Immature Renal Tubules Are Resistant to Prolonged Anoxia

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ABSTRACT. Very few data are available regarding the decreased susceptibility of the developing kidney to anoxia. Therefore, the purpose of this study was to develop an experimental system that would allow comparison of an anoxic insult in immature and mature proximal tubule segments and to investigate the hypothesis that the developing kidney is resistant to anoxia as compared with the mature kidney. Suspensions of proximal tubules from immature (age 8–10 d) and mature (8–10 wk) rats were obtained. The purity of the tubule suspension from the immature rats was documented by villin staining. A common buffer solution was developed to compare results from the immature and mature tubules. To study the response of the tubules to anoxia, we subjected the tubule suspension from both the immature and mature rats to 15, 30, 45, and 60 min of anoxia. Lactate dehydrogenase release was measured to assess plasma membrane damage, and ATP levels were determined as an index of cellular energy. After a short anoxic insult (15 or 30 min), the percentage of lactate dehydrogenase release was not significantly different from mature tubules. After prolonged anoxia (45 and 60 min) lactate dehydrogenase release continued to increase, whereas membrane integrity stabilized in the immature tubules. ATP levels decreased in both immature and mature tubules after anoxia, but the decline of ATP was greater in the mature tubules, with a plateau at 20% of basal ATP levels as compared with 40% in the immature tubules. Therefore, the developing kidney is resistant to prolonged anoxia. (*Pediatr Res* 35: 152–156, 1994)

Abbreviations

LDH, lactate dehydrogenase

On the basis of clinical observations, the immature kidney has been considered to be more resistant to an ischemic or hypoxic injury than an adult kidney. This assumption is based on the fact that acute renal failure seldom develops in premature infants with respiratory distress syndrome despite severe hypoxemia, limited renal perfusion, and oxygen delivery. Studies of tissues other than the kidney of newborns have documented resistance to anoxic injury (1). However, few data are available regarding the putative resistance or decreased susceptibility of the developing kidney to oxygen deprivation. Therefore, the objectives of this study were as follows: 1) to develop an experimental system that would allow an accurate and reproducible comparison of an anoxic insult to immature and mature proximal tubule segments,

and 2) to investigate the hypothesis that the developing kidney is less susceptible to anoxia as compared with the mature kidney.

MATERIALS AND METHODS

Preparation of Suspensions Enriched in Tubule Segments. To obtain tubule segments from immature and mature rat kidneys, we used a modification of our previous procedures (2). Mature rats (8–10 wk of age) were anesthetized with Inactin, and the aorta was carefully cannulated with PE-90 tubing distal to the origin of both renal arteries. To initiate the harvest procedure, we incised the left renal vein and flushed the kidneys with 15 mL of buffered Ringer's solution containing metabolic substrates that had been equilibrated with 95% O₂/5% CO₂ at 37°C. The kidneys were perfused with a collagenase solution [60 mg collagenase (Sigma Chemical Co., St. Louis, MO), 6 mg protease (Sigma) dissolved in 60 mL of buffered Ringer's solution heated to a temperature of 46°C] for 20 min and then flushed with 10 mL of cold buffered Ringer's solution. Both kidneys were rapidly excised and placed on ice while the renal capsules were removed and the cortex was gently scraped. The tubules were suspended in 25 mL of buffered Ringer's solution, placed on ice, and stirred for 15 min. The suspension was filtered through a 115-μm fabric mesh (Tetko) and then washed and spun at 500 rpm three times for 2 min. The tubule suspension was equilibrated with 95% O₂/5% CO₂ and kept at 4°C until studied. The final pellet was resuspended with the buffered Ringer's solution to a concentration of approximately 2 mg protein/mL.

To obtain tubules from the immature rats (8–10 d of age), we anesthetized the pups with ether, excised the kidneys, and placed them in a buffered Ringer's solution. The capsules were removed, and the kidneys were immersed in a collagenase solution (20 mg collagenase, 2 mg protease dissolved to 10 mL buffered Ringer's solution) and placed in a shaking water bath at 46°C. After 15 min, the kidneys were placed on ice, and the cortex was gently scraped. Tubules were suspended in 25 mL of cold buffered Ringer's solution and stirred for 15 min. The suspension was filtered through a 50-μm fabric mesh (Tetko Inc., Briarcliff Manor, NY) to eliminate glomeruli. The suspension was equilibrated with 95% O₂/5% CO₂, then washed, spun at 500 rpm three times for 2 min, and kept at 4°C until studied.

Documentation of Purity of Enriched Proximal Tubule Suspension. The components of the tubule suspension were assessed by two independent observers. Proximal tubules were identified with staining for villin, which is a protein specific to the brush border of proximal tubule cells (3) (Fig. 1). A suspension of tubule segments was fixed in paraformaldehyde-lysine-periodate for 1 h at room temperature. The tubules were spun at lowest speed in a clinical centrifuge to form a pool, were resuspended in PBS, and washed three times. The tubules were then placed in PBS 10% DMSO for 10 min for cryoprotection and frozen in liquid N₂ for storage. The tubules were thawed and labeled for apical villin expression. Tubules were blocked for nonspecific binding in a solution of PBS 1% BSA, 0.8% gelatin for 30 min

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Fig. 1. Confocal images of a segment of isolated immature proximal tubule. *Left*, Optical slice of the intact tubule segment showing the lumen and optical labeling with polyclonal antivillin antibody. *Right*, Pseudophase image of the intact tubule segment. Magnification $\times 750$.

at room temperature. Exposure to rabbit primary polyclonal antibody to villin (courtesy M. Mooseker, Department of Biology) was carried out for 1 h at room temperature on a carousel rotator. The suspension was washed 3 times and then exposed to secondary antirabbit IgG conjugated to rhodamine (Cappel, Durham, NC). The tubules were again washed 3 times, mixed with a 75- μ L volume of Aquamount (Lerner Labs, Pittsburgh, PA), and transferred to a slide with a micropipet and covered.

To view intact segments of tubule, which were in effect whole mounted on the slide, we used confocal microscopy. The resulting images are optical sections of the tubule yielding high resolution with simultaneous fluorescent signal and phase-contrast identification. Confocal microscopy was performed using an MRC-600 laser scanning microscope (Bio-Rad Laboratories, Richmond, CA). To achieve consistency in sampling technique, we set the variable confocal aperture size to a predetermined setting to give an optical section thickness exhibiting an acceptable signal/noise ratio. All subsequent samples were viewed at this setting and also with the same objective lens (Leitz water immersion 50 $\times$ numerical aperture 1.0; Leica, Deerfield, IL), and raster-controlled zoom factor (1.5 $\times$). Images thus created possess an identical "volume" of fluorescence signal and are analyzed using the MRC system software for pixel intensity so that relative changes are displayed under equivalent conditions.

Preparation of a Common Buffer Solution. Recent investigations have indicated that the amino acid content of the suspension solution may have profound effects on the stability of a suspension of tubules and the severity of an injury to renal tubules (4, 5). Moreover, the common buffer solutions used for tubules from mature rats are different from those used for immature tubules. The standard buffered Ringer's solution used to study adult tubules contains lactate, alanine, and butyrate (5, 6), and glutamine and malate are frequently added in studies of immature tubules (7). Therefore, we studied the stability of both mature and immature tubules in three buffer solutions. Buffer A contained 4 mM lactate, 6 mM alanine, and 3 mM butyrate. Buffer B contained 5 mM lactate, 4 mM alanine, 1 mM butyrate, 1 mM glutamine, and 2 mM malate. Buffer C contained 4 mM lactate, 2 mM alanine, 3 mM butyrate, 2 mM glutamine, and 5

mM malate. In each case, these substrates were dissolved in a buffered Ringer's solution containing 5.5 mM glucose, 112 mM NaCl, 3 mM KCl, 1.2 mM MgSO_4 , 1 mM KH_2PO_4 , 1.5 mM CaCl_2 , and 25 mM NaHCO_3 , with a final osmolarity of 305–310 mosmol/L and a pH of 7.4.

Studies of the response of the immature and mature tubules to anoxia. The tubule suspensions from both the immature and mature rats were subjected to 15, 30, 45, and 60 min of anoxia. Before the anoxic injury, the tubule suspension was preequilibrated at 37°C and bubbled with 95% O_2 /5% CO_2 for 10 min to allow for steady state equilibration. Anoxia was produced by gassing an aliquot of the suspension with 95% N_2 /5% CO_2 for 2 min, by which time the PO_2 fell to zero as measured by a polarographic oxygen electrode (Clark, Instech Lab. Inc., Hershamp, PA). The microtube was capped and incubated at 37°C in a shaking water bath for the designated anoxic interval. All studies were performed at the end of the anoxic period.

Tubule integrity. LDH release was determined as an index of plasma membrane damage to assess the integrity of the suspension of tubules. LDH was assayed by the procedures described by Takano *et al.* (8). Released LDH (supernatant LDH) is expressed as a percentage of total LDH (supernatant LDH plus cellular LDH, which is determined after sonication).

Determination of cellular ATP levels. ATP levels were measured as an index of the energy state of the tubules. Intracellular ATP concentration was measured with a modification of the luciferase assay described by Uchida and Endou (9). Adenine nucleotides were extracted in a 1:1 ratio of 0.5 mL perchloric acid. ATP was measured in a luminometer (Turner TD 20e, Turner Designs, Mountain View, CA) using an ATP bioluminescent assay kit (Sigma) in 100- μ L samples.

DNA content. To allow a direct comparison between immature and mature tubules, the data are expressed per mg DNA because the DNA would not be expected to be altered with maturation. The DNA content of the tubule suspension was measured by the mithramycin-binding assay described by Hill and Whately (10).

Response of Immature Glomeruli to Anoxia. Because approximately 3–5% of the suspensions of both mature and immature tubules were contaminated with glomeruli, LDH release was

measured in suspensions of pure glomeruli obtained from the microfilter (Tetko). The LDH release was measured before and after 15 and 45 min of anoxia.

Statistics. All values are reported as means $\pm$ SEM. For statistical comparisons the analysis of variance and Newman-Keuls multiple comparison test were used. Values were considered significantly different if $p < 0.05$.

RESULTS

Documentation of Purity of the Enriched Tubule Suspension.

The distribution of components in the suspensions of immature and mature tubules was similar. Intact proximal tubules confirmed by villin staining (Fig. 1) accounted for $>90\%$ of the formed elements in the suspensions. The proportion of distal tubules and collecting ducts totaled approximately 6–8%. Suspensions obtained from immature kidneys had 3–5% glomeruli, whereas glomeruli were seen less frequently in suspensions from mature kidneys (1–2%). Suspensions of tubules were inspected after each preparation and remained consistent with this distribution.

Development of a Common Buffer. Because of the potential effect of substrates, particularly amino acids, on the stability of tubules in suspensions and the severity of an anoxic injury (4, 5), it was necessary to develop a common buffer solution so that mature and immature tubules could be studied under the same circumstances and in the same milieu. As shown in Figure 2, the common buffer solution, buffer C, provides good substrates for support of both mature and immature tubules. In control, oxygenated, and noninjured conditions, LDH released in buffer C is equivalent to that obtained in either buffer solution A (designed specifically for adult tubules) or buffer solution B (designed for immature tubules). In buffer C, ATP levels measured for mature tubules were $100 \pm 2\%$ of those obtained in buffer A, and for immature tubules ATP levels measured were $96 \pm 4\%$ of those obtained in buffer B. Similarly, the severity of 45 min of anoxia in buffer C was equivalent to that in buffer A for mature tubules and buffer B for immature tubules. Therefore, all subsequent studies were carried out using the common buffer solution, buffer C, for both mature and immature tubule suspensions.

Studies of the Response of the Immature and Mature Tubules to Anoxia. Tubule integrity. No statistical difference was observed in LDH released under baseline oxygenated conditions after rewarming (0 min of anoxia) between the immature ($12.6 \pm 0.4\%$) and the mature tubules ($11.4 \pm 0.7\%$) (Table 1). Therefore, both groups of tubules demonstrated a similar level of stability before anoxia. After the induction of anoxia, the pattern of injury was quite different in immature and mature tubules (Table 1,

Fig. 2. Percentage LDH released from mature tubules (open bars) suspended in standard buffer A and from immature tubules (hatched bars) suspended in buffer B specifically designed for developing tubules and compared with common buffer C.

Table 1. Time course of anoxic-induced injury in immature and mature tubules*

Anoxic interval (min)	Percentage of LDH release		ATP nmol/mg DNA	
	Immature tubules	Mature tubules	Immature tubules	Mature tubules
0	12.6 $\pm$ 0.4 (14)	11.4 $\pm$ 0.6 (12)	65.3 $\pm$ 6 (20)	244.8 $\pm$ 30† (16)
15	20.2 $\pm$ 1.5‡ (10)	23.2 $\pm$ 1.3‡ (9)	33.4 $\pm$ 4‡ (15)	61.1 $\pm$ 10†‡ (10)
30	23.0 $\pm$ 0.9‡ (9)	27.2 $\pm$ 1.2‡ (8)	26.0 $\pm$ 5‡ (15)	51.5 $\pm$ 10†‡ (12)
45	24.0 $\pm$ 1.1‡ (8)	35.0 $\pm$ 0.7†‡ (8)	24.5 $\pm$ 3‡ (8)	50.1 $\pm$ 7†‡ (10)
60	26.8 $\pm$ 1.2‡ (9)	45.4 $\pm$ 1.3†‡ (9)	23.8 $\pm$ 5‡ (8)	46.7 $\pm$ 7†‡ (12)

* Values are mean $\pm$ SEM. Numbers in parentheses represents number of animals.

† $p < 0.05$ between immature and mature tubules at same anoxic interval.

‡ $p < 0.05$ compared with 0 min in same group.

Fig. 3. Percentage LDH released from mature tubules (open circles) and immature tubules (closed squares) after 0, 15, 30, 45, and 60 min of anoxia.

Fig. 3). After a short anoxic insult (15 min or 30 min) the percentage of LDH release was not statistically different in immature compared with mature tubules, although the absolute values were consistently higher in mature tubules. However, after prolonged anoxia (45 min or 60 min), LDH release continued to increase in mature tubules, whereas the cellular integrity of the immature tubules stabilized. At both 45 min and 60 min of anoxia, LDH release in suspensions of immature tubules was significantly less than that of suspensions of mature tubules.

Because glomeruli were seen more frequently in suspensions from immature kidneys, LDH release from anoxic immature glomeruli were determined to be certain that the lower levels of LDH release obtained in the immature tubules was not due to glomerular contamination. The percentage LDH released from a suspension of immature glomeruli was $44 \pm 1.2\%$ and $55 \pm 1.5\%$ after 15 and 45 min of anoxia, respectively, as compared with $20 \pm 1.5\%$ and $24 \pm 1.2\%$ in the immature tubule suspension. Therefore, any glomeruli in the immature tubule suspension would serve to bias the data toward increased LDH release rather than diminish LDH release, which was observed.

Cellular ATP Levels. Under basal oxygenated conditions, after rewarming (0 min of anoxia), the cellular ATP levels in immature tubules were significantly lower, when expressed per mg DNA,

than those in the mature tubules (Table 1). Additional warming and equilibration under oxygenated conditions did not alter these basal ATP levels.

As expected, cellular ATP levels fell in both immature and mature tubules after anoxia (Table 1). Because of the differences in basal ATP concentrations, a comparison of the respective patterns of diminished ATP is best accomplished by evaluating the proportional decline in ATP at each anoxic interval (Fig. 4). In mature tubules, cellular ATP decreased to 20% of control (0 min of anoxia) values after 15 min of oxygen deprivation and remained at that level even when the period of anoxia was extended to 30, 45, and 60 min. In comparison, the cellular ATP level in the immature tubules was 50% of control values after 15 min of anoxia and plateaued at 40% of control values at each subsequent anoxic interval. Thus, cellular ATP was maintained at 2-fold higher levels relative to basal (0 min of anoxia) ATP concentrations in the immature tubules.

DISCUSSION

For many years, investigators have noted differences between the response of developing and mature animals to oxygen deprivation. As early as 1670, Boyle (11) demonstrated that day-old killifish continued three times longer in the exhausted receiver than other animals of that size probably would have. Much work has been concentrated on the developing nervous system. It has been well documented that the neonatal brain is more resistant to oxygen deprivation than that of the adult (1). The underlying mechanisms for this tolerance is unclear, but it is postulated that the newborn can decrease the metabolic rate to preserve cellular ATP. In rat brain slices, ATP levels in the young animal after anoxia are higher than those in the adult (12).

Experimental studies that address the susceptibility of the developing kidney to anoxia, however, are few. Zager and Alpers (13) evaluated the effects of aging on the susceptibility to an ischemic renal insult in the rat and concluded that aging, even the transition from the adolescent to the mature state, increases the susceptibility to severe ischemic acute renal failure. However, adolescent, mature, and aged rats rather than immature animals were used. In the present study, immature rats were defined as 8–10 d of age, and mature rats were 8–10 wk old. Larsson *et al.* (14) has shown that the superficial nephrons are formed around d 7. Aperia and Larsson (15) established that these nephrons reach full structural maturation between d 30 and 40. Celsi *et al.* (16) have also demonstrated that Na-K-ATPase could not be increased in the immature proximal tubules in the 10-d-old rat

to the level observed in adult rats, age 40 d, by pharmacologic doses of hormones.

The present study clearly demonstrates that immature tubules are more tolerant of anoxia with diminished cellular injury and improved preservation of intracellular ATP than mature tubules. The release of LDH has been used as a standard for assessing cellular injury in suspensions of enriched proximal tubules (17). The percentage release of LDH measured in the mature tubules in this study is consistent with a similar time course of anoxia-induced LDH release documented by Doctor and Mandel (6). After both a short (15 or 30 min) and prolonged (45 or 60 min) anoxic insult, a progressive increase occurred in LDH release in mature tubules. This finding is in marked contrast to the pattern of response of the immature tubules. When exposed to short intervals of anoxia (15 or 30 min), the percentage LDH released by immature and mature tubules was similar, whereas a lesser degree of cellular damage occurred after prolonged (45 and 60 min) anoxia in immature tubules. Thus, in a manner similar to that documented in the nervous system, immature renal tubules tolerate prolonged anoxia better than mature tubules.

Because a rapid reduction in cellular ATP is the classic response to anoxia, changes in cellular ATP were measured and compared in the mature and immature animals. Because hyperplasia predominates over hypertrophy in the developing renal tubule until d 14 (14, 18), a given volume of tubule suspension obtained from the immature rat would be expected to contain more tubules per milligram of protein than that from a mature rat. Therefore, data are expressed per milligram DNA to allow an accurate comparison between immature and mature tubules. Under basal oxygenated conditions, ATP levels were significantly lower in the immature tubules as compared with the mature tubules. However, if we express ATP levels as moles per milligram protein, the baseline value would be greater in the immature tubules as compared with the mature tubules: 8.5 nm ATP/mg protein and 5.4 nm ATP/mg protein, respectively. The data are then consistent with the observation of Fischer and Isselhard (19), who measured basal ATP levels expressed as micromoles ATP per gram dry weight of kidney tissue and found ATP levels in the kidneys of the newborn rabbits significantly higher compared with adult rabbits. However, our finding that cellular ATP is lower in immature tubules is consistent with a lower intracellular inorganic phosphate concentration observed by Barac-Nieto *et al.* (20) in the immature guinea pig kidney using nuclear magnetic resonance spectroscopy and chemical determinations in both *in vivo* and in isolated perfused kidneys. Because inorganic phosphate is required for phosphorylation of ADP in the mitochondria to generate ATP, one would expect a lower ATP level in immature tubules. In addition, Na-K-ATPase activity is lower in the developing proximal tubule than in the mature tubule in rats (16) and in rabbits (21). Also, Barac-Nieto and Spitzer (7) have suggested that active (ouabain-sensitive) transport occurs at lower levels in the immature tubule.

Not surprisingly in the present study, cellular ATP levels decreased significantly in both the immature and mature tubules after both short (15 and 30 min) and prolonged (45 and 60 min) anoxia. This dissociation of the percentage release of LDH and cellular ATP levels can be explained by previous observations from our laboratory in which we demonstrated that the degree of cellular damage is related to a combination of the duration and degree of ATP depletion (22). However, in comparison with the mature tubules, cellular ATP, as percentage of control values, was 2-fold higher in the immature tubules at each anoxic interval. Fischer and Isselhard (19) reported similar findings in the rabbit after 10, 20, and 40 min of ischemia. In the adult rabbit, ATP levels decreased to 20% of control values after 10 min of ischemia and remained at that level at all ischemic periods. In contrast, ATP levels in the newborn rabbit decreased to 47% of control values and plateaued at 23% of control values after prolonged ischemia. Therefore, the enhanced tolerance of immature tubules

Fig. 4. Percentage intracellular ATP in the mature tubules (open circles) and immature tubules (closed squares) after 0, 15, 30, 45, and 60 min of anoxia.

to prolonged ischemia is associated with preservation of cellular ATP levels.

Several possible explanations exist for the preservation of intracellular ATP in the immature tubules. Increased glycolytic ATP production could exist in the immature tubules as compared with mature tubules, as suggested by others. As early as 1941, Fazekas *et al.* (23) showed that immature animals tolerate anoxia longer than adults by mechanisms that depend on energy from glycolysis. Whittam (24) demonstrated that kidney cortex slices of neonatal rabbits have a 5-fold greater rate of glycolysis during anaerobic incubation as compared with adult tissue. This increased capacity for anaerobic glycolysis fell to adult rates by 4 wk of age. Dicker and Shirley (25) confirmed these observations in the newborn rat and correlated the presence of glycogen, oxidative enzymes, and their progressive disappearance during 2–3 wk after birth with the initial high rate of anaerobic glycolysis and its subsequent decrease to levels found in the adult animal. Fischer and Isselhard (19) studied the metabolic states of tissues of newborn rabbits before and after varying periods of ischemia and found that the glycolytic activity of the kidneys during ischemia was much higher in newborn rabbits.

An alternative explanation for these observations could be related to maturational development and cellular energy distribution. Previous studies from our laboratory (26) and Sakarcian *et al.* (27) have shown that, under basal conditions, cellular energy is distributed equally to transport (ouabain-sensitive) and nontransport (ouabain-insensitive) activities in the proximal tubule of the mature rat. After an ischemia (26) or toxic (27) insult, when cellular ATP declines, energy for nontransport activity is minimally reduced, and a greater and preferential reduction in energy directed for transport function occurs. Because Na-K-ATPase levels (16, 21) and active (ouabain-sensitive) transport (7) are lower in the immature tubule, we would expect that under basal conditions a smaller proportion of energy is devoted to active transport processes (26). Thus, we would anticipate that after anoxia higher ATP levels would be found in the immature tubules because a larger proportion of basal energy is devoted to nontransport processes and because energy related to transport processes is preferentially reduced after a renal insult (26).

The data in the present study demonstrate the following: 1) a stable suspension of immature proximal tubules can be obtained, studied, and compared with mature tubules and can provide information about the impact of development on acute renal failure, and 2) the developing kidney is more tolerant of an anoxic injury documented by the preservation of cellular ATP and membrane integrity.

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