Renal Carbonic Anhydrase Activity in DBA/2FG-pcy/pcy Mice with Inherited Polycystic Kidney Disease

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Abstract: DBA/2FG-pcy/pcy (D2-pcy) mice are a hereditary murine model of slowly progressive polycystic kidney disease (PKD) and characterized by the persistent excretion of acidic urine, in association with polyuria, after weaning. In this study, the activity of carbonic anhydrase (CA) and its histological distribution in the kidney of D2-pcy mice were investigated by immunohistochemistry. Significantly higher CA activity was detected in the cytosolic, but not membrane, fraction of kidney homogenates in 5-week-old D2-pcy mice than in age-matched, control DBA/2 (D2) mice, and a more rapid rate of urine acidification was noted in 11-week-old mice when acetazolamide, an inhibitor of the enzyme, was administered orally. By immunohistochemistry for the major renal CA isoenzyme (CA II), epithelial cells in the distal straight tubules and the cortical collecting ducts were stained intensely, whereas those of the proximal convoluted tubules had only weak and diffuse staining. The glomeruli, the proximal straight tubules and the ascending thin limb of Henle's loop were almost free from staining. In the cells lining cysts and/or dilated tubules, CA II activity was well preserved, although the staining intensity was considerably reduced in fully-flattened, lining cells of cysts, but no difference was found between D2-pcy and D2 mice in any segmental localization of renal CA II activity. From these results it seems that D2-pcy mice in the early stages of the cystic disease continue to secrete excess protons through the CA-mediated reaction that is stimulated for regulation of acid-base balance in the distal portion of the nephron and the collecting duct in kidney. It also suggests that monitoring urine pH may be useful in predicting the effects of early interventions on the progression of slowly developing renal cysts.

Key words: murine inherited polycystic kidney disease, renal carbonic anhydrase, immunohistochemistry, urine pH

(Received 24 June 1998; Accepted 25 February 1999)
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Introduction

Human polycystic kidney disease (PKD) is characterized by the development of numerous fluid-filled cysts derived from the nephron and collecting ducts in the cortex and medulla of the kidney, leading to fatal renal failure in many patients [15]. The most common inherited form of PKD is autosomal dominant PKD (ADPKD), in which cysts grow very slowly for decades resulting in a complete distortion of the normal renal architecture. Clinically, certain functional abnormalities including hypertension and defects in urinary concentrating capacity, urinary acidification and urinary ammonia excretion have been known to occur in early-stage ADPKD [5, 15].

The DBA/2FG- pcy/pcy (D2-pcy) mouse has a form of renal cystic disease that appears similar in many respects to that seen in human ADPKD, although the murine disease is transmitted as an autosomal recessive trait [19]. Cyst formation can be detected in the kidneys of fetal and newborn mice, as in human ADPKD [5]. The increase in renal Na\(^+\), K\(^+\)-ATPase activity and the excretion of acidic urine, as well as polyuria, are also characteristic of young D2-pcy mice after weaning [10, 11]. Changes in basolateral Na\(^+\), K\(^+\)-ATPase activity and the apical chloride efflux in the renal tubular epithelium have been proposed to play an important role in cyst formation and fluid accumulation in the cyst cavity in human ADPKD [5, 6, 15]. On the other hand, urine pH is finally determined by the protons derived from the carbonic anhydrase (CA)-mediated reaction for bicarbonate reabsorption in the collecting ducts [21]. In this connection, the present study was undertaken to clarify whether CA activity and its segmental distribution in the kidney may be responsible for the excretion of acidic urine in D2-pcy mice.

Materials and Methods

Animals: D2-pcy male mice used were those from the colony that had been kept at the laboratory animal room of Hokkaido College of Pharmacy for a decade since introduction of the origin from the Laboratory Animal Center of Fujita Health University. Age-matched DBA/2 (D2) male mice were used as a normal control. Animals were fed a commercially available diet (Oriental-CMF) and water ad libitum. The animal room was maintained under controlled conditions at 23 ± 1°C, in a relative humidity of 50 ± 5% and on a 12-hr light-dark cycle.

Determination of renal CA activity: Kidneys from 5-week-old mice were perfused thoroughly with cold saline to wash out blood, weighed, then homogenized in 3.3% with 50 mM mannitol-2 mM Tris-HCl buffer at pH 7.0. Cytosolic and membrane fractions were obtained from the homogenates by a modification of the differential precipitation method [14], and total CA activity (enzyme unit per mg protein) was determined as follows. Time required for a decrease in pH from 8.0 to 6.3 caused by the addition of 1.0 ml of CO\(_2\)-saturated water to 5.0 ml of the sample solution or medium alone and enzyme unit was evaluated with the formula \([t_f - t_o]/t\), where \(t_f\) and \(t_o\) were the time of reaction in the presence and the absence of CA [22].

Measurements of urine pH and urinary ammonium nitrogen: Mice at 12 weeks of age were placed individually in glass metabolism cages and urine samples were collected for 24 hr under paraffin oil in an ice-cooled flask before and after oral administration of acetazolamide at 200 mg/kg. Urine pH was determined with a pH meter (SEI700GC, Fuji Kagaku Keisoku Co., Ltd., Tokyo) on a small quantity of the urine sample freshly obtained from the bladder by gently pressing on the abdomen with the fingers before and at 1, 2 and 24 hr after the administration. Urinary ammonium nitrogen was quantified with a Wako kit for ammonia testing (Wako Pure Chemicals Co., Ltd., Tokyo).

Immunohistochemistry: Kidneys from mice at 5 weeks and 11 weeks of age were fixed overnight in Carnoy's fluid, dehydrated through 100% ethanol and chloroform, embedded in paraffin and transversely cut at 4 μm. To investigate the segmental distribution of Type II CA (CA II) of the major CA isozyme in the kidneys [21], the following method was employed [9]. Sections were deparaffinized and treated with normal rabbit serum to block non-specific reaction for 20 min. Then the sections were incubated with 1:1500 diluted primary anti-human CA II sheep serum (Biogenesis, USA), followed by biotinylated secondary rabbit anti-sheep IgG and an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Lab., Inc., USA). Each incubation for staining was performed for 30 min. To visualize, the tissue sections were treated with the substrate 3,3'-diaminobenzidine for 10 min.
The sections were also counterstained with hematoxylin. Specificity of the anti-serum for the mouse kidney CA II was confirmed by western blot analysis (Fig. 1). In addition, to identify the distal tubule and collecting duct segments, the staining method with peanut agglutinin-horseradish peroxidase was applied [7].

Statistics: The data were expressed as means ± S.E. Statistical significance was determined at 5% or less by Student’s t-test.

Results

Renal CA activity: The total CA activity was much higher in the cytosolic fraction of kidney homogenates from D2-pcy mice at 5 weeks of age than in that from age-matched D2 mice, but there was no significant difference between the two strains in the membrane fractions (Fig. 2).

Effects of CA-inhibition on urine pH and urinary excretion of ammonium nitrogen: As shown in Fig. 3, urine was rapidly alkalized in both strains of mice 1 and 2 hr after oral administration of acetazolamide at 200 mg/kg, but the pH of 24-hr accumulated urine of D2-pcy mice was significantly lower than that of D2 mice. The treatment also caused noticeable increases in the amounts of urine and urinary ammonium nitro-

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**Fig. 1.** Specificity of anti-human type II CA antiserum detected by western blot analysis. The mouse kidney cytosols showed a single band of the 31.0 kd position corresponding to CA. M means the lane for marker proteins.

**Fig. 2.** Carboxic anhydrase activities in cytosolic and membrane fractions from kidney homogenates in D2-pcy and D2 mice. Bars mean standard errors of the means (5 determinations for each mouse strain). Total CA activity was determined by the method described in the text. *P<0.05
Fig. 3. Changes in urine pH after oral administration of acetazolamide to D2-ncy and D2 mice. A single dose of 200 mg/kg was used. Bars mean standard errors of the means (4 determinations for each mouse strain). **P<0.01

Table 1. Effects of acetazolamide on urine volume and urinary excretion of ammonium nitrogen in two strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Urine volume (mL/24 hr)</th>
<th>Ammonium nitrogen (µg/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>Untreated</td>
<td>1.28 ± 0.23</td>
<td>43.3 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>2.22 ± 0.15*</td>
<td>194.0 ± 6.2**</td>
</tr>
<tr>
<td>D2-ncy</td>
<td>Untreated</td>
<td>4.05 ± 0.87</td>
<td>68.0 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>5.75 ± 0.82</td>
<td>195.4 ± 31.6**</td>
</tr>
</tbody>
</table>

Mice 11 weeks old were orally given acetazolamide at 200 mg/kg and urine was collected for 24 hr. Values are the mean ± S.E. for 5 animals per group. *P<0.05, **P<0.01.

In both strains (Table 1), but the increase in urine volume was not significant in D2-ncy mice with congenital polyuria.

Localization of renal CA II activity: In the cortex of the 5-week-old control D2 mouse kidney (Fig. 4A), the distal straight tubule (ascending thick limb of Henle’s loop) and the collecting duct showed intense staining for CA II. It is reported that the epithelium of the collecting duct in mouse kidney consists of two types of cells, principal cells and intercalated cells [20, 21]. In D2 mice the intercalated cells were stained much more heavily than the principal cells. In contrast, the epithelium of the proximal convoluted tubule was weakly stained, whereas that of the straight segment was heavily stained. The glomerulus was negative for CA II staining. In the innermedulla (Fig. 4B) the staining for cellular CA II was intense in the distal straight tubules, but was less and negative in the descending and ascending thin limbs of Henle’s loop, respectively. The papillary collecting ducts were almost all negative for CA II. The segmental distribution of renal CA II was similar in D2 and D2-ncy mice at 5 weeks of age, and was in close agreement with that in the mouse kidneys described in the literature [4, 20]. Nevertheless, a number of cysts derived mostly from the distal straight tubules developed in both the cortex (Fig. 5A) and the medulla (Fig. 5B) of the 5-week-old D2-ncy mouse kidneys. The lining cells of the cysts were heavily stained with the anti CA II antibody. The cells of proximal convoluted tubules were weakly stained as seen in the intact cells, and inconsistently in intensity from cell to cell. In the 11-week-old D2-ncy mouse kidneys, the dilated tubules and cysts of various forms and sizes increased in number in the cortex (Fig. 6A) and medulla (Fig. 6B). The lining cells of cysts, especially the intercalated cells of collecting ducts remained heavily staining, but fully-flattened cells of largely expanded cysts exhibited an overall reduction in the intensity of staining.
Fig. 4. Immunohistochemical localization of type II carbonic anhydrase (CA II) in the 5-week-old control D2 mouse kidney. In the cortex (A), cells of distal straight tubules (DST) are heavily in CA II stained, but cells of proximal convoluted tubules (PCT) are stained only weakly and diffusely. Cortical collecting ducts (CCD) comprise two cell types, large intercalated cells (arrows) with heavy CA II staining and principal cells with poor staining. Glomeruli (G) are not stained. In the innermedulla (B), distal straight tubules are stained heavily, but CA II activity is weak and negative in the descending thin limbs (DTL) and ascending thin limbs (ATL) of Henle’s loop, respectively. Magnification is ×100 in each case.
Fig. 5. Immunohistochemical localization of CA II in the 5-week-old D2-pecy mouse kidney. In the cortex (A), enlarged cysts of distal tubule origin (DST) are lined by epithelial cells strongly positive for CA II activity. Slightly dilated collecting ducts (CCD) are lined heterogeneously with CA II-rich intercalated cells (arrows) and CA II-poor principal cells. In the medulla (B), the distal straight tubules (DST) form cysts enclosed by cells showing strong positive CA II activity. The descending thin limbs of Henle’s loop (DTL) are weakly stained. Magnification is × 50 in each case.
Fig. 6. Immunohistochemical localization of CA II in 11-week-old D2-pecy mouse kidney. In the cortex (A), largely expanded cysts of distal tubule origin (DST) are delineated by cells showing strong positive CA II activity. Epithelium of the mildly dilated collecting duct (CD) contains intercalated cells (arrows) which are heavily stained with the anti-CA II antibody. Proximal convoluted tubules (PCT) also show signs of faint to moderate staining. In the medulla (B), cysts develop mostly from distal straight tubules (DST). CA II activity is weak and negative in the descending thin limbs (DTL) and ascending thin limbs (ATL) of Henle’s loop, respectively. Magnification is ×50 in each case.
Discussion

In ADPKD renal epithelia, basolateral Na\(^+\)-K\(^+-\)ATPase and apical chloride channels may play the main role in fluid accumulation within cysts [6]. Clinically, Na\(^+\)-K\(^+-\)ATPase is known to be greatly increased in the early stage in ADPKD kidneys [23]. In D2-pcy mice, the enzyme is localized most abundantly in the distal tubule segment, whereas a very low activity is detectable only in normal collecting ducts [12]. During maturation, the cellular distribution of Na\(^+\)-K\(^+-\)ATPase in the kidney changes profoundly in association with a decrease in the number of the enzyme-positive cells, whereas CA activity increases [8]. In C57BL/6J-cpk cpk mice, a murine model for rapidly progressive PKD in humans [17], the renal Na\(^+\)-K\(^+-\)ATPase activity is characterized by a marked increase in the earliest fetal stages of cyst formation of the proximal tubules before the collecting duct enlarges enormously after birth [1]. In the postnatal D2-pcy mouse kidney, CA as well as Na\(^+\)-K\(^+-\)ATPase [10] are both increased to modest but a significantly larger extent, as compared to the respective normal levels, suggesting a possible role of CA in the physiological regulation of excess Na\(^+\), K\(^+-\)ATPase activity. The collecting duct epithelium consists of two types of cells: principal cells with Na\(^+\)-K\(^+-\)ATPase activity in the basolateral site and intercalated cells rich in cytoplasmic CA II, together with H\(^+\)-ATPase and Cl\(^-\)/HCO\(_3\)- exchanger in the luminal site [21].

Interestingly, congenitally CA II-deficient (CAR 2 null) mice are known to exhibit renal distal tubular acidosis with alkaline urine pH and reduced serum bicarbonate, and failure to acidify urine after ammonium-chloride loading, probably because of the absence of intercalated cells in the collecting ducts [2, 3]. Usually, chronic metabolic acidosis increases renal CA and its mRNA expression in the intact kidney, in association with increased density of Type A intercalated cells in the collecting ducts [13, 18]. In addition, cultured Madin Darby canine kidney (MDCK) cells, which originate in the collecting duct segment, are known to form liquid-filled domes by predominant assemblies of cells with the highest density of CA activity [16], and the dome formation is stimulated considerably by the addition of the cyst fluids from D2-pcy mice or patients with ADPKD added to the culture medium [6, 24]. Increased CA activity in the collecting duct may contribute to the persistent excretion of acidic urine in D2-pcy mice after weaning. Whether this may be a sign of adaptation to a special necessity to excrete protons or a direct indication of progress of cyst formation during and after maturation of the kidney in D2-pcy mice is to be further studied. Factors causing increases in acidic urine excretion and ammonia production in the kidney, such as dietary high protein intake and chronic potassium deficiency, may contribute to the exacerbation of renal pathology including PKD [15]. Therefore, monitoring urine pH may be useful in obtaining information in the study of early intervention that delays the progression of slowly developing renal cysts.

In conclusion, the CA activity of the cytosolic fraction from the kidney homogenate was significantly higher in the kidney of the 5-week-old D2-pcy mice than in the age-matched, control D2 mice and the enzyme inhibition caused by orally administration of acetazolamide led to acidification of urine at a more rapid rate. By immunohistochemistry for the major renal CA isoenzyme CA II, the intense staining was detected in the epithelial cells of the distal straight tubules and the cortical collecting duct. In the lining cells of cysts, CA II activity was well-preserved, although it was reduced in fully-flattened lining cells of the cysts in the 11-week-old D2-pcy mouse kidneys as a whole, but no difference between D2-pcy and control D2 mice at 5 weeks of age was found in segmental distribution of CA II.

References