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# Pseudohypoaldosteronism Due to Renal and Multisystem Resistance to Mineralocorticoids Respond Differently to Carbenoxolone

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**Type I pseudohypoaldosteronism (PHA) is a hereditary syndrome of salt wasting resulting from unresponsiveness to mineralocorticoids. PHA is manifested in two clinically and genetically distinct forms, affecting either only the kidney or multiple target organs of aldosterone. We examined the mineralocorticoid effect of carbenoxolone (CBX) in young PHA patients with either renal or multisystem resistance to aldosterone to find out whether CBX may help reduce the requirement for a high-salt diet. CBX did not show any significant salt-retaining effect in two patients with multiple PHA, and did not affect the renin-aldosterone system. In contrast, CBX significantly suppressed the renin-aldosterone system in a renal PHA patient for the whole duration of treatment, but without a long-term salt-retaining effect. On CBX treatment, urinary cortisone levels decreased and the cortisol:cortisone ratio increased, indicating that CBX inhibited 11β-HSD activity that metabolizes cortisol to cortisone. The complete lack of effect of CBX on the renin-aldosterone system in multisystem PHA patients indicates that CBX does not exert an effect via mineralocorticoid (MR) or glucocorticoid receptors. Examination of the structure and expression of the MR gene by Southern blot analysis and polymerase chain reaction (PCR) showed no abnormality. Whereas multiple PHA results from a spectrum of mutations in the mineralocorticoid activated epithelial sodium channel subunits, the genetic basis of renal PHA is still unknown. The response to CBX suggests that there is at least a partly functional MR in renal PHA patients. © 1997 Elsevier Science Ltd.**

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## INTRODUCTION

Type I pseudohypoaldosteronism (PHA) is a hereditary salt-wasting disease that includes two major distinct entities with different modes of inheritance [1]. The mild form stems from an isolated target organ defect with diminished renal tubular responsiveness to aldosterone, and is inherited as an autosomal dominant trait. In contrast, the severe form of PHA is characterized by multiple target organ resistance to aldosterone with a high rate of mortality in infancy as a result of excessive loss of electrolytes from sweat, salivary glands, colonic mucosa and distal renal tubules. This form is inherited as an autosomal recessive trait [1–3].

Because many of the other steroid resistance syndromes were found to result from mutations in the steroid receptor [4–6], the resistance to mineralocorticoids in PHA was also thought to be the result of a defect in mineralocorticoid receptor (MR). Studies of the MR using binding assays and anti-receptor antibodies indicated abnormalities in MR expressed in the mononuclear leukocytes of patients with both renal and multisystem PHA, suggesting that there may be a defect in MR expression in PHA [7, 8]. However, examination of the MR genes has not revealed any defect in this gene in PHA patients with diverse characteristics [9–13]. Recently, the genetic defect in multiple PHA has been located in the ami-

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loride-sensitive sodium channel [14, 15]. However, the possibility has not been eliminated that in renal PHA the disease may result from a defect in the function of the MR [13].

The therapy of renal PHA usually requires salt supplementation for the first 2–3 years of life, which becomes unnecessary later. In contrast, multisystem PHA patients require very high amounts of salt in the diet life-long (as high as 45 g NaCl/day) [1, 16]. Although the affected children may have an increased appetite for salt, they do not usually tolerate very high amounts of salt in the diet, and their poor dietary compliance frequently precipitates life-threatening salt-losing episodes.

A recent study showed that carbenoxolone (CBX) treatment for 2 weeks exerted a mineralocorticoid effect in patients with an apparently isolated renal tubular defect [17]. CBX, a derivative of glycyrrhetinic acid in liquorice, can exert a mineralocorticoid effect in healthy individuals by inhibiting 11$\beta$-hydroxysteroid dehydrogenase (HSD) activity [18]. This enzyme is present in high levels in aldosterone-responsive target tissues, and it prevents the mineralocorticoid action of cortisol by metabolizing cortisol to its inactive form, cortisone [19]. CBX inhibition of 11$\beta$-HSD allows unmetabolized cortisol to bind to mineralocorticoid receptors and to activate them just as aldosterone does [18].

In this study we examined the mineralocorticoid effect of CBX in young PHA patients with both renal and multisystem resistance to aldosterone, to find out whether CBX affects the two forms of PHA differently, and if it may help reduce the high-salt diet in multisystem PHA patients. We also examined the structure and expression of the MR gene in our patients using Southern blot analysis, and polymerase chain reaction (PCR) to see if there is an abnormality in this gene.

## SUBJECTS AND METHODS

### *Subjects*

Two patients with multisystem and one patient with renal tubular unresponsiveness to aldosterone were examined. The basic clinical and laboratory data of these three patients at diagnosis are shown in Table 1. In patients 1 and 2 with multisystem unresponsiveness to aldosterone, sweat and salivary electrolytes were persistently elevated [20]. In these patients, who were products of consanguineous marriages, the family pedigrees were consistent with autosomal recessive inheritance [1, 20]. In patient 3 sweat and salivary electrolytes were normal, indicating an isolated renal tubular defect. He is an asymptomatic member of a family whose pedigree was consistent with an autosomal dominant mode of inheritance (see pedigree of kindred I, generation III, patient 4) [1].

Carbenoxolone therapy was started in three patients at a dose of 10–12 mg/kg, a dose known to exert a mineralocorticoid effect in normal individuals. Clinical and biochemical parameters were evaluated before and periodically during CBX treatment for up to 5 months. The study protocol was approved by E. Wolfson hospital local ethics committee operating under the Helsinki accords, and informed consent was obtained from all the parents before the trial. Carbenoxolone was generously provided by Miss L. Baxendale of Biorex Laboratories (Enfield, Middx, U.K.).

*Patient 1.* In this 6-year-old girl the therapeutic effects of CBX were analysed in three stages. In Stage 1 CBX was administered for 2 months at a dose of 150 mg/day, while the patient was maintained on a high-salt diet of 12 g/day. In Stage 2 the CBX dose was increased to 200 mg/day for an additional 3 months without a change in NaCl supplementation. In Stage 3 the CBX dose was maintained at 200 mg/day for 2 days while the total salt supplementation was reduced by half to 6 g/day.

*Patient 2.* In this 8-year-old patient CBX was given at a dose of 250 mg (10 mg/kg) for 2 months. She was maintained on 20 g NaCl during the study period.

*Patient 3.* In this 5.5-year-old boy with renal PHA, CBX was given at a dose of 150 mg (12 mg/kg) for 68 days.

### *Assays*

Plasma renin activity (PRA), and serum aldosterone concentrations were measured in duplicate by RIA kits as previously described [1]. In patient 1 the inhibitory effect of CBX on 11$\beta$-HSD and on the

*Table 1. Laboratory data in three PHA patients at diagnosis*

| Patient no. | Age | Sex | Serum Na (nmol/l) | Serum K (nmol/l) | Aldosterone (ng/dl) | PRA (ng/ml/h) | Sweat, saliva (Na, Cl) |
|---|---|---|---|---|---|---|---|
| 1 | 9 day | F | 125 | 10.0 | 1427 | 150 | High |
| 2 | 10 day | F | 116 | 12.3 | 1914 | 21 | High |
| 3 | 10 months | M | 140 | 5.8 | 52 | 6.5 | Normal |
| Normal* | | | 136–146 | 3.5–4.8 | <100 | <10 | |

*Normal aldosterone and PRA in infants up to 3 months. In infants older than 6 months aldosterone: <15 ng/dl, PRA: <3 ng/ml/h..

peripheral metabolism of cortisol was shown by measuring the levels of urinary free cortisol and cortisone [21]. Fractional excretions of sodium and potassium were calculated according to the following formulae:

$$FE_{Na} = \frac{[Na]urine \times [Cr]blood}{[Cr]urine \times [Na]blood} \times 100$$

$$FE_K = \frac{[K]urine \times [Cr]blood}{[Cr]urine \times [K]blood} \times 100$$

*Lymphoblast cell lines.* Immortalized lymphoblast cell lines were generated by Epstein–Barr virus transfection of peripheral blood lymphocytes, obtained from the patients with the recessive PHA according to previously described methods [22,23].

*Southern blot analysis.* Genomic DNA was isolated from fresh blood samples as described [24]. For Southern blot analysis, the restriction enzyme cut genomic DNA samples were electrophoresed on a 1% agarose gel, electro-transferred to GeneScreen Plus (New England Nuclear), and hybridized with radiolabelled MR cDNA at 42°C, according to the instructions in the GeneScreen Plus manual. MR cDNA, generously provided by Dr J. L. Arriza [12], was labelled by the "random primed" method using a Boehringer-Mannheim kit and ($\alpha$-$^{32}$P)dCTP (3000 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.).

*RNA isolation and PCR analysis.* RNA was isolated from lymphoblast cell lines grown in culture. The cells were harvested, washed and the RNA isolated with Tri Reagent LS (Molecular Research Center, Cincinnati, OH, U.S.A.) at a yield of 200–400 $\mu$g total RNA per litre culture. First-strand cDNA was synthesized from 10 $\mu$g total RNA with a Stratagene kit. PCR was carried out in heat-sealed 10 $\mu$l glass capillary tubes in a thermal cycler (Robocycler, Idaho Technology) programmed as follows: an initial 120 s at 94°C, 30 cycles with denaturation at 92°C for 0 s, annealing at 60°C for 0 s, and extension at 72°C for 12 s, and a final step of 180 s at 72°C. The following primer sequences were selected based on the human MR cDNA sequence [25].

Forward 4587 : 5′–
– *CGCTCAACCGCTTAGCAGGCAAAC*

Reverse 4588 : 5′–
– *GGTCCTTCTGGGTGTGGAACAAC*

PCR products were separated on a 1.5% agarose gel. The PCR bands were eluted and sequenced to verify their identity with the human MR sequence.

## RESULTS

*Effect of carbenoxolone on electrolytes*

*Patient 1.* While on 12 g NaCl/day, treatment with CBX did not result in any significant change in serum, or urine electrolyte values or in urine $Na^+$:$K^+$ ratios. The fractional excretion of sodium and potassium also did not change significantly. A tendency towards higher serum $Na^+$, lower serum $K^+$ and lower fractional excretion of sodium was observed at 20 days of treatment, a change which did not persist. Saliva and sweat electrolyte values and $Na^+$:$K^+$ ratios showed no significant change (data not shown). The blood pressure remained normal, and the patient did not gain weight (Fig. 1).

When we halved the dose of NaCl while the dose of CBX was 200 mg/day (Stage 3), the blood urea nitrogen rose to 62 mg/dl and the sodium dropped to 134 meq/l. The patient lost 400 g and appeared moderately dehydrated (sunken eyes, dry mucosal membranes). This sequence of events confirmed the dependence of the patient on a high dose of NaCl and the lack of mineralocorticoid effect of CBX.

*Patient 2.* Similarly to patient 1, CBX did not show any mineralocorticoid effect. There were no significant changes in serum, saliva, sweat and urine electrolyte values and saliva and urine $Na^+$:$K^+$ ratios. The fractional excretion of sodium and potassium also did not change significantly during the study except for a temporary decrease in the fractional excretion of sodium during the third week (data not shown). The blood pressure remained normal. The patient did not gain weight.

*Patient 3.* In this renal PHA patient CBX treatment for 68 days did not change serum electrolytes. There was no reduction of fractional excretion of sodium in urine. Saliva and urine $Na^+$:$K^+$ ratios also did not change. There was no weight gain or elevation in blood pressure (Fig. 2).

*Effect of carbenoxolone on renin-angiotensin-aldosterone system*

*Patient 1.* On CBX treatment serum aldosterone levels remained very high (5 to 33-fold higher than normal). Plasma renin activity (PRA) also remained high (two- to 13-fold higher than normal). A further increase in CBX dosage to 200 mg/day did not alter the clinical or laboratory findings during the ensuing 3 months (Fig. 1). In Stage 3, PRA rose significantly within 48 h from 6.3 to 77 ng/ml/h (1.74–21.3 ng/(l.s)) and aldosterone from 329 to 684 ng/dl (9130–18970 pmol/l). Resuming the high salt diet resulted in a lowering of both aldosterone and PRA (Fig. 1).

*Patient 2.* The serum aldosterone values before CBX ranged from 437 to 652 ng/dl (12120–18090 pmol/l) and were not suppressed by CBX, remaining 39- to 73-fold higher than normal. Plasma

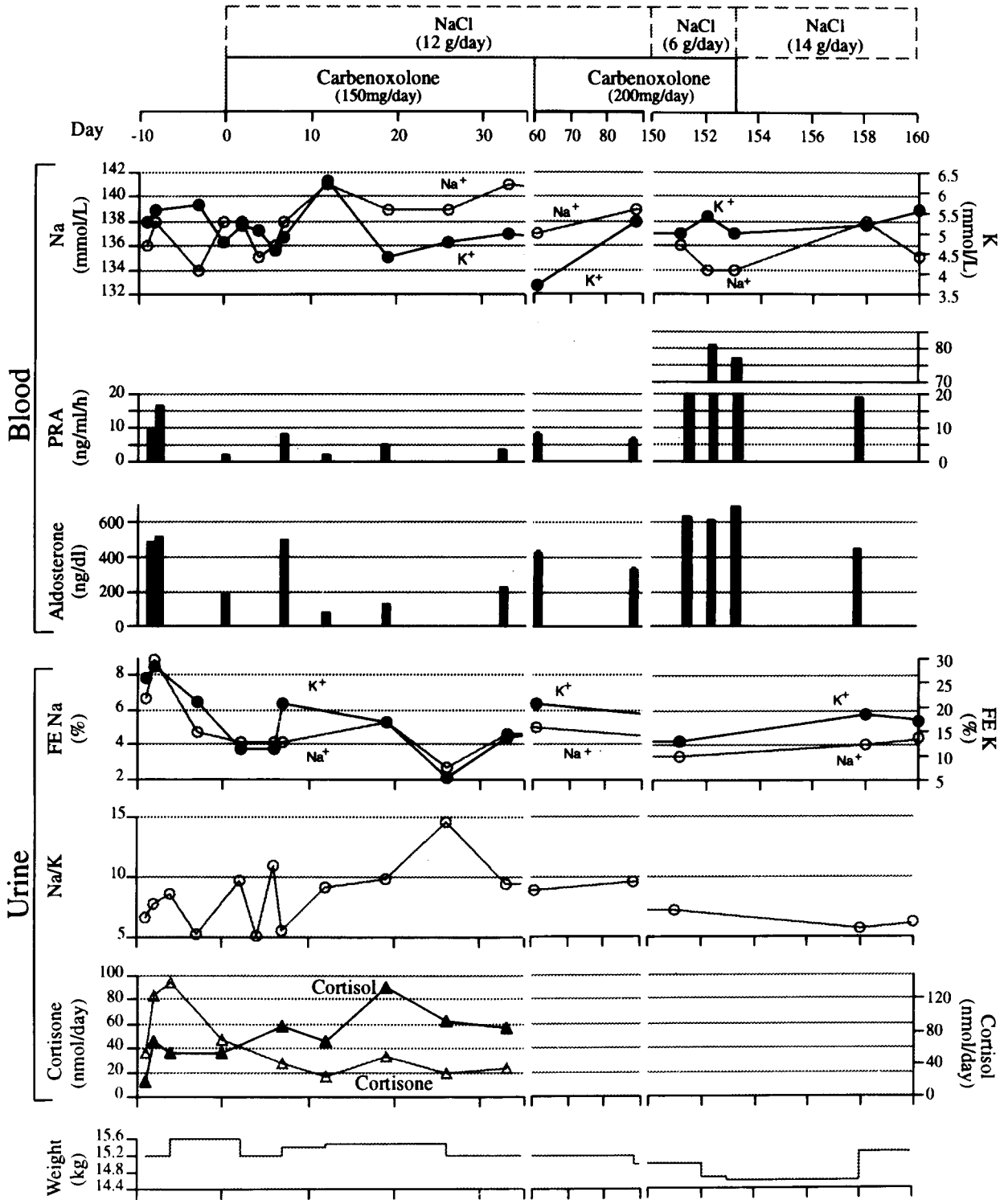


**Fig. 1. Effect of carbenoxolone on blood and urine electrolytes, renin, aldosterone and urinary free cortisol and cortisone in a patient with multisystem PHA during high (12 g/day) and reduced (6 g/day) NaCl supplementation. Normal values in children beyond the age of 1 year: Aldosterone, 55.5–388 pmol/l (2–15 ng/dl); PRA, 0.14–0.4 ng/(l.s), (0.5–1.5 ng/ml/h). For additional details see the Methods section. FE, fractional excretion. To convert ng/dl to pmol/l multiply by 27.74. To convert ng/ml/h to ng/[l.s] multiply by 0.2778.**

renin activity also remained high throughout the study period (7.8–33.4 ng/ml/h; 2.2–9.2 ng/(l.s)).

*Patient 3.* In this patient, in contrast to the multisystem PHA patients, aldosterone values became undetectable within two days of commencing CBX (from 34 to <5 ng/dl) and remained so throughout the study period. Plasma renin activity was similarly suppressed from 11 before treatment to 1 ng/ml/h two days after starting CBX and remained low (0.4–1 ng/ml/h) during the study. After the discontinuation of

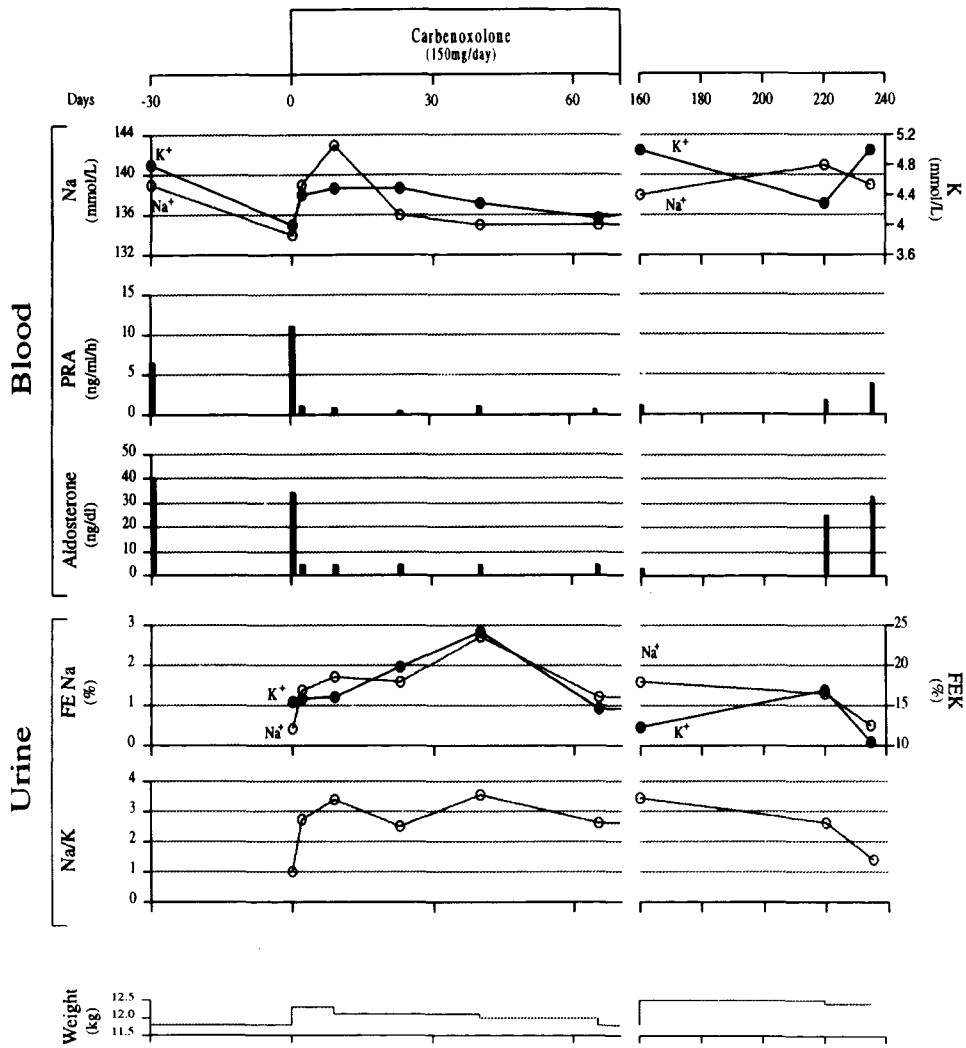


**Fig. 2. Effect of carbenoxolone on blood and urine electrolytes, renin and aldosterone in a patient with renal PHA. This patient received carbenoxolone 150 mg/day for 68 days. For normal values see legend of Fig. 1.**

CBX, both aldosterone and plasma renin activity reached the pretreatment values in about five months (Fig. 2).

*Effect of carbenoxolone on cortisol metabolism*

To ascertain that CBX inhibited 11$\beta$-HSD we determined the urinary cortisol and cortisone levels in patient one. On CBX treatment urinary cortisone levels decreased significantly, and the urinary cortisol:cortisone ratio increased (by day seven and on) (Fig. 1). These findings indicated inhibition of 11$\beta$-HSD activity [21].

*Structure and expression of the MR gene*

To compare the structure of the MR gene of the patients with that of normal individuals, the genomic DNA was cut with restriction enzymes EcoRI, and PvuII and subjected to Southern blot analysis. The EcoRI restriction patterns of the genes using MR cDNA probes showed two bands that did not appear in all subjects (Fig. 3). However, there was no apparent association of these patterns with the PHA inheritance (Fig. 3). The Southern blot analyses of genomic DNA from both dominant and recessive PHA patients thus did not reveal a major defect in the MR genes.

PCR analysis of the lymphoblast RNA isolated from the multisystem PHA patients showed that the MR gene is expressed in these patients as in healthy controls (Fig. 4). Sequencing of the PCR products did not reveal any sequence difference from the previously published MR cDNA [25].

## DISCUSSION

*The response to carbenoxolone in renal and multisystem PHA patients*

The present findings revealed a major difference in the response to CBX of renal and multisystem PHA patients. CBX at a dose that exerts mineralocorticoid effect in normal individuals [18] showed no salt-retaining effect in any patient, and no effect in PRA

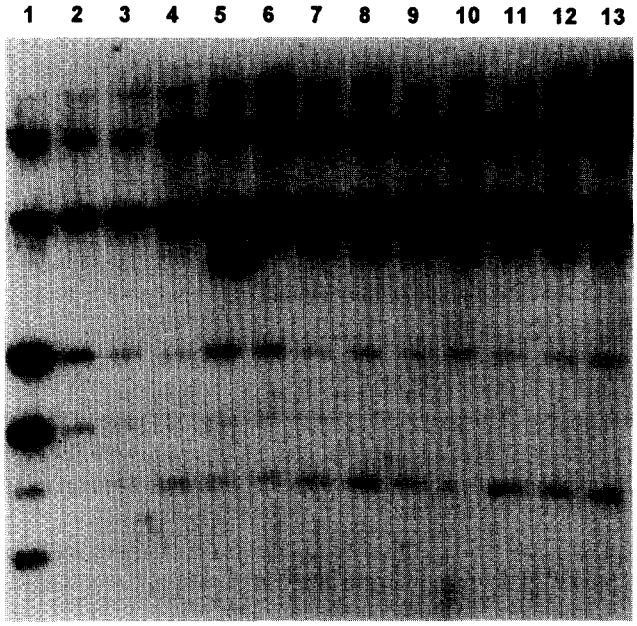


**Fig. 3. Southern blot analysis of genomic DNA from PHA patients and normal family members. Genomic DNA (4 μg/ lane) was cut with EcoRI, electrophoresed on 1% agarose gel, transferred to a nylon membrane (Genescreen Plus) and then hybridized with radiolabelled mineralocorticoid receptor cDNA (MR 3750). DNA samples 1–4 from the family of patient 3: 1. father (unaffected); 2. mother (affected, asymptomatic); 3. sister (affected, symptomatic); 4. patient 3 (affected, asymptomatic). DNA samples 5–7 from family of a renal PHA patient: 5. mother (affected, symptomatic); 6. sister (affected, symptomatic); 7. brother (affected, asymptomatic). DNA samples 8–13 from family of multisystem PHA patient 1: 8. father; 9. mother; 10. sister; 11. sister; 12. brother; 13. patient 1.**

or aldosterone levels in patients with multiple end-organ unresponsiveness to aldosterone. In contrast, CBX readily suppressed the renin-aldosterone system in a patient with renal PHA.

In patients with multisystem PHA the lack of responsiveness to CBX was complete, as even an attempt to reduce the amount of NaCl by 50% while the patient was maintained on a high dose of CBX did not prevent a salt-losing crisis. Our results differ from those of a recent report showing a salt-retaining effect of CBX in a 17-year-old boy with multisystem unresponsiveness to aldosterone [26]. One possible explanation for this difference is an age-dependent change in mineralocorticoid responsiveness. The boy, who responded to CBX at the age of 17, also responded to another mineralocorticoid, Florinef, in high doses, although he had failed to respond to Florinef in childhood [27]. In this boy the combined treatment of CBX plus dexamethasone suppressed cortisol secretion and eliminated the salt-retaining effect of CBX. These data and the lack of responsiveness to CBX in our multisystem PHA patients suggest that the mineralocorticoid effect of CBX is purely dependent on mineralocorticoid "responsiveness" which may be age-dependent.

In contrast to our multisystem PHA patients, CBX significantly suppressed the renin-aldosterone system in our renal PHA patient for the whole duration of treatment. Despite the suppression of renin and aldosterone other laboratory and clinical findings did not indicate a long-term salt-retaining effect. In healthy volunteers the maximal effects of both liquorice and CBX was observed in days 7–10 of the experiment. After this period mineralocorticoid escape occurred in most individuals [18, 28]. In four renal PHA patients from the same family CBX treatment for two weeks exerted a mineralocorticoid effect causing a significant reduction in Na excretion [17]. However, the renin-aldosterone system was not evaluated in these patients.

*Molecular basis of the differential responses of renal and multisystem PHA patients*

In PHA patients the mineralocorticoid action of CBX was proposed to be mediated by the glucocorticoid receptor [17]. However, the complete lack of effect of CBX on the renin-aldosterone system in our multisystem PHA patients suggests that CBX does not exert an effect via the glucocorticoid receptor. Moreover, combined treatment with both CBX and dexamethasone was shown to suppress cortisol secretion, and reversed the beneficial effect of CBX in a patient with multisystem PHA, further supporting the idea that CBX effect is mediated by the inhibition of

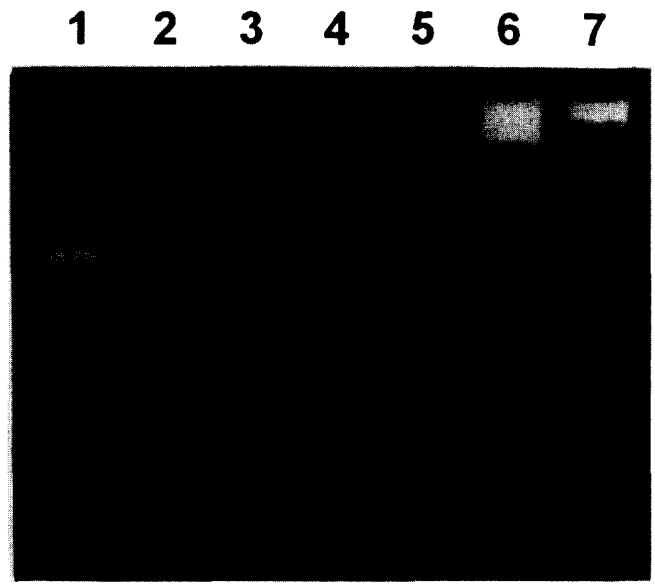


**Fig. 4. Expression of the mineralocorticoid receptor RNA in the lymphoblast cells of multisystem PHA patients. Lymphoblast RNA was reverse transcribed and subjected to PCR as described in Methods. The lanes contained the following PCR samples: 1. healthy control; 2. patient 1; 3. patient 2; 4. and 5. twin patients described in ref. [18]; 6. bluescript plasmid containing human MR cDNA [25]; 7. same as lane 6 but without Taq polymerase. The size of the major band in lanes 1–6 is 717 bp as expected based on the human MR sequence.**

the metabolism of cortisol that acts via the mineralocorticoid receptor [26].

The different mineralocorticoid responsiveness of renal and multisystem PHA patients indicates a difference in their MR function. The partial response to CBX in renal PHA suggests that there is at least a partly functional MR. This is also supported by the observation that spironolactone, a mineralocorticoid antagonist, aggravated sodium loss in several patients with renal PHA [29,30].

In recent immunofluorescence studies, MR was detected in the monocytes of renal PHA patients, but not in the monocytes of two multisystem PHA patients, thus suggesting an abnormality in MR expression [8]. However, our PCR results (Fig. 4) did not reveal a defect in the expression of the MR gene, at least in the lymphoblasts of our patients.

The present results, together with our recent studies showing that there is no linkage between multisystem PHA and MR gene locus, [12] rule out an abnormality in the MR gene or its expression in multisystem PHA. Multisystem PHA is caused by mutations in the mineralocorticoid-activated epithelial sodium channel subunits [14,15]. These channels are expressed in all mineralocorticoid responsive epithelial tissues [31–33]. Aldosterone binding abnormalities in the monocytes of PHA patients [7,8] may reflect secondary effects of high levels of aldosterone. However, the negative findings still do not exclude abnormalities in the MR gene in the case of renal PHA [13]. Identification of the mutations that cause renal PHA is necessary to understand fully the responses of the two forms of PHA.

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