Utilization of a Mutagenesis Screen to Generate Mouse Models of Hyperaldosteronism

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Primary aldosteronism is considered to be responsible for almost 10% 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, underlying mechanisms still remain unclear. In an attempt to define novel genetic loci involved in the pathophysiology of primary aldosteronism, a mutagenesis screen after treatment of mice with the alkylating agent *N*-ethyl-*N*-nitrosourea was established for the parameter aldosterone. As the detection method we used a time-resolved fluorescence immunoassay that allows the measurement of aldosterone in very small murine sample volumes. Based on this assay, we first determined the normal aldosterone values for wild-type C3HeB/FeJ mice under baseline conditions [92 $\pm$ 6 pg/ml for females (n = 69) and 173 $\pm$ 16 pg/ml for males (n = 55)]. Subsequently, aldosterone measurement was carried out in more than 2800 F_1 offspring of chemically mutagenized C3HeB/FeJ mice, and values were compared with aldosterone levels from untreated animals. Persistent hyperaldosteronism (defined as levels +3 SD above the mean of untreated animals) upon repeated measurements was present in seven female and two male F_1 offspring. Further breeding of these founders gave rise to F_2 pedigrees from which eight lines with different patterns of inheritance of hyperaldosteronism could be established. These animals will serve for detailed phenotypic and genetic characterization in the future. Taken together, our data demonstrate the feasibility of a phenotype-driven mutagenesis screen to detect and establish mutant mouse lines with a phenotype of chronic hyperaldosteronism. **(*Endocrinology* 152: 326–331, 2011)**

Arterial hypertension represents one of the most common diseases in developed countries. According to recent epidemiological studies, about 20% of the adult population is diagnosed with any form of increased blood pressure (1). The most frequent cause of secondary arterial hypertension is primary aldosteronism. Primary aldosteronism develops from hypersecretion of the mineralocorticoid aldosterone in which aldosterone production is inappropriately high, relatively autonomous from angiotensin II stimulation and, thus, nonsuppressible by sodium load. The large majority of primary aldosteronism is caused either by an aldosterone-producing adrenal adenoma or by bilateral adrenal hyperplasia, with two thirds of patients being diagnosed with bilateral and one third with unilateral disease (2, 3). Despite its high prevalence, so far, the genetic causes of

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1081 Received September 16, 2010. Accepted October 8, 2010.
First Published Online November 10, 2010

Abbreviation: ENU, *N*-ethyl-*N*-nitrosourea; TASK, TWIK-related acid-sensitive K^+ channel (KCNK3-potassium channel, subfamily K, member 3).

primary aldosteronism have been elucidated only in rare familial forms of the disease.

Animal models can provide important information on the physiology of aldosterone regulation and on the pathophysiology of autonomous aldosterone secretion. Furthermore, these models can also be used to investigate cardiovascular and metabolic consequences of unopposed aldosterone secretion and potential restoration of these parameters through pharmacological interventions. So far, however, only a few mouse models have been described with a phenotype similar to that of primary aldosteronism. A mouse with TWIK-related acid-sensitive K^+ channel (KCNK3-potassium channel, subfamily K, member 3) (TASK) channel deletion (4, 5) and a circadian clock-deficient cry-null mouse model showing an up-regulation of adrenal *Hsd3b6* expression (6) are such examples.

An approach to develop new mouse models for a specific phenotype is to induce genetic variation by random mutagenesis of the mouse genome using *N*-ethyl-*N*-nitrosourea (ENU) as mutagen (7, 8). ENU is an alkylating agent that causes ethylation of nucleic acids, which ultimately results in point mutations. Specifically, ENU exerts mutagenic action on DNA of premeiotic spermatogonial stem cells (9, 10) *i.e.* A-T base pair substitutions and/or small intragenic lesions (11–13). Many of the mutant offspring derived from 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 (14, 15). This allows the production of a large number of F_1 founder animals from a single treated male, minimizing the number of animals required and the handling of ENU. As assessed by the specific-locus test (9, 16) 1 in 1000 F_1 animals is heterozygous for a functionally relevant mutation of interest, and good quantitative screening tools are required to detect what can be a fairly subtle effect (17). Several studies have demonstrated that the number of induced mutations depends on the dosage of ENU administered (18, 19) and on the number of exons and length of coding sequences (20). In the case of phenotype-driven genetics, the genomic association is accomplished using common single nucleotide polymorphisms.

In this study we tested whether a dominant ENU-induced mutagenesis screen can be used to generate new models of hyperaldosteronism, and we describe the identification of eight mouse lines with inheritable hyperaldosteronism.

Materials and Methods

Animals and housing conditions

All animal studies were performed according to protocols examined and approved by the Regierung von Oberbayern and according to the German Animal Protection Law. Mice were kept in a specific pathogen-free animal facility area at an ambient temperature of 22 ± 2 C (relative humidity 60 ± 5%) on a 12-h light, 12-h dark circle. The animals were fed with chow no. 1314 (Altromin, Lage, Germany) *ad libitum* with free access to tap water. C3HeB/FeJ mice (The Jackson Laboratory, Bar Harbor, ME) involved in the experiments were maintained in groups of three individuals per cage.

ENU injection and initial breeding

In the Munich ENU project, which takes place at the Institute of Experimental Genetics of the Helmholtz Center Munich, C3HeB/FeJ male mice were treated with three weekly injections of 90 mg/kg body weight ENU at approximately 10–14 wk of age. Upon injection individual sterility periods of several weeks to months were expected, and around 50% of all treated males did not regain fertility after treatment. Only F_1 litters deriving from ENU-treated males that underwent a minimal sterility period of 100 d were chosen for phenotyping to ensure that they were generated with mutagenic treatment-derived sperms.

Blood sampling

The collection of blood samples had to take place under stress-free conditions. To avoid any influence of the examined parameters through hypothalamic-pituitary-adrenal axis activation, handling of the animals until blood sampling was kept to an interval of less than 1 min. Blood sampling took place between 0800 h and 1100 h for all animals. After effective ether anesthesia, the retro-orbital vein plexus of the mouse was punctured with a glass capillary and from each animal 0.25 ml of blood was collected in Li-heparin coated tubes to avoid coagulation. After centrifugation (10,000 × *g*, 10 min) plasma was separated and kept at −20 C until measurement.

Aldosterone measurement

Aldosterone was determined with an in-house, time-resolved fluorescent-immunoassay as described in detail elsewhere (21).

Statistical analysis

For a dominant trait of inheritance, offspring of mutants and wild-type animals should be affected in 50% of cases, which is defined as 100% phenotypic penetrance. Thus, the observed penetrance in individual mouse lines was calculated by number of affected animals/(all animals/2) and expressed as percent (22). Statistical analysis was carried out with the Prism 3.02 (GraphPad Software, La Jolla, CA). All results are expressed as mean ± SEM. Statistical significance was determined using the Mann-Whitney test. Statistical significance was defined as $P < 0.05$ indicated by *asterisks* in the figures.

Results

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 is used in general for the Munich ENU mutagenesis screen. Measurement of baseline aldosterone levels in 12-wk-old male (n = 55) and female (n = 69) mice revealed baseline al-

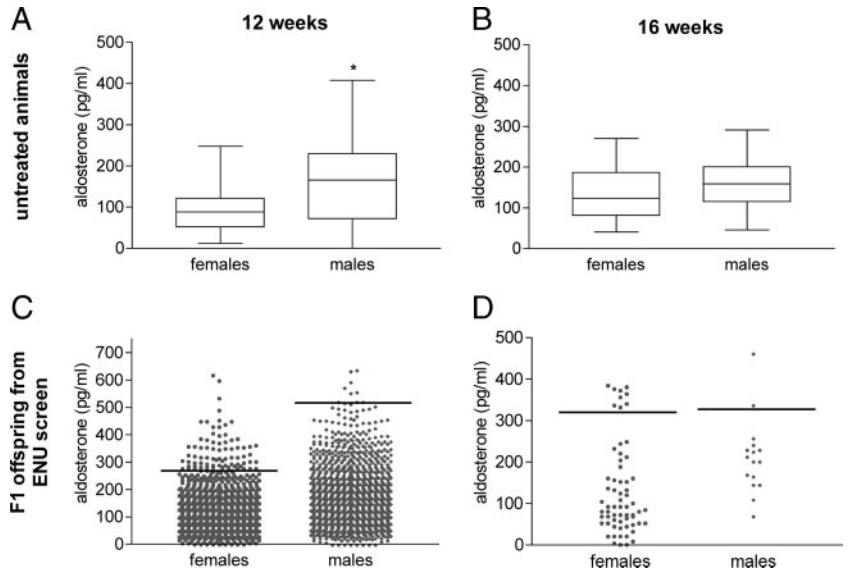

FIG. 1. Range of aldosterone values (mean ± 3 SDs) in wild-type animals at 12 wk (A) and 16 wk (B) of age (females, n = 69; males, n = 55). Aldosterone values of F_1 offspring of ENU-treated mice at 12 wk (females, n = 1414; males, n = 1450; panel C) and at the age of 16 wk (females, n = 65; males, n = 18; panel D), respectively. *Star* denotes significant differences of aldosterone levels between the genders. *Bars* represent cut-off levels defined as 3 SDs above the mean of gender- and age-matched controls. For conversion to SI units: pg/ml × 2.775 = pmol/liter.

dosterone values of 173 ± 16 pg/ml and 92 ± 6 pg/ml, respectively, which resulted in a statistically significant difference of aldosterone levels between the genders ($P <$ 0.001, Fig. 1A). 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 wk old. Therefore, aldosterone values under baseline conditions were also defined in 16-wk-old C3HeB/FeJ animals. In comparison with the 12-wk-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 (136 ± 11 pg/ml *vs.* 92 ± 6 pg/ml; $P < 0.001$, Fig. 1B).

Initial screen and distribution of cohorts

For the mutagenesis screen for the phenotype hyperaldosteronism, all F_1 offspring from ENU-treated males and wild-type females were checked for their aldosterone levels at the age of 12 wk. The aldosterone values received were then compared with 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 SDs of the aldosterone values of the wild-type population. According to this definition, for 12-wk-old mice the upper normal limit was set at 518 pg/ml for males and at 251 pg/ml for females, respectively. The offspring tested presented with a wide range of aldosterone values. Many more females were characterized by abnormal values in comparison with male animals. All animals that showed once pathological aldosterone levels were retested at the age of 16 wk. In the initial screen 2864 mice took part: 1450 male and 1414 female animals. Of this cohort 83 animals initially displayed high aldosterone levels (18 males and 65 females; Fig. 1C). After the confirmation measurement only 11 mice had sustained high aldosterone values (two male and nine female; Fig. 1D). Two of the female mice with abnormal aldosterone levels died, due to undefined reasons, before further breeding could take place. All remaining animals were mated to wild-type animals of the opposite gender. From this breeding eight lines with affected offspring could be generated. Breeding of the ninth animal gave rise only to unaffected offspring, and this mouse line was thus excluded from further phenotypical characterization. Taken together, of all offspring tested for the phenotype hyperaldosteronism, 2.9% showed initially high aldosterone values of which 13.2% of these had sustained hyperaldosteronism. Of these, 81.8% were females and only 18.2% were males. In total, 0.38% of all mice screened had confirmed hyperaldosteronism.

Pattern of inheritance and phenotypical penetrance of established lines

Further breeding of the established lines by mating affected F_2 animals to wild-type mice of the opposite gender provided us with the opportunity to gather first evidence for possible patterns of inheritance. In all but two established pedigrees, the founder was a female animal, which originated in each case from a different ENU-treated male parent. Thus, the presence of different mutations as the genetic cause of the common phenotype, hyperaldosteronism, is highly likely. Accordingly, different patterns of inheritance between individual pedigrees were observed (Fig. 2 and Table 1): In line I, male and female animals were both affected in approximately 35% of cases. In line III the pathological phenotype was present in both male and female mice in 27% and 22% of animals, respectively. Additionally, the total male to female ratio was 1.5:1. In line V only male animals displayed the pathological phenotype with hyperaldosteronism at a rate of 38%. Another discrepancy observed in this mouse line was the total male to female ratio, which was found to be 4:3. From line

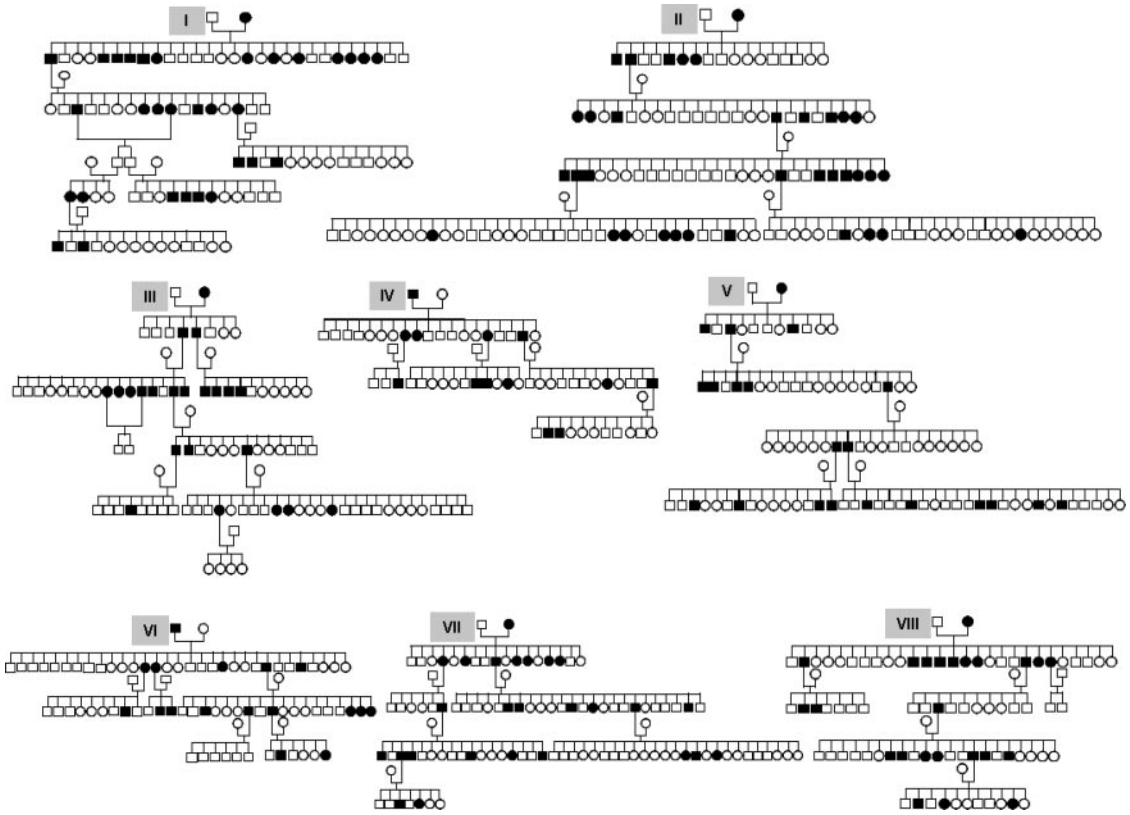

FIG. 2. Pedigrees with hyperaldosteronism generated from the ENU screen. *Black symbols* denote affected animals.

VIII 30% of the males and 28% of the females showed hyperaldosteronism. However, in this mouse line the total male to female ratio was 2:1, potentially due to excess mortality in female offspring.

A penetrance of above 50% in general is considered as suitable for an ENU screen (22); on this basis in our cohort, all but one mouse lines showed a suitable penetrance in either one or both genders (Table 1). Considering the fact that penetrance can also vary with increasing age, further aldosterone measurements in older animals (20 wk old) revealed a tendency to higher penetrance with increasing age (data not shown). In mouse line IV, where penetrance is 40–45%, in females and males, respectively, breeding over two generations showed so far preservation of the abnormal phenotype. In all eight lines, the numbers of animals exhibiting plasma aldosterone levels above the cut-off level were significantly (chi-square-test, $P < 0.001$) higher than the expected 0.3% of the C3H mouse population (using as cut-off: mean ± 3 SD).

Discussion

In the present study we defined the normal aldosterone values in male and female mice of the strain C3HeB/FeJ. We were able to demonstrate a significant difference in aldosterone values between genders with aldosterone levels in male mice being higher than those of females of the

TABLE 1. Percentages of affected male and female mice and their respective penetrances

Mouse line	Males affected	Females affected	Penetrance males	Penetrance females
I	34.9% (15/43)	34.7% (16/46)	70%	70%
II	25.4% (16/63)	28.1% (18/64)	51%	56%
III	27.5% (14/51)	21.9% (7/32)	55%	44%
IV	22.6% (7/31)	20.0% (5/25)	45%	40%
V	38.3% (23/60)	0.0% (0/46)	77%	0%
VI	20.0% (9/45)	25.0% (7/28)	40%	50%
VII	26.9% (14/52)	25.0% (11/44)	54%	50%
VIII	30.6% (15/49)	27.6% (8/29)	62%	55%

Phenotypic penetrance (100%) is defined in the case of the appearance of 50% offspring exhibiting hyperaldosteronism after mating phenotypic mutants to wild-type mice. The observed penetrance in individual mouse lines was calculated by number of affected animals: (all animals: 2) and expressed as percent (22).

same age. Gender-dependent differences in aldosterone levels are suggested to be influenced by other hormones with modulating effects on the renin-angiotensin-aldosterone system. Progesterone presents one putative candidate, which, through its weak mineralocorticoid activity, might contribute to lowering aldosterone secretion. So far, however, only conflicting data have been reported on the role of progesterone as negative feedback on aldosterone output *in vivo* (23). Thus, it remains unclear to date whether gender-dependent differences in steroid secretion could be causative for the observed lower aldosterone levels in female animals. Interestingly, another study has reported lower aldosterone values in female C57BL/6xNMRI mice but without significant difference in comparison with males (21). These observations, thus, provide indirect evidence for the presence of strain-dependent genetic modulators that can affect gender-related differences in aldosterone regulation. We further observed a significant age-dependent variation of aldosterone values in C3HeB/FeJ. As reported earlier (21), there are differences in the mean aldosterone values in younger animals from 3–11 wk of age. Consistently, evaluation of adrenal zonation displays an evolving pattern during puberty (24). So far, variation of aldosterone values in adult mice has not been reported. Here we demonstrate that aldosterone values in 16-wk-old male mice are overall lower than those in 12-wk-old animals. Interestingly, in female mice the opposite phenomenon could be observed. 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 consistent with reduced activities of biosynthetic enzymes, adenyl cyclase, and L-type calcium channels and the expression of $P450_{scc}$ enzyme in zona glomerulosa cells (25). Overall, these observations demonstrate that both gender- and age-related differences in aldosterone output must be taken into account for interpretation of aldosterone levels in rodent animal models.

Mutagenesis screens 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 for various phenotypes. In the Munich Mutagenesis Project, after screening of more than 14,000 mice for a large number of clinically relevant parameters, 182 mouse mutants with a variety of phenotypes have been recovered (26). Whereas in one study, three mutant lines of 100 tested F_1 (~3%) were established (27), during other screens, only three mutated lines (0.04%) could be maintained from 6400 F_1 mice tested (28). In our study we have established eight mutated lines from 2864 F_1 animals screened. The resulting proportion of 0.28% is, thus, within the lower expected range of retrieved mutants and might reflect the very strict selection criteria applied: all animals tested had to present with aldosterone values above 3 SDs 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. After the second test, the number of mice adhering to all predefined criteria was low, but the heritability test performed in those mice has so far proved that offspring from all but one candidate mutant preserved the pathological phenotype.

So far, in the F_1 generation we could identify significantly more female animals displaying the pathological phenotype. Although this observation could be due to chance, specific molecular reasons for this finding could also 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 (4) or TASK1 and TASK3 (5) resulted in a phenotype of primary aldosteronism. Interestingly, only female animals displayed this phenotype with concomitant zonation defects in the adrenal cortex, which was reversible upon testosterone treatment. Thus, 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. Interestingly, in our screen the eight established lines displayed a different pattern of inheritance of the pathological trait. In line V only male mice showed the pathological phenotype, whereas in line I the affected male to female ratio was 1:1. Additionally, in line VIII, the total male to female ratio was 2:1. Because all established lines 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 V is consistent with an X-linked trait, whereas for most of the other lines an autosomal locus is likely to cause this phenotype. Future linkage analysis will be necessary to determine the causative mutations.

Taken together, in this study we have demonstrated the utility of a phenotype-driven mutagenesis screen for the identification of mouse lines with chronic hyperaldosteronism. These mouse lines can further serve not only for genetic characterization and identification of involved mutations but also as *in vivo* models for intervention studies.

Acknowledgments

We thank Brigitte Mauracher and Igor Shapiro (Department of Medicine, Endocrine Research, University Hospital Innenstadt,

Ludwig Maximilians University) and Elfi Holupirek and Sandra Hoffmann (Institute of Experimental Genetics, Helmholtz Center Munich) for excellent technical assistance.

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This work was supported by Deutsche Forschungsgemeinschaft Grants BE2177/8-1 (to F.B) and HR12/3-1(to M.H.), and in part by Grant 01GS0851 from the German National Genome Network (to E.W.).

Disclosure Summary: The authors have nothing to disclose.

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