Carbonic Anhydrase in the Human Fetal Kidney

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Summary

Kidney tissue from human fetuses of gestational ages ranging 12-26 wk were studied by histochemical and biochemical methods. Clear staining for carbonic anhydrase activity was found in both proximal and distal parts of some nephrons already at gestational age of 12-15 wk. The staining pattern in the inner cortex of the fetuses of 24 and 26 wk did not differ markedly from that in adult renal cortex, except for the absence of capillary staining; proximal and distal convoluted tubules as well as initial collecting tubules exhibited distinct enzyme activity. In the outer cortex, where the nephronogenic zone is located, newly formed nephrons with no or little staining were found in all kidneys. Loops of Henle were absent or sparse in the medulla of the fetuses of 12-17 wk. Fully developed loops of Henle were relatively few also in the fetuses of 24 and 26 wk; the thick ascending limb and part of the thin limb were stained in such loops. The collecting ducts in all kidneys contained a varying number of intensely stained cells interspersed among unstained or weakly stained ones.

The catalytic activity and the immunoassayable amount of carbonic anhydrase were determined in homogenates of renal cortex from three fetuses (19, 21, and 26 wk). The kidney from the fetus of 26 wk showed the highest values with an enzyme activity and an immunoassayable amount of HCA-C corresponding to 204 enzyme units and 0.14 mg enzyme per g wet weight of tissue, respectively. These values are similar to those of adult renal cortex and indicate that all of antigenically determined HCA-C is also catalytically active in the kidney at this gestational age.

As judged from their content of carbonic anhydrase many nephrons might be fully able to reabsorb bicarbonate and secrete hydrogen ions in human fetuses of 24-26 wk. Other nephrons are much less developed at this stage, however. This nephronic heterogeneity might at least partly explain the reduced capacity for urinary acidification which has been reported for premature newborn infants.

The enzyme carbonic anhydrase (carbonate dehydratase EC 4.2.1.1.) is an extremely efficient catalyst of the forward and reverse reaction: $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3$. It is present in erythrocytes and many electrolyte transporting epithelia (e.g., stomach, pancreas, ciliary epithelium) where it is thought to have an effect on the transfer of CO_2 , H^+ , HCO_3^- and Cl^- (19). Histochemical (13) and biochemical (21, 37, 40) studies of the adult human kidney have shown that the enzyme is present in many different parts of the nephron. Carbonic anhydrase is critical for efficient operation of the urinary acidification process; inhibition of the enzyme markedly reduces the rate of renal bicarbonate reabsorption and hydrogen ion secretion (35).

The distribution and function of carbonic anhydrase in the human fetal kidney have apparently not been studied. Several authors, however, have reported that the capacity for urinary acidification is reduced in newborn human infants, especially prematures (1, 4, 9, 29, 32, 33).

We have presently studied the distribution of carbonic anhydrase in the kidneys of human fetuses with a gestational age of 12-26 wk by a histochemical technique and determined the activity and isoenzyme pattern of the enzyme in renal cortex homogenates from fetuses of 19-26 wk by means of a new radioimmunosorbent method. The aim of the study was to contribute to the understanding of the urinary acidification process in the fetus and the premature newborn infant.

Detailed observations on the structures of the nephronogenic zone are not included in the present report. They will be presented in a forthcoming paper.

MATERIALS AND METHODS

Tissue preparation. Kidney tissue was obtained from eight human fetuses, removed by cesarean section. In seven cases (gesta-

Fig. 1. Renal cortex from a fetus of 12 wk. The nephronogenic zone under the renal capsule (*above*) shows no or little staining. Several intensely stained tubules are seen in the juxtamedullary zone of the cortex (*below*). Unfixed, 4- μm thick section. Incubation time, 12 min. Counterstaining with hematoxylin and eosin (H & E). $\times 200$.

tional ages 12, 15, 17, 19, 21, 23, and 24 wk) legel abortion was performed in healthy women for non-medical reasons; these women had not been taking any drugs regularly. In one case (gestational age 26 wk) the fetus was removed because of severe mitral valve disease in the mother. This woman had taken digoxin, quinidine, warfarin, and phenytoin throughout the pregnancy. Gestational age was determined from the patient history and the fetal crown-heel length (36). Tissue preparation was begun within 30 min after removal of the fetus. Some pieces of kidney tissue were frozen immediately, whereas others were fixed by immersion in 2.5% glutaraldehyde + 0.1 M phosphate buffer (pH 7.4) at 4°C for about 24 h. After a brief rinse in distilled water, some pieces of fixed tissues were rapidly frozen, whereas others were embedded in the water-soluble resin JB-4 (Polysciences, Warrington, PA, USA) as described by Ridderstråle (27). Frozen tissues were stored at -70°C until used.

Histochemical staining procedure. Kidney tissue from all fetuses except one (gestational age 19 wk) was stained for carbonic anhydrase activity. Only frozen sections were available from the fetuses of 12, 15, and 17 wk; in the former case only unfixed sections, and in the latter two both fixed and unfixed. Both resin-embedded and frozen (fixed and unfixed) sections from the other fetuses were stained for carbonic anhydrase.

The histochemical method of Hansson was used (7, 8). In this method sections are floated on a medium containing 157 mM NaHCO₃, 1.75–3.5 mM CoSO₄, 11.7 mM KH₂PO₄ and 53 mM H₂SO₄. The pH of the medium is 5.8 immediately after mixing.

Fig. 2. Renal cortex, juxtamedullary zone (detail of Fig. 1). P is probably a proximal tubule, and the other stained structures distal tubules and/or initial collecting tubules. $\times 800$.

Fig. 3. Renal medulla from a fetus of 12 wk. One stained and two unstained collecting ducts (C) are seen. The surrounding interstitium is unstained. Staining as Figure 1. $\times 600$.

Carbonic anhydrase catalyzes the dehydration of HCO₃⁻ to CO₂ and OH⁻. Continuous local OH⁻ formation at sites of enzyme activity causes deposition of a basic cobalt-phosphate complex, which is converted to CoS. Thus, a black precipitate is formed where carbonic anhydrase is present. Sections, 4–8 μ m thick, of frozen tissues were sectioned in a cryostat at -20°C and incubated for 1–12 min with 1.75 mM CoSO₄ in the medium. Unfixed sections were supported on Millipore filters (8). Sections 1–2 μ m thick of resin-embedded tissues were incubated for 1–8 min with 1.75 mM CoSO₄ in the medium (27). Some sections were counterstained with hematoxylin and eosin (H&E). The sections were mounted in Eukitt.

Biochemical methods. Renal cortex from three fetuses (gestational ages 19, 21, and 26 wk) was assayed. After thawing and gentle blotting on absorbent paper, the tissue was homogenized in nine parts of distilled water, which contained 1 mM EDTA (sodium salt) to protect the enzyme from inactivation by heavy metal ions. An aliquot of the homogenate was taken for assay of carbonic anhydrase activity. The homogenate was centrifuged at 100,000 $\times$ g for 60 min. The supernatant was assayed for carbonic anhydrase activity and for its content of protein, hemoglobin, and the two human carbonic anhydrase isoenzymes HCA-B and HCA-C. In one case the pellet was resuspended and assayed for carbonic anhydrase activity. Hemoglobin and carbonic anhydrase activity were determined in a blood sample from each fetus. These data were used to correct for contamination of the tissues with blood.

Carbonic anhydrase activity of the tissue homogenates was measured by the changing pH method of Philpot and Philpot (25).

Carbonic anhydrase proteins were assayed by a radioimmunosorbent technique (39), using antibodies selective against the human erythrocyte isoenzymes HCA-C (anti-HCA-C) and HCA-B (anti-HCA-B). The sensitivity of this method is 0.2 ng enzyme protein/ml of tissue fluid and the precision is 5% in duplicate determinations. Protein was assayed by the method of Lowry *et al.* (12) and hemoglobin by the cyan-methemoglobin technique (10). Further data on the biochemical methods used are given in a recent paper by Lönnérholm and Wistrand (17).

RESULTS

Histochemical findings. In a fetus of 12 wk, little or no staining was found in the nephrogenic zone located under the renal capsule (Fig. 1). In the inner cortex, *i.e.*, its juxtamedullary part, a relatively small number of nephrons were seen. The glomeruli were unstained, but some tubular structures showed distinct staining for carbonic anhydrase activity (Figs 1 and 2). The different parts of the cortical tubules could not be identified with certainty because only unfixed tissue was available from this kidney. Probably, the large tubules with predominantly luminal staining represent proximal tubules with intensely stained brush-border, whereas the smaller ones represent distal segments of the nephron, *i.e.*, distal tubules or initial collecting tubules (Fig. 2).

In the medulla only few tubular structures were found, sur-

Fig. 4. Inner cortex from a fetus of 15 wk. *P* is a proximal tubule continuous with its glomerulus (*G*). Other stained proximal tubules are also seen. The two small tubules to the right of *G* (arrows) are probably initial collecting tubules. Fixed, 8- μ m thick section. Incubation time, 12 min. Counterstaining with H & E. $\times 500$.

Fig. 5. Inner cortex (*above*) and outer medulla (*below*) from a fetus of 17 wk. Most tubular structures in the medulla are collecting ducts. Fixed, 8- μ m thick section. Incubation time 12 min. $\times 200$.

rounded by abundant interstitial tissue. They were identified as collecting ducts. In the outer medulla a few stained collecting ducts were seen (Fig. 3). In the inner medulla, close to the renal pelvis, no staining was observed.

The staining pattern in the kidneys of a fetus of 15 wk was similar to that of the 12-wk fetus. Figure 4 shows a proximal tubule continuous with its glomerulus; the whole tubular cells, including the brush-border region, are intensely stained for carbonic anhydrase activity.

In a fetus of 17-wk the staining of the cortex was similar to that described above, with clear staining of some proximal tubules as well as of some distal segments of the nephron (Fig. 5). Most of the tubular structures in the medulla could be identified as collecting ducts. Their enzyme activity varied; some were unstained, whereas others contained a number of intensely stained cells interspersed among unstained ones (Fig. 6).

In a fetus of 21 wk the renal cortex was thicker and contained a larger number of nephrons than in the more immature kidneys (Fig. 7). Because both resin-embedded and frozen sections were available from this kidney, a more detailed analysis could be made than in the kidneys described above, from which only frozen sections were available.

In the cortex, distal convoluted tubules could be identified. Most of their staining was located at the cell membranes (basal, lateral, and apical) in resin-embedded sections (Fig. 8). In frozen sections, however, stain deposits were found in the whole of the distal tubular cells. Initial collecting tubules were also identified.

Fig. 6. Renal medulla (detail of Fig. 5). One collecting duct in the center shows staining of most cells, while another duct has only a few stained cells (arrows). An unstained collecting duct is seen to the left. Note the relative sparsity of tubular structures and the abundance of interstitium. $\times 500$.

They contained a mosaic of intensely stained and unstained cells (Fig. 8, arrows); intermediate forms were also found. Many proximal convoluted tubules showed heavy staining, especially in the brush-border region (Fig. 8).

In the medulla several loops of Henle were seen. Thick ascending limbs of loops of Henle showed staining similar to that of distal convoluted tubules, *i.e.*, distinct staining at basal, lateral, and apical cell membranes in resin-embedded sections (Fig. 9), and more diffuse staining in frozen sections. Unstained tubules, probably identical with thin limbs of loops of Henle, were sometimes found (Fig. 9). The collecting ducts showed a varying number of stained cells as described above.

In the kidneys of a fetus of 23-wk the cortical structures were particularly well preserved. Figure 10 shows proximal convoluted tubules with patent lumen and well preserved brush border. Most of the staining was found at the brush-border of these tubules. Because they were located close to the nephronogenic zone, they probably belong to newly formed nephrons. Proximal tubules located in the deeper layers of the cortex and belonging to more mature nephrons exhibited distinct cytoplasmic staining in addition to the stained brush-border (not shown, but *cf.* Figs. 4 and 8). The distal tubules and initial collecting tubules were stained as described for the fetus of 21 wk.

The renal medulla of a fetus of 24 wk contained a greater

number of tubular structures than that of the fetus of 17 wk (*cf.* Fig. 11 with Fig. 5). Compared to the adult kidney, the interstitium of the medulla was still relatively abundant in the fetus of 24 wk, however. Stained thick ascending limbs and collecting ducts were found in the outer medulla. Stained and unstained thin limbs of loops of Henle were also found in the medulla. They were identified with certainty in the inner medulla (Figs. 12 and 13), where thick limbs are absent (34). It could not be decided whether the stained segments represented the descending or the ascending part of the thin limb. The staining, when present, was weak and located basally in the cells of the thin limb (Fig. 12). In the collecting ducts the number of stained cells decreased from the outer to the inner medulla (Fig. 12). Collecting ducts in the tip of the papilla were completely unstained (Fig. 13).

In a fetus of 26 wk, formation of new nephrons was still evident in the nephronogenic zone of the cortex. Little or no staining was found in the tubules of these nephrons. In the deeper layers of the cortex, the tubules showed a staining pattern similar to that described above for the fetuses of 21–24 wk. The staining of the medulla was similar to that seen in the fetus of 24 wk.

The renal vessels, the interstitial cells, the glomeruli and Bowman's capsule were unstained in all kidneys. Red blood cells were unstained in the fetuses of 12–17 wk, whereas weak staining was sometimes found in the fetuses of 21–26 wk.

Fig. 7. Renal cortex from a fetus of 21 wk. There is little staining in the nephronogenic zone under the renal capsule (above). Unstained glomeruli, surrounded by stained proximal, distal and initial collecting tubules, are seen in the deeper parts of the cortex. Resin-embedded, 2- μ m thick section. Incubation time, 8 min. Counterstaining with H & E. $\times 200$.

Fig. 8. Renal cortex (detail of Fig. 7). The lumens of two proximal tubules (*upper right*) are filled with intensely stained brush border. In the distal tubule (*above D*) the staining is restricted to the cell borders. In the cortical collecting tubule a few unstained cells (*arrows*) are seen among heavily stained ones. $\times 1000$.

Throughout the study the specificity of the staining procedure was checked by incubation of sections in the presence of $10\text{ }\mu\text{M}$ acetazolamide (Diamox, American Cyanamid Company, Pearl River, New York), a specific inhibitor of carbonic anhydrase (19). This concentration of acetazolamide completely abolished visible staining (Fig. 14), whereas the presence of $10\text{ }\mu\text{M}$ of the inactive control substance C1 13850 (18), a N^5 -t-butyl analogue of acetazolamide (American Cyanamid Company), did not interfere with the staining. Sections incubated in the medium without any substrate, *i.e.*, sodium bicarbonate, remained unstained.

Biochemical findings. The carbonic anhydrase activity in homogenates of renal cortex was higher in the fetus of 26 wk than in those of 19 and 21 wk (Table 1). The difference was about 5-fold in whole homogenates and about 2.5-fold in the supernatant fraction. The data from one kidney (gestational age 26 wk) suggest that most of the enzyme is in a soluble form because 70% of the total activity was recovered in the supernatant fraction and 10% in the pellet.

Large amounts of the carbonic anhydrase isoenzyme HCA-C were found in the supernatant fraction of all kidneys (Table 1). Only small amounts of HCA-B were found, and they were probably attributable to contaminating erythrocytes.

The immunoassayable amounts of HCA-C, 1.86 mg per g protein in the supernatant fraction from the fetus of 26 wk corresponds to about 0.14 mg HCA-C per g tissue. Because the

specific activity of pure HCA-C is 1500 enzyme units per mg HCA-C (38) it may be calculated that the cortex of this fetus should contain an enzyme activity of 210 units per g tissue, which agrees well with the measured value of 204 units per g tissue (Table 1). This implies that the majority of antigenically active HCA-C is also catalytically active in these developing kidneys.

The mean enzyme activity in the blood from the three fetuses was 14.7 enzyme units per ml whole blood (range 11.8–16.6). The mean HCA-C concentration in fetal blood was 131 (range 115–159) and the mean HCA-B concentration 73 (range 34–123) ng enzyme per mg hemoglobin.

DISCUSSION

Evidence for the validity of the histochemical method used here has been produced by several investigators (7, 8, 14, 28). Muther (23, 24) has questioned the specificity of the method, but the controversy caused by his criticism has been fully resolved by recent work in our (15) and other laboratories (11, 20, 30, 31). In the present study tissues were prepared in different ways for the incubation. The findings were similar (although resin-embedded tissue allowed more detailed observations than frozen tissue), which argues against artifacts caused by diffusion or inactivation

Fig. 9. Renal medulla from a fetus of 21 wk. A collecting duct contains two heavily stained cells (*arrow heads*). Two thick ascending limbs of loops of Henle show staining at the cell borders, especially basally. The unstained tubule (*arrow*) is probably a thin limb. The structures in the interstitium lack staining. Resin-embedded, $2\text{-}\mu\text{m}$ thick section. Incubation time, 8 min. Counterstaining with H & E. $\times 800$.

Fig. 10. Renal cortex, close to the nephrogenic zone from a fetus of 23 wk. The proximal tubules (*lower left*) are stained mainly at the brush border. *D* is a distal tubule. *IC* is an initial collecting tubule. Resin-embedded, 1- μ m thick section. Incubation time, 8 min. $\times 1400$.

of the enzyme. The only clear-cut exceptions were thick ascending limbs and distal convoluted tubules, where the degree of cytoplasmic staining differed between frozen and resin-embedded sections. It is not clear at present whether the more pronounced cytoplasmic staining in the frozen sections was due to diffusion artifacts or to better preservation of cytoplasmic enzyme activity.

These fetal human kidneys studied histochemically cover a wide range of gestational ages, 12–26 wk. Clear staining for carbonic anhydrase activity was found both in proximal and distal parts of the nephron already at gestational ages of 12 and 15 wk. Some proximal tubules in these kidneys, as well as in the more developed ones, showed intense staining both in the cytoplasm and in the brush-border region, which is similar to the staining of proximal convoluted tubules in the adult human kidney (13). Distal tubules and initial collecting tubules could be identified with certainty in the fetuses of 21–26 wk, and the staining was similar to that observed in adults. More extensive invaginations of the stained basolateral cell membranes are seen in distal convoluted tubules of adult kidneys, however (16).

The staining pattern in the inner cortex of the fetuses of 24 and 26 wk did not differ markedly from that in adult renal cortex, except for the absence of capillary staining. Enzyme staining was widely distributed in the kidney tubules, as discussed above. In the outer cortex, however, newly formed nephrons with little or

no carbonic anhydrase activity were found. Thus, a marked nephronic heterogeneity was apparent in the cortex of these fetuses.

A few stained collecting ducts were found already in the renal medulla of the fetus of 12 wk. Loops of Henle were absent or sparse in the medulla of the fetuses of 12–17 wk, but increased in number with gestational age. The renal medulla of the fetuses of 24 and 26 wk differed from adult renal medulla mainly by its smaller number of loops of Henle. This is explained by the fact that newly formed nephrons in the fetal kidney have short loops, which only penetrate the outer medulla, or do not even extend below the corticomedullary junction (26). The thin limb of such short loops was usually unstained, whereas the thin limb of fully developed loops, extending into the inner zone of the medulla, often had a stained segment. Thus, nephronic heterogeneity was observed also in the medullary parts of the kidney tubules in the fetuses of 24 and 26 wk.

The lack of staining in the capillaries of all fetal kidneys studied is worthy of note because many capillaries in the adult human kidney are clearly stained for carbonic anhydrase activity, especially in the cortex (13, 16). The functional significance of the lack of enzyme in the fetal capillaries cannot be judged at present because the function of carbonic anhydrase in the renal capillaries of adults is unknown.

Fig. 11. Inner cortex (*above*) and outer medulla from a fetus of 24 wk. The stained structures in the medulla are collecting ducts and loops of Henle. Fixed, 8- μ m thick section. Incubation time, 12 min. Counterstaining with H & E. $\times 175$.

Fig. 12. Inner medulla from a fetus of 24 wk. Collecting ducts (C) show only a few intensely stained cells. A thin limb (at right of H) exhibits weak staining in the basal part of the cells. Resin-embedded, 2- μ m thick section. Incubation time, 8 min. $\times 500$.

Fig. 13. Renal papilla from a fetus of 24 wk. A few stained thin limbs of loops of Henle are seen close to the papillary tip. Note the unstained collecting ducts (arrows) and papillary surface epithelium. Fixed, 8- μ m thick section. Incubation time, 12 min. Counterstaining with H & E. $\times 140$.

The data for enzyme activity and isoenzyme concentrations from the fetus of 26 wk (virtually all of the enzyme in the supernatant fraction from all fetuses was in the form of the isoenzyme HCA-C) are similar to those reported for adult renal cortex (37). This is in agreement with the findings of Day and Franklin (3) and Berfenstam (2), who reported that the level of carbonic anhydrase activity in the renal cortex of premature infants was similar to or only slightly lower than in full term infants and adults. The enzyme activity of whole kidney homogenates from fetuses of 2–5 months was considerably lower than in full term infants, however (2).

The mean carbonic anhydrase activity in whole blood from the fetuses of 19–26 wk was only about 3% of that in whole blood from adults, which explains the weak staining of the red blood cells. The HCA-C concentration was about 8% and the HCA-B concentration about 0.6% of that in adult blood (6, 39). These low values are not surprising because the concentrations of HCA-B and HCA-C in the blood of newborn full-term infants are known to be less than 10% of those in adult blood, with even lower values in premature infants (22).

In summary, the present data show that some nephrons possess considerable carbonic anhydrase activity already during early fetal life, i.e., at 12–15 wk of gestation. The amount of carbonic anhydrase in the kidney increases with gestational age. As judged from their content of the enzyme, many nephrons might be fully able to reabsorb bicarbonate and secrete hydrogen ions at a

Table 1. *Carbonic anhydrase in homogenates of fetal human renal cortex*

Gestational age (wk)	E.u./g wet weight of tissue ^{1,2}	Corrected for blood contamination		
		E.u./mg protein in supernatant ³	HCA-B ng/mg protein in supernatant ⁴	HCA-C ng/mg protein in supernatant ⁵
19	40	1.0	0.3	725
21	45	1.1	3.0	1020
26	204	2.6	0.4	1860

¹ E.u. is enzyme units.

² The enzyme activity in the whole homogenate could not be corrected for blood contamination because turbidity prevented determination of the hemoglobin concentration.

³ Less than 1% of the total activity of the supernatant fraction was attributable to carbonic anhydrase from red blood cells.

⁴ 32–88% of the total amount was attributable to red blood cells.

⁵ Less than 1% of the total amount was attributable to red blood cells.

gestational age of 24–26 wk. Other nephrons are much less developed at this stage, however, regarding both cell structure and carbonic anhydrase content. This nephronic heterogeneity might at least partly explain the reduced capacity for urinary acidifica-

Fig. 14. Complete inhibition by 10 μ M acetazolamide. Inner cortex (above) and outer medulla from a fetus of 26 wk. Resin-embedded, 2- μ m thick section. Incubation time, 8 min. Counterstaining with H & E. $\times 200$.

tion which has been reported for premature newborn infants (29). Bicarbonate titration studies in 1-month-old infants have indicated that nephronic heterogeneity still persists and probably explains the low plasma threshold of bicarbonate found at this age (5).

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