cardiovascular phenotype in the different mouse lines.

4.5 Perspectives

As described above, we could identify a number of female mice with chronically elevated aldosterone levels upon continuous measurements which have been or will serve as founder animals for further breeding studies. However, individual lines of animals have to fulfill several functional standards before detailed genomic characterization can be considered. Morphological analysis might reveal zona glomerulosa hypertrophy or the presence of adrenal nodules/tumors. *In vivo,* autonomy of aldosterone secretion has to be further substantiated by sodium load and fludrocortisone treatment. Moreover, ACTH or ANGII responsiveness of aldosterone secretion (as utilized in the clinical setting with the upright posture test) would give indirect evidence on autonomous aldosterone secretion due to an adenoma or bilateral hypertrophy. Finally, unilateral vs. bilateral overproduction of aldosterone has to be evaluated by comparison of aldosterone output from individual adrenals (e.g. as *in vitro* organ cultures) and expression levels of aldosterone synthase.

As the identified mouse lines most likely represent mutations in different genetic loci crossbreeding strategies between the lines will help to define the pathophysiological relevance of modulating effects or interdependency of individual mutations. Although detailed molecular analysis of adrenals from affected animals might help to pinpoint a pathway with potential impact in dysregulated aldosterone secretion in the individual mouse line, the specific underlying genetic cause is unlikely to be determined solely on the basis of phenotypic techniques. Thus, a genetic approach will be required for the identification of the mutation induced by the initial chemical mutagenesis. For backcross mapping/sequencing and phenotypic characterization of mutant mouse lines a technology platform based on SNP markers and MALDI-TOF mass spectrometry has been implemented at the Helmholtz Center Munich, which allows chromosomal mapping in a high-throughput manner. Identification of genes involved in autonomous aldosterone secretion opens the possibility to explore the potential impact of these pre-defined candidate genes in human disease. Finally, these mouse models can be used for pharmacological intervention studies. Comparison of therapeutic effects in different mouse lines of hyperaldosteronism might provide indirect evidence of genetic modifiers of treatment response and outline pharmacogenetic prospects for future clinical intervention trials.

5. Summary

According to recent epidemiological studies, primary aldosteronism is considered to be responsible for almost 10-15% of all cases of arterial hypertension. The genetic background of this common disease, however, has been elucidated only for the rare familial types whereas in the large majority of sporadic cases genetic causes or modifiers still remain unclear. In an attempt to define novel genetic mechanisms of hyperaldosteronism we utilized a random mutagenesis screen after treatment with the alkylating agent N-ethylnitrosourea (ENU) and phenotypically characterized affected mice for their blood aldosterone levels. As the detection method we used a time resolved fluorescent immunoassay which allows the measurement of aldosterone in very small murine plasma volumes. Using this assay we determined the normal aldosterone values for C3HeB/FeJ wild type mice under baseline conditions and following specific stimulation and suppression tests. We could demonstrate the expected increase in aldosterone response upon ACTH stimulation, a high potassium diet and an angiotensin II injection, as well as the decrease after a dexamethasone suppression test, a normal saline load test and a fludrocortisone suppression test.

On the molecular level, the expression of aldosterone synthase showed a similar pattern with a fast response to the investigated stimuli. These tests should later be applied to the mouse lines derived from the ENU screen, in order to investigate potential abnormal response to these stimuli in comparison to wild type animals. Furthermore, aldosterone measurement was carried out in more than 2000 F1 offspring (of both genders) of chemically mutated inbred C3HeB/FeJ mice. From these tested F1 offspring, aldosterone levels were consistently elevated (defined as levels above 3 SD over the mean of untreated animals) upon repeated measurement in 9 animals (8 females and 1 male). Further breeding of affected female animals gave rise to F2 pedigrees from which four established lines displayed high aldosterone values. These animals served for a detailed phenotypic characterization and showed an increased aldosterone to renin ratio, low potassium values and normal renal function in line with the presence of primary aldosteronism. In addition, the investigation of their cardiac phenotype showed increased collagen deposits and subsequently cardiac fibrosis, as also observed in patients suffering from primary aldosteronism. In the future, genetic SNP analysis can be performed to identify underlying genetic loci, responsible for this trait. Taken together, these data demonstrate the feasibility of a phenotype-driven mutagenesis screen to detect and establish mutant mouse lines with a high aldosterone phenotype.

Zusammenfassung

Neueren epidemiologischen Daten zufolge stellt der primäre Hyperaldosteronismus in 10% aller Fälle die Ursache arterieller Hypertonie dar. Die genetischen Ursachen dieser Erkrankung sind dabei nur für die seltenen Formen des familiären Hyperaldosteronismus näher untersucht, während für die weitaus häufigeren sporadischen Fälle solche Untersuchungen fehlen. Zur Aufklärung neuer genetischer Mechanismen etablierten wir einen Mutagenese-Screen nach Behandlung mit N-Ethylnitrosurea (ENU) und charakterisierten Mäuse phänotypisch bezüglich ihrer Aldosteron Werte. Mit Hilfe eines Fluoreszenz-Immunoassays, der die Messung von Aldosteron in sehr kleinen Plasma-Volumina ermöglichte, bestimmten wir zunächst den Aldosteron-Normbereich für C3HeB/FeJ Wildtyp Mäuse unter basalen Bedingungen und unter spezifischen Stimulations- und Suppressionsexperimenten. Hierbei konnten wir sowohl den erwarteten Aldosteron Anstieg nach ACTH-Stimulation, Angiotensin II Stimulation und einer Hochkalium-Diät nachweisen, als auch die Reduktion des Aldosteron-Spiegels nach Dexamethason-Hemmtest, Kochsalzbelastungstest und Fludrocortison Suppressionstest.

Auf molekularer Ebene erfolgte eine rasche Regulation der Aldosteron Synthase Expression nach dem gleichen Muster in Abhängigkeit von den jeweiligen Stimuli. Die so etablierten Tests sollen des Weiteren für die Charakterisierung der durch den ENU Screen erhaltenen Mauslinien Verwendung finden, um mögliche pathologische Antworten ENU behandelter Mäuse im Vergleich zu Wildtyp Mäusen anzuzeigen. Darüber hinaus wurden Aldosteron-Messungen bei mehr als 2000 Nachkommen innerhalb der F1-Generation ENU-behandelter C3HeB/FeJ Inzuchtmäuse durchgeführt. Unter diesen F1-Nachkommen konnten nach wiederholten Messungen bei 9 Tieren (8 Weibchen und 1 Männchen) konsistent erhöhte Aldosteronwerte festgestellt werden (definiert als Aldosteron-Wert drei Standardabweichungen über dem Mittelwert unbehandelter Mäuse). Weitere Züchtung auffälliger Weibchen führte zu F2-Nachkommen, von denen 4 etablierte Mauslinien hohe Aldosteron Spiegel aufwiesen. Diese Tiere wurden einer detaillierten phänotypischen Charakterisierung unterzogen und wiesen hierbei erhöhte Aldosteron/Renin Quotienten, erniedrigtes Serum Kalium bei unauffälliger Nierenfunktion, entsprechend der klinischen Symptomatik des primären Hyperaldosteronismus auf. Darüber hinaus zeigte die Untersuchung des Herzgewebes erhöhte Kollagenablagerungen und nachfolgende kardiale Fibrosen, wie sonst in Patienten mit primärem Hyperaldosteronismus beobachtet werden kann. Für die Zukunft sind nun genetische SNP Analysen geplant, die zur Identifizierung der involvierten genetischen loci führen sollen. Zusammenfassend demonstrieren die präsentierten Daten den erfoglreichen Einsatz eines chemischen Mutagenese-Screens zur Generierung mutierter Mauslinien mit primärem Hyperaldosteronismus.

6. References

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7. Appendix

7.1 Abbreviations

ACTH	Adrenocorticotropic Hormone
mRNA	Messenger Ribonucleic Acid
PRA	Plasma Renin Activity
ENU	Ethylnitrosourea
RAA	Renin Angiotensin Aldosterone
GFR	Glomerular Filtration Rate
ACE	Angiotensin Converting Enzyme
ADH	Antidiuretic Hormone
StAR	Steroidogenic Acute Regulatory protein
scc	Side chain cleavage enzyme
HSD	Hydroxysteroid Dehydrogenase
et al.	et alii
MAPK	Mitogen Activated Protein Kinase
ECG	Electrocardiogram
l	litre
DNA	Desoxyribonucleic Acid
SNP	Single Nucleotide Polymorphism
h	hour
ng	nanogram
pg	picogram
ml	millilitre
µl	microlitre
EDTA	Ethylenediaminetetraacetic Acid
DCM	Dichloromethane
PEG	Polyethylene Glycol
V	Volt
nm	nanometre
min	minute
PCR	Polymerase Chain Reaction
TASK	TWIK-related Acid-Sensitive K^+
CaMK	Calmodulin Kinase
SD	Standard Deviation
SEM	Standard Error of the Mean

7.2 Acknowledgments

I would like to express my sincere gratitude to my supervisor Prof. Dr. med. Felix Beuschlein for his constant support and encouragement. Thank you for having faith in me, for your endless patience, for sharing your knowledge with me and teaching me so much over the past two years.

I am deeply indebted to Prof. Dr. med. Martin Reincke for giving me the opportunity to carry out my thesis in the Medizinische Klinik Innenstadt and for his precious advice and support.

I would also like to acknowledge Sibylle Wagner for the perfect organisation of our cooperation and the excellent communication we had. Thanks for answering all my questions and for your everyday support.

I have to say a huge thank you to Jenny not only for developing and sharing the aldosterone assay, without it the whole project would never take place, but also for being such a supportive and patient friend.

I would also like to say a big thank you to Conny not only for her assistance and useful advice in the lab and with the animal experiments but also for the long discussions, the confessions and the plans we have made together.

Thanks to Martin Bidlingmaier for his valuable comments and advice, but also for providing me with the necessary facilities in the lab.

I am also thankful to Prof. Dr. Hrabé de Angelis for giving me the opportunity to take part in the ENU mutagenesis screen and for the excellent cooperation.

Thanks a lot to Brigitte, my great lab-mum who always had a solution for my problems and encouraged me everyday and to everyone else in the lab for their support and especially Inga, Rita, Juliane and Sarina.

Last but not least, I would like to say a big thank you to my special ones, my mother, my brother and Dimitris for being there for me.

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PUBLICATION

A. Spyroglou, J. Manolopoulou, S. Wagner, M. Bidlingmaier, M. Reincke, F. Beuschlein: Short term regulation of aldosterone secretion after stimulation and suppression experiments in mice. Journal of Molecular Endocrinology (2009) 42, 407-413