Mineralocorticoid Receptor Mutations and a Severe Recessive Pseudohypoaldosteronism Type 1

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ABSTRACT

Pseudohypoaldosteronism type 1 (PHA1) is a rare genetic disease of mineralocorticoid resistance characterized by salt wasting and failure to thrive in infancy. Here we describe the first case of a newborn with severe recessive PHA1 caused by two heterozygous mutations in NR3C2, gene coding for the mineralocorticoid receptor (MR). Independent segregation of the mutations occurred in the family, with p.Ser166X being transmitted from the affected father and p.Trp806X from the asymptomatic mother. Whereas the truncated MR166X protein was degraded, MR806X was expressed both at the mRNA and protein level. Functional studies demonstrated that despite its inability to bind aldosterone, MR806X had partial ligand-independent transcriptional activity. Partial nuclear localization of MR806X in the absence of hormone was identified as a prerequisite to initiate transcription. This exceptional case broadens the spectrum of clinical phenotypes of PHA1 and demonstrates that minimal residual activity of MR is compatible with life. It also suggests that rare hypomorphic NR3C2 alleles may be more common than expected from the prevalence of detected PHA1 cases. This might prove relevant for patient’s care in neonatal salt losing disorders and may affect renal salt handling and blood pressure in the general population.


Type 1 pseudohypoaldosteronism (PHA1) is a rare genetic disease of mineralocorticoid resistance. Patients present with neonatal salt wasting and failure to thrive, associated with hyponatremia, hyperkalemia, and metabolic acidosis, despite extremely high levels of plasma renin and aldosterone. Generalized and renal forms of the disease have been described, these are distinguished at the clinical and genetic level. Infants with generalized PHA1 present multi-organ salt loss from the kidney, colon, sweat and salivary glands. This severe recessive form of PHA1 is caused by homozygous or compound heterozygous loss of function mutations in genes encoding the three subunits of the epithelial sodium channel ENaC. Renal PHA1 is a mild, autosomal dominant form of mineralocorticoid resistance, with a phenotypic expression restricted to the kidney. Patients can be asymptomatic or pres-
ent a salt-losing syndrome in the neonatal period; systematically, symptoms improve with age. To date, all cases of familial or sporadic renal PHA1 have been associated with heterozygous inactivating mutations in the NR3C2 gene coding for the mineralocorticoid receptor (MR) highlighting the importance of aldosterone and the MR for the maintenance of fluid and electrolyte homeostasis.

Here we report a unique pedigree, in which the index case (case 00_01) was referred at age 2 and 3 mo with failure to thrive. He was the second child of a nonconsanguineous family and was born at full term from an uneventful pregnancy with normal birth weight (3970 g, +1.1 SD Score (SDS)) and birth length (53 cm, +0.8 SDS). Biochemical examination showed hyponatremia (133 mmol/L) and hyperkalemia (5.7 mmol/L). As rapid weight gain followed without treatment on both occasions, the child was discharged without further evaluation. At the age of 9 mo, he represented with failure to thrive (weight from –1 SDS to –3 SDS), dehydration and malnutrition (Body Mass Index –4 SDS, skinfold, sunken eyes and decreased muscle mass). Biochemical measures were highly suggestive of PHA1 (Table 1) and the diagnosis was confirmed by genetic testing of the NR3C2 gene, which showed a frameshift mutation in exon 2 (c.497_498delCT) leading to replacement of Ser166 by a stop codon (p.Ser166X) (Figure 1B). The infant was treated with salt supplementation (5 mEq/kg/d) and ion exchange resins, with rapid remission of symptoms. Currently, at the age of 4 yr, clinical and biochemical assessments are normal with a salt supplementation of 2 g/d.

Two years later, his younger sister (patient 00_04) presented with an extremely severe phenotype (Table 1). She was born at full term with normal birth weight (3540 g, –0.1 SDS) and length (51 cm, –0.2 SDS). At day 5, her weight was 3240 g and she showed moderate hyperkalemia (5.8 mmol/L) and increased urinary sodium excretion (52 mmol/L). At the age of 8 d, she developed vomiting and severe dehydration with weakness and hypotonia. Her weight was 3060 g and she had profound hyponatremia, very marked hyperkalemia with altered ECG (peaked T waves and widening of the QRS complex), metabolic acidosis (alkaline reserve 13 mmol/L) and increased urinary sodium excretion (112 mmol/L) (Table 1). Plasma aldosterone and renin levels were extremely elevated, despite salt supplementation and ion exchange resins (Table 1). Normalization of electrolyte balance was particularly challenging because of unusually high salt needs: 30 mEq/kg/d given by nasogastric tube then by percutaneous endoscopic gastrostomy. Addition of indomethacin and fludrocortisone was unable to reduce her salt requirement. Currently, at 2 yr of age, 31 mEq/kg/d of sodium chloride is required to achieve a normal salt balance and the child has growth retardation (–1.9 SDS) and moderate psychomotor delay.

Although asymptomatic, the father (patient 01_10) and an older sister (patient 00_03) reported increased salt appetite, and the father had a history of poor weight gain during infancy. The family was screened for the p.Ser166X mutation, which was transmitted from the father (01_10) to the index-case (00_01) and the younger sister (00_04), while the mother (01–20), the half-brother (00_02), and the older sister (00_03) had a wild-type NR3C2 sequence at this position (Figure 1A). The parents then elected to participate in the clinical research protocol “PHACARV—Cardiovascular Evaluation of Adult PHA1 Patients” (PHRC P070139, NCT00646828). It appeared that both the father and the mother, who did not carry the p.Ser166X mutation, had a phenotype suggesting PHA1, i.e. increased plasma aldosterone levels with normal or low mean 24h blood pressure (BP) on ambulatory 24h monitoring and normal potassium levels (Table 1). This unexpected phenotype prompted us to sequence the entire NR3C2 coding sequence and intron-exon junctions in patient 01_20. A nonsense mutation was identified in exon 6 (c.2418G>A/p.Trp806X). Genetic screening for this second mutation in the pedigree revealed its transmission from the mother to the asymptomatic

Figure 1. Identification of NR3C2 mutations. A. Pedigree of the PHA1 family explored in this study. The index case is indicated by an arrow. B. Results of NR3C2 sequencing on lymphocytes’ DNA, showing the two nonsense mutations found in the different family members.
sister (00_03) and to the younger sister (00_04) who was thus
compound heterozygous for both familial nonsense mutations
(Figure 1B).

Given the severity of the phenotype and the therapeutic
difficulty encountered, we estimated the residual MR function
associated with the two NR3C2 mutations. Both alleles were
expressed in lymphocytes from patients 01_10, 01_20 and
00_04, as demonstrated by the presence of both nonsense mu-
tations on cDNA amplification products of exon 2 and exon 6
in the absence of emetine (Figure 2A), indicating that these
mutations did not induce Nonsense-Mediated mRNA De-
cay. We tested whether mutations p.Ser166X and p.Trp806X
induced MR protein degradation. Western blot analyses were
performed on protein extracts from renal RCSV3 cells tran-
siently transfected with mutant MR proteins. Compared with
MRWT transfected cells expressing a 110 kD product, an addi-
tional approximately 90 kD protein was detectable in cells
transfected with MR806X, corresponding to the expected size
for this truncated protein (Figure 2B). In contrast, no protein
was detected at approximately 20 kD, the expected size of
MR166X, indicating that this mutant is probably degraded by
the proteasome.

The MR is functionally organized in three domains, in-
volved in DNA binding, ligand binding and transcriptional
activation. As expected, truncation at the very beginning of
the MR ligand binding pocket completely abolished hor-
mone binding by MR806X (Figure 3A). The ability of MR806X to
activate transcription was assessed by transient transfection in
RCSV3, COS-7 and HEK293T cells. Aldosterone increased
transactivation by the MRWT in a dose-dependent manner
(ED50 of approximately 5 × 10^{-11}M; Figure 3B and Fig. S1A).
Surprisingly, MR806X was constitutively active in RCSV3 (Fig-
ure 3B) and COS-7 (Fig. S1A) cells, possessing approximately
25% and 45% of transcriptional activity observed with the
MRWT at 10^{-8}M aldosterone, respectively; transcriptional ac-
tivity was lower (approximately 7%) in HEK293T cells (Fig.
S1A). Similar results were observed in RCSV3 cells in the pres-
ence of cortisol, a physiologic MR ligand, with MR806X having
a constitutive activity of about approximately 25% of that ob-
erved with the MRWT in the presence of 10^{-7}M cortisol (Fig.
S1A).

The mechanisms whereby MR806X had acquired partial
ligand-independent transcriptional activity, despite the
complete loss of ligand binding and of the activation func-
tion (AF)-2 domain, required for ligand-dependent tran-
scriptional activation were investigated. First, the intracel-
ular localization of MR806X was studied in COS-7 cells
transiently transfected with eGFP_MRW\textsubscript{WT} or eGFP_MR806X.
In contrast to MR\textsubscript{WT}, which translocates from the cyto-
plasm to the nucleus in an aldosterone-dependent manner
within 30 min (Figure 3C), MR\textsubscript{806X} was both cytoplasmic and
nuclear in the absence of hormone, with no further modifica-
tion of intracellular localization upon hormone addition (Figure 3C).

The ability of MR\textsubscript{806X} to recruit tran-
scriptional coactivators was studied using a mammalian two

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<th>Table 1. Biological characteristics of members of the PHA1 family explored in this study</th>
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<td><strong>Normal values</strong></td>
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*Normal values refer to normal laboratory values for plasma aldosterone and plasma renin. To convert plasma renin from pmol/L to pg/ml, divide by 0.0237; to convert plasma aldosterone from pmol/L to ng/dl, divide by 0.0277. Biochemical data at diagnosis; BP = blood pressure; SBP = systolic BP; DBP = diastolic BP.
hybrid assay. Ligand-dependent recruitment of the steroid receptor coactivator 2 (SRC-2) was observed with the MRWT in all cell lines, whereas MR806X was unable to recruit SRC-2 even in the presence of aldosterone (Figure 3D and Fig. S1B). Thus, the partial nuclear localization of MR806X in the absence of hormone is a prerequisite for its ability to interact with DNA and activate transcription. Loss of the AF-2 domain17 diminishes the repertoire of transcriptional coactivators recruited to the MR806X; however, truncation of part of the ligand binding domain also eliminates some inhibitory functions associated with this domain, which are usually relieved by ligand binding, similar to what observed for the MR splice variant, MR

The functional consequences of the two mutations explain the phenotypic differences observed within the family, with carriers of the p.Ser166X mutation presenting clinical PHA1 due to haploinsufficiency, while carriers of p.Trp806X are asymptomatic. In patient 00_04, the combined MR defect leads to an extremely severe phenotype comparable to autosomal recessive generalized PHA1 due to ENaC mutations. In contrast to generalized PHA1, recessive PHA1 linked to MR mutations was not associated with airway disease6 or with cutaneous lesions due to excessive salt loss from sweat glands.19 Sweat test at the age of 3 yr was borderline (chloride concentration 55 mmol/L, with normal test <45 mmol/L, positive test >60 mmol/L), although salt supplementation may interfere with this test.20 Although MR is expressed in sweat glands,21 the absence of a cutaneous phenotype in patient 00_04 suggests that minimal MR function is sufficient to regulate sweat salt balance or alternatively, MR is dispensable for Na+ reabsorption in this tissue.

Given the low prevalence of PHA1 (approximately 10 cases out of 800,000 births/yr diagnosed in France, PHA1.NET, coordinator M.-C. Zennaro22), the estimated probability of a compound heterozygous mutation is in the order of 10−11. Our findings suggest that heterozygous carriers of NR3C2 mutations may be more frequent in the general population than expected, due to the large phenotypic variability of PHA1. This might prove relevant for patient’s care with wider screening for PHA1 indicated in neonates with renal salt losing syndromes and/or failure to thrive. Importantly, the possible existence of rare hypomorphic NR3C2 alleles in the general population puts a new perspective on BP regulation and the development of hypertension, as the combined effects of rare independent mutations (including in genes whose products mediate or regulate renal salt reabsorption) are expected to account for a substantial fraction of individual BP variation.23 The 1000 genomes Project pilot data indicate that each person carries approximately 250 to 300 loss-of-function variants in annotated genes.24 For NR3C2, 24 variations in coding exons have been catalogued. 13 of them are nonsynonymous; bioinformatic predictions using the Alamut software (Interactive Biosoftware, Rouen, France) suggest that several variants are deleterious (data not shown).

As for certain renal transporters, further studies will need to address if rare independent NR3C2 mutations in the general population could result in undiagnosed salt losing disorders in infancy and modulate the individual risk for developing hypertension.
Figure 3. In vitro characterization of $MR_{806X}$. A. $MR_{WT}$ and $MR_{BO6X}$ were analyzed for their capacity to bind aldosterone. Specific aldosterone binding was determined after incubation of $MR_{WT}$ or $MR_{BO6X}$ with $10^{-8}$M tritiated aldosterone with or without a 100-fold excess of unlabeled aldosterone. B. Transcriptional properties of $MR_{BO6X}$. Aldosterone dose-response curves showing the transcriptional activation by $MR_{WT}$ and $MR_{BO6X}$ in RCSV3 cells. Cells were transiently transfected with expression vectors of $MR_{WT}$ or $MR_{BO6X}$ together with GRE2-luciferase reporter plasmid. Relative transcriptional induction is represented relative to the induction obtained for $MR_{WT}$ stimulated with $10^{-8}$M aldosterone. Results represent mean ± SEM of at least three independent experiments performed in triplicate. ***P < 0.001. Insets represent negative controls transfected with empty vector. C. Intracellular trafficking of wild type and mutant receptors in living cells. COS-7 cells were transiently transfected with chimeric receptor proteins eGFP-$MR_{WT}$ or eGFP-$MR_{BO6X}$ and grown in steroid-free medium. Intracellular localization of fluorescence proteins was then observed in the absence (no Aldo) or presence of $10^{-8}$M aldosterone during 30 min. D. Recruitment of transcriptional co-regulator SRC-2 by $MR_{WT}$ and $MR_{BO6X}$ in mammalian two-hybrid assays. RCSV3 cells were transiently transfected with the fusion proteins VP16-$MR_{WT}$ or VP16-$MR_{BO6X}$ and the GAL4 DNA-binding domain fused to the N-term and the receptor interacting domain of SRC-2; cells were incubated in triplicate with increasing concentrations of aldosterone or vehicle. After harvesting the cells, the luciferase activities were measured and normalized by the values obtained by vehicle. The results are the means ± SEM of three independent experiments. ***P < 0.001. Insets represent negative controls transfected with empty vector.
CONCISE METHODS

NR3C2 Sequencing
For NR3C2 sequencing please see supplemental appendix.

Lymphocyte Purification, Culture and Treatments
Blood samples were collected and stored at 4 °C. Lymphocytes were purified by density gradient centrifugation on UNI-SEPmaxi columns (Novamed, Chicago, Illinois) following the manufacturer’s protocol. Lymphocytes were transferred into six well plates (Costar, Washington, DC) and cultured in a complete medium containing RPMI, 10% charcoal-stripped fetal calf serum, 100 UI/mL, penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California) for 24h at 37 °C in a 5% CO2 humidified atmosphere, in the presence or absence of emetine, an inhibitor of Nonsense-Mediated mRNA Decay,13 a cellular mechanism of mRNA surveillance which prevents the expression of truncated or erroneous proteins.

RNA Isolation and RT-PCR Experiments
Total RNA was extracted from lymphocytes with a Qiagen RNeasy mini kit according to the manufacturer’s recommendations. After DNaseI treatment (Invitrogen, Carlsbad, Ca, USA), 500 ng of total RNA were retro-transcribed using Superscript II RT (Invitrogen) and random hexamers (Promega, Madison, WI, USA). For PCR, please see supplemental appendix.

Plasmids and Transactivation Assays
For plasmids and transactivation assays, please see supplemental appendix.

Mammalian Two-Hybrid Assays
For mammalian two-hybrid assays, please see supplemental appendix.

Intracellular Localization of MR
COS-7 cells were transiently transfected with lipofectamine with plasmids coding for chimeric eGFP_MRW1 and eGFP_MRa06X proteins. Cells were seeded in Lab-Tek culture chambers at a density of 100,000 cells/well and cultured in steroid-free medium. After addition of 10 nM aldosterone, intracellular fluorescence was observed by confocal microscopy at constant temperature, pH and CO2 (Zeiss LSM 510 laser scanning confocal microscope (CarlZeiss, Thornwood, New York)).

Western Blot Analyses
Given the limited amount of biologic material available from patients, Western blot analyses were performed on protein extracts from renal RCSV3 cells transiently transfected with plasmids coding for mutant MR proteins MRa06X and MRa806X. Total protein extracts were prepared from cells lysed at 4 °C and sonicated (protein extraction buffer, Cell Signaling, Danvers, Massachusetts.). Lysates were subfractionated by SDS–PAGE on 4 to 15% gradient acrylamide gel (Biorad, Hercules, California). Immunoblots were incubated overnight with the 1D5 anti-MR antibody (kindly provided by C. Gomez-Sanchez, University of Mississippi Medical Center, Jackson, Mississippi), and with an anti-β actin antibody (Sigma Aldrich, St. Louis, Missouri) followed by a peroxidase-conjugated goat anti-mouse antibody. Proteins were visualized by an ECL + detection kit (GE healthcare, Buckinghamshire, UK).

Aldosterone Binding Assays
For aldosterone binding assays, please see supplemental appendix.

Statistical Analyses
Differences between wild type and mutant MR in cell culture experiments were analyzed by one-way or two-way ANOVA with Bonferroni post hoc tests or with nonparametric Kruskall-Wallis test. In vitro results are shown as the mean ± SEM. Statistical analysis of all of the in vitro results were performed with Graph Pad Prism version 5 (GraphPad software Inc, San Diego, California).

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DISCLOSURES
None.

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