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Carbonic anhydrase isozymes IV and II in urinary membranes from carbonic anhydrase II-deficient patients

(osteopetrosis/renal tubular acidosis/phosphatidylinositol-glycan/brush borders)

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ABSTRACT **Carbonic anhydrase II (CA II) deficiency has been shown to be the primary defect in the recessively inherited syndrome of osteopetrosis with renal tubular acidosis. Until now, the absence of CA II in kidney of CA II-deficient patients has not been shown directly, and the status of the membrane-associated CA in kidney of CA II-deficient patients has been unclear. To address these questions, we analyzed urinary membranes and soluble fractions from normal and CA II-deficient subjects. The CA activity in membrane fractions of normal urine was found to comprise two components—(*i*) a vesicle-enclosed, sodium dodecyl sulfate (SDS)-sensitive fraction, which was shown immunochemically to be the 29-kDa CA II, and (*ii*) an SDS-resistant fraction, which was due to native and cleaved forms of the 35-kDa, membrane-anchored isozyme CA IV. Urinary membranes from CA II-deficient patients showed little or no SDS-sensitive activity and no immunoreactivity for CA II, providing direct evidence that their mutation, which produces CA II deficiency in erythrocytes, also affects CA II in kidney. CA IV activity and immunoreactivity were present in normal amounts in urinary membranes from CA II-deficient patients. We conclude from the enzymatic and immunological evidence presented that both CA II and CA IV are present in urinary membranes from normal subjects, that renal CA IV is present but renal CA II is absent in urinary membranes from patients with the CA II-deficiency syndrome, and that the methods presented should be useful in studying renal CA II and renal CA IV in other disorders of impaired bicarbonate reabsorption.**

Human kidney contains two carbonic anhydrases (CAs) (1–5). One is the 29-kDa isozyme CA II, which is found in the cytosol of certain cells of renal tubules and collecting ducts. The other is a 35-kDa membrane-associated CA (CA IV), which is found in the brush border of the proximal tubules and basolateral membranes of certain cells (3–5). A role for CA II in proximal and distal urinary acidification was suggested by the findings in patients with the recessively inherited syndrome of osteopetrosis with renal tubular acidosis (6–8). All such patients so far identified have shown virtually nondetectable CA II in erythrocytes (9). Deficiency of CA II in the kidney of these patients has been inferred to explain the renal tubular acidosis (6, 8), although the unlikely possibility remained that the renal abnormalities were secondary consequences of the absence of CA II in erythrocytes (10).

Although the membrane-associated CA accounts for less than 5% of the total activity in kidney (2, 3), it plays a major role in HCO_3^- reabsorption by the proximal tubule. This conclusion was first suggested by micropuncture studies in which dextran-bound inhibitor—which could gain access to the luminal (membrane-associated) CA and not the cytosolic CA II—nevertheless inhibited 80% of HCO_3^- reabsorption by the proximal tubule (11). We suspected that the membrane-associated CA was functionally normal in CA II-deficient patients, since they showed a dramatic bicarbonaturic response to infused acetazolamide comparable to that seen in controls, which we could explain by inhibition of the membrane-associated CA in the proximal tubule (8). However, one could argue that this simply meant that the renal CA II was not affected by the mutation that produced the CA II deficiency in erythrocytes. In this work, we sought to address that question directly.

We previously purified CA IV from human lung and kidney and presented evidence that the 35-kDa membrane-anchored enzymes in both organs are identical (5). Since CA IV should be present in brush borders, and small amounts of several brush border enzymes appear in normal urine in membrane-associated and soluble forms (12), it seemed possible that we might demonstrate CA IV in normal urine and establish its presence or absence in CA II-deficient patients. That proved to be the case. In addition, the unexpected finding that normal urinary membrane vesicles also contained the cytosolic isozyme CA II permitted us to obtain direct evidence that renal CA II is absent in patients with recessively inherited osteopetrosis and renal tubular acidosis.

MATERIALS AND METHODS

Experimental subjects included eight healthy volunteers (6–50 years of age) and two sisters with the CA II-deficiency syndrome (29 and 36 years of age) (6). Urinary "brush border" membranes were isolated as described (12). Twenty-four-hour urine samples were collected in refrigerated containers in 1 mM benzamidine and stored at 4°C. After centrifugation at 3000 × *g* for 30 min to remove whole cells, membranes were sedimented by centrifugation at 100,000 × *g* for 1 hr. The supernatant at 100,000 × *g* was saved as the "soluble fraction." The pellet (crude membrane fraction) was resuspended in 1.35 ml of buffer A (25 mM triethanolamine, pH 8.1/59 mM Na_2SO_4/1 mM benzamidine), and 0.15 ml of 1% saponin was added.

Assay of CA. CA assay of the crude membrane fraction was based on the end-point titration method of Maren (13) as described (14). Membrane fraction (100 μl) was added to 4 ml of phenol red solution at 4°C into which CO_2 (600 ml/min) was bubbled. After 30 sec, 2 ml of barbitol buffer was added, and timing was begun. Timing was stopped when the indicator color matched that of the pH 7.2 standard. To measure SDS-resistant CA activity, SDS was added to the enzyme to 0.2% final concentration, and the mixture was incubated at room temperature for 30 min prior to assay of CA activity.

The inhibitor-affinity column was described by Osborne and Tashian (15). *p*-Aminomethyl benzenesulfonamide (0.56 g) in 0.1 M Mops buffer (pH 7.5) was coupled to Affi-Gel 10

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Abbreviations: CA, carbonic anhydrase; HLCA, human lung carbonic anhydrase.

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(Bio-Rad) according to the manufacturer's instructions. To prepare the antibody-affinity column, 50 mg of IgG purified from rabbit anti-human lung CA IV antiserum was coupled to 25 ml of Affi-Gel 10 according to the manufacturer's instructions. To purify CA IV from membrane fractions, membranes were solubilized by adding an equal volume of 10% SDS and incubating at room temperature for 30 min. After addition of 6 vol of buffer A, 1 vol of 5% deoxycholate, and 1 vol of 5% Triton X-100, the membrane fraction was applied to 1.0 ml of one of the two affinity columns (0.5 × 5.0 cm). The soluble fractions were applied directly onto the affinity columns without SDS. After the columns were washed with 20 ml of buffer A containing 0.1% Brij 35, enzyme was eluted from the inhibitor-affinity columns with 0.5 M $NaClO_4$ and from antibody-affinity columns with 3 M NaSCN. Four 1-ml fractions were collected, dialyzed separately twice against 2 liters of 10 mM Tris sulfate (pH 7.5) containing 1 mM benzamidine, and concentrated by use of Centricon 10 (Amicon).

Immunoblotting. Reducing SDS/PAGE was carried out as described by Laemmli (16). Nonreducing SDS/PAGE was done similarly except that 2-mercaptoethanol was omitted from the sample buffer. After SDS/PAGE, proteins were transferred to nitrocellulose membranes by use of Polyblot (American Bionetics, Emeryville, CA). After transfer, the immunoblots were stained with rabbit anti-human lung CA IV antiserum (anti-HLCA) (1:1000) and then with goat antiserum to rabbit IgG conjugated with alkaline phosphatase as described (5).

Phosphatidylinositol-specific phospholipase C treatment of urinary membranes was as described (17). The purified enzyme from *Bacillus thuringiensis* was the generous gift of Martin Low (Columbia University, New York). Triton X-100 phase separation was carried out as described (18).

RESULTS

CA Activity in Urinary Membrane Fraction. Urinary membranes might contain CA II and CA IV of renal origin and CA I and CA II from erythrocytes in urine. We found that CA I and CA II were promptly and completely inhibited by 0.2% SDS, in which purified CA IV was stable for at least 2 hr at room temperature. This provided a specific assay for CA IV. Further evidence that the SDS-resistant activity was CA IV was provided by differential inhibition studies with antiserum to CA IV (data not shown). Table 1 presents analyses of SDS-sensitive and SDS-resistant activities in urinary membranes from eight healthy volunteers. The SDS-sensitive activity ranged from 2.8–7.6 units/day per 1.73 m^2. The SDS-sensitive activity appeared to be CA II and not CA I, since less than 20% of the SDS-sensitive activity was inhibitable by 7.5 mM sodium iodide, a concentration that inhibits CA II by only about 20% but inhibits CA I completely (14).

Table 1. SDS-sensitive and SDS-resistant CA activity in urinary membrane fractions from healthy volunteers

Volunteer	Age, yr	Sex	CA activity, unit(s)/day per 1.73 m^2	
			SDS-sensitive	SDS-resistant
1	37	M	5.4*	1.7†
2	34	M	2.8	2.2
3	50	M	4.5	2.5
4	31	M	3.5	1.8
5	37	F	4.8	1.3
6	10	F	3.0	1.4
7	10	M	4.4	2.3
8	6	M	7.6	1.3

M, male; F, female.
*5.42 ± 0.94 (n = 11, mean ± SD).
†1.68 ± 0.41 (n = 29, mean ± SD).

The CA II activity appeared to be membrane-enclosed, since extensive washing of the urinary membranes by resedimentation in normal saline or 0.4 M KCl did not release the CA II activity unless the vesicles were permeabilized by adding saponin to the buffers. Furthermore, the rate of CO_2 hydration by the resuspended membranes was consistently stimulated by added saponin. Since neither purified CA II nor CA IV are stimulated by saponin, we interpret the stimulation of the membrane activity by saponin to result from permeabilization of the vesicles, which allows the HCO_3^- produced by the reaction to be released. We suggest that in the absence of saponin, the CO_2 hydration reaction becomes product-inhibited by the HCO_3^- accumulated in the vesicles.

The SDS-resistant CA IV activity in membranes from healthy volunteers ranged from 1.3 to 2.5 units/day per 1.73 m^2. The mean for 29 collections from volunteer 1 was 1.68 ± 0.41 units/day per 1.73 m^2. Thus, both CA II and CA IV were readily demonstrated enzymatically in the membrane fractions from all control subjects.

Table 2 presents the data from studies of two adult sisters known to have osteopetrosis with renal tubular acidosis and erythrocyte CA II deficiency (6). SDS-sensitive activity was near the lower limits of detection, suggesting the absence of CA II in the urinary membranes. SDS-resistant activity was present in at least normal levels in membranes from all three 24-hr urine collections from both subjects, suggesting that CA IV expression was not affected by the mutation producing CA II deficiency.

Immunodetection of CA II and CA IV in Urinary Membranes. Fig. 1 presents immunoblot data on CA II in the crude membrane fractions from three controls and from the two patients with CA II deficiency. CA II was detected as a prominent 29-kDa band in membrane fractions from control subjects but was nondetectable in the membrane fraction from the CA II-deficient patients. Thus, the immunological data support the enzymatic data showing the presence of CA II in normal urinary membranes and the absence of CA II from urinary membranes from these patients.

Fig. 2 presents immunoblotting data on CA IV from membrane fractions from two 24-hr urine collections from volunteer 1, of which one was purified over an inhibitor-affinity column and the other was purified over the antibody-affinity column. Both samples were analyzed by immunoblotting after reducing SDS/PAGE. Three bands were identified. The faintest is the band at 35 kDa, the position of native CA IV. Two more prominent bands are seen at 18.0 and 16.8 kDa. A smear of lower molecular weight material is also seen in the two-peak fractions. These data suggest that most of the CA IV in urinary membranes has been cleaved into 18.0- and 16.8-kDa fragments and some further degraded.

Triton X-114 phase separation before SDS/PAGE demonstrated that all three bands partition into the detergent phase, indicating that all three bands seen on reducing SDS/PAGE gels of the urinary membrane (Fig. 2) are from enzyme anchored to membrane (data not shown). However, more

Table 2. SDS-sensitive and SDS-resistant CA activity in urinary membrane fractions from sisters with CA II deficiency

Patient	Age, yr	CA activity,* unit(s)/day per 1.73 m^2	
		SDS-sensitive	SDS-resistant
K.K.	36	0.8 (0.2–1.4)	2.0 (1.7–2.2)
D.K.	29	0.2 (−0.7–0.9)	1.8 (1.5–2.2)

*Values are means of three measurements; numbers in parentheses show the range of three measurements.

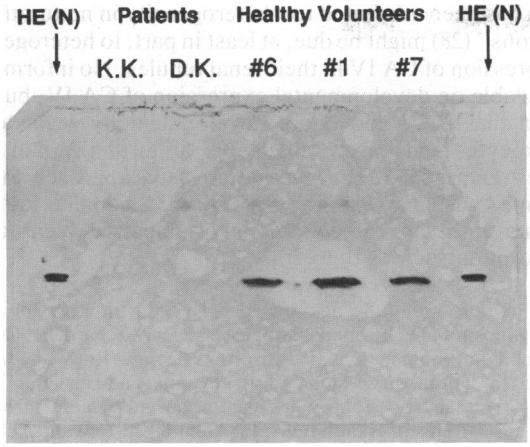

FIG. 1. Immunochemical detection of CA II in membrane fractions from CA II-deficiency patients and healthy volunteers on reducing SDS/10% PAGE. Twenty microliters of the membrane fractions were loaded. Lanes: HE(N), dehemoglobinized hemolysate extract from a healthy volunteer; K.K. and D.K., initials of the patients; #6, #1, and #7, healthy volunteers as shown in Table 1. The first antibody was rabbit anti-human CA II from Richard Tashian (Ann Arbor, MI).

than 90% of the SDS-resistant catalytic activity was released when the urinary membranes were treated with phosphatidylinositol-specific phospholipase C (data not shown). This result indicated that most of the CA IV in urinary membranes is attached to the membranes by a phosphatidylinositol glycan anchor, as is true for other brush border enzymes (19).

We suspected that the three major bands seen on reducing SDS/PAGE represent the 35-kDa membrane-anchored native CA IV and two fragments produced by proteolytic cleavage of the 35-kDa enzyme that are still held together by disulfide bonds and anchored to membrane through one of the peptide fragments. If this interpretation is correct, one should see only the 35-kDa form on nonreducing SDS/PAGE. Fig. 3 shows the experiment that tested this prediction. Here one sees only a single 35-kDa band on nonreducing SDS/PAGE, both in the inhibitor-purified enzyme and in the

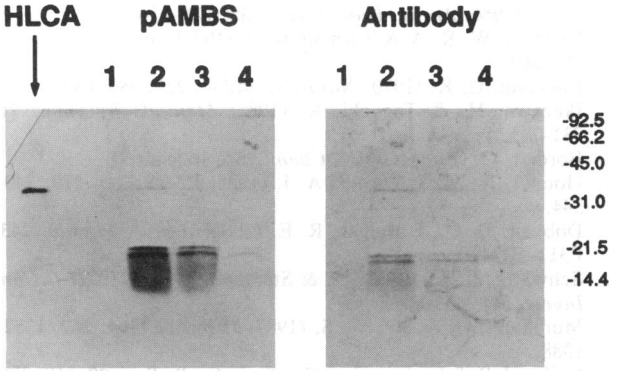

FIG. 2. Immunochemical detection of urinary membrane CA IV from healthy volunteers on reducing SDS/15% PAGE. Urinary membrane CA IV was purified through inhibitor (pAMBS)- and antibody (anti-HLCA IV)-affinity columns after SDS solubilization as described. The 1-ml fractions were dialyzed, concentrated to 50–100 μl by Centricon 10. One-third of the retentates from inhibitor-affinity column and one-sixth of the retentates from antibody-affinity column were loaded onto SDS/15% PAGE and analyzed by immunoblotting. All samples were loaded after reduction and denaturation. Lanes: HLCA, CA IV (5 ng) purified from human lung; pAMBS 1–4, fractions eluted from the inhibitor-affinity column; antibody 1–4, fractions eluted from antibody-affinity column. The migration positions of molecular mass markers in kDa (Bio-Rad) are shown to the right of the gel.

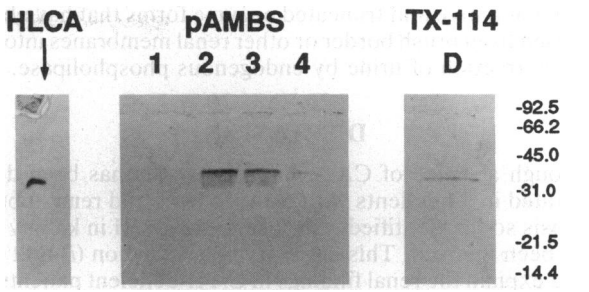

FIG. 3. Immunochemical detection of urinary membrane CA IV from healthy volunteer on "nonreducing" SDS/15% PAGE. HLCA IV (5 ng) was loaded onto SDS/PAGE after reduction and denaturation. Affinity-purified CA IV fractions from a 24-hr urine collection (lanes pAMBS 1–4) and some CA IV in the detergent phase from the Triton X-114 extraction (lane D) were loaded on SDS/PAGE after "nonreducing" denaturation (denaturation in SDS without 2-mercaptoethanol).

detergent-rich phase after Triton X-114 phase separation. These results supported the hypothesis that the 18.0- and 16.8-kDa bands represent the cleaved N-terminal and C-terminal fragments of CA IV in urinary membranes. Other experiments (data not shown) indicated that the cleaved enzyme is anchored to membranes through the 16.8-kDa polypeptide fragment.

Fig. 4 presents immunoblot data on enzyme purified by antibody-affinity columns from membrane fractions from single 24-hr urine collections from each of two CA II-deficient patients. Both CA II-deficient patients showed the same three specifically stained bands that were seen in membranes from healthy controls. Although less of the 35-kDa CA IV was cleaved to smaller fragments than in the membranes from the controls, the total amount of immunoreactive CA IV was similar to that seen in membrane for controls. The immunoblot data confirmed the enzymatic analyses, which indicated at least normal levels of CA IV in membranes from CA II-deficient patients.

Immunodetection of CA IV in the Soluble Fractions from Urine. When the soluble fractions (100,000 × *g* supernatants) from normal and CA II-deficient patients were purified by inhibitor-affinity or antibody-affinity chromatography and analyzed by immunoblot after SDS/PAGE, 35-kDa and 33.7-kDa bands were observed in all samples (data not shown). Both the 35- and 33.7-kDa bands partitioned into the aqueous phase on Triton X-114 separation, indicating that they lacked the hydrophobic membrane anchor. These two bands may

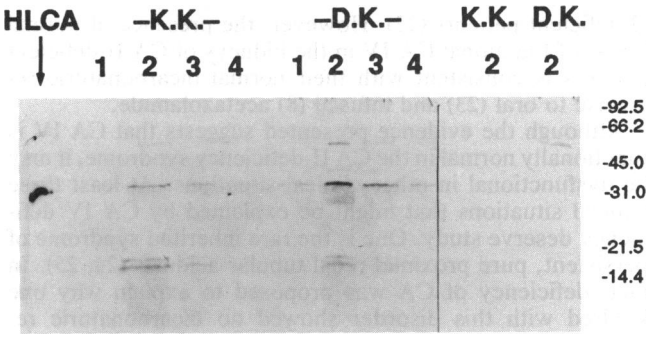

FIG. 4. Immunochemical detection of urinary membrane CA IV from CA II-deficiency patients on reducing SDS/15% PAGE. Samples were purified through antibody-affinity column, eluted in four 1-ml fractions, and concentrated after dialysis. One-third of the retentates were loaded onto SDS/15% PAGE. Lanes: HLCA, CA IV (5 ng) purified from human lung; K.K. 1–4 and D.K. 1–4, fractions eluted from antibody-affinity column from 24-hr urine collection; K.K.2 and D.K.2, preimmune serum was used instead of anti-HLCA IV in the immunoblotting of fraction 2.

represent native and truncated enzyme forms that have been released from brush border or other renal membranes into the soluble fraction of urine by endogenous phospholipase.

DISCUSSION

Although absence of CA II in erythrocytes has been demonstrated in all patients with osteopetrosis and renal tubular acidosis so far identified, the absence of CA II in kidney has only been inferred. This inference was based on (*i*) that one could explain the renal findings in CA II-deficient patients by CA II deficiency in the kidney (8), and (*ii*) that transfusion of CA II-deficient patients with CA II-replete erythrocytes failed to correct their urinary abnormalities, making it less likely that they were secondary to erythrocyte CA II deficiency (10). The unexpected finding of CA II in urinary membranes from normal humans made it possible for us to address this question directly. This opportunity was unexpected because CA II is a cytosolic enzyme that is not found in renal brush borders (1, 20). Its presence in normal urinary membranes suggests that the urinary membranes include, in addition to brush border membranes, vesicles from the distal tubules and collecting ducts, where cytosolic concentrations of CA II are the greatest (1). The absence of CA II in the same fractions from the two patients with osteopetrosis and renal tubular acidosis provided the first direct evidence that CA II is absent in their kidneys as well as their erythrocytes.

The absence of CA II in proximal tubules can explain the proximal component of the renal tubular acidosis seen in many patients with this syndrome. CA II is presumably required in the cytosol of the proximal tubule cells to regenerate H^+ ions from absorbed CO_2 at a rate sufficient to sustain maximal rates of HCO_3^- reabsorption (9). The absence of CA II in distal tubules would explain the distal acidification defect. Here CA II is required in the cytosol to titrate the OH^- produced on the cytosolic face of the membrane by the proton translocating Mg^{2+}-ATPase which translocates H^+ ions across the apical membrane into the lumen (9). In the absence of CA II to remove the OH^- ions by forming HCO_3^-, the distal tubular cells have a limited ability to generate an H^+ ion gradient and acidify the lumen. A similar CA requirement for acidification of the lumen has been demonstrated in certain cells of the turtle bladder (21).

The finding of at least normal levels of CA IV activity and immunoreactivity in the urine of patients with CA II deficiency indicates that the mutation producing CA II deficiency does not affect the expression of CA IV. This result could not have been predicted *a priori*, since salivary CA (CA VI) expression was observed to be markedly depressed in CA II-deficient patients (22). However, the presence of normal levels of functional CA IV in the kidneys of CA II-deficient patients is consistent with their normal bicarbonaturic response to oral (23) and infused (8) acetazolamide.

Although the evidence presented suggests that CA IV is functionally normal in the CA II-deficiency syndrome, it may be dysfunctional in other clinical situations. At least three clinical situations that might be explained by CA IV deficiency deserve study. One is the rare inherited syndrome of persistent, pure proximal renal tubular acidosis (24, 25). In fact, deficiency of CA was proposed to explain why one kindred with this disorder showed no bicarbonaturic response to infused acetazolamide (24). Second, congenital, transient, pure proximal renal tubular acidosis, which tends to improve with age (26, 27), could be due to a mutational alteration in the developmental program of CA IV expression. In other words, CA IV might fail to be induced at the normal time and only appear later in infancy or childhood. Finally, the "physiologic" renal tubular acidosis of infants, which has been attributed to "heterogeneity in maturation of nephrons" (28) might be due, at least in part, to heterogeneity in expression of CA IV in their renal tubules. No information is available on developmental expression of CA IV, but it is known that CA I and II are developmentally regulated in erythrocytes and barely detectable at birth (29, 30). The studies reported here suggest a noninvasive approach and the reagents to use in examining CA IV expression in urine from patients with clinical conditions of impaired bicarbonate reabsorption such as these.

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