Adenosine Production in the Ischemic Kidney

WAYNE L. MILLER, ROSEMARY A. THOMAS, ROBERT M. BERNE, AND RAFAEL RUBIO

SUMMARY We conducted experiments to determine (1) tissue, blood, and urine levels of adenosine produced by the ischemic kidney under conditions of renal artery occlusion, and (2) the site(s) of production and release of adenosine by the kidney. Concentrations of adenosine, inosine, and hypoxanthine in the dog urine were found to increase after 2 minutes of renal artery occlusion as were concentrations of these metabolites in renal tissue after 10 minutes of renal artery occlusion. Renal venous plasma levels of inosine and hypoxanthine also were elevated after 3 minutes of arterial occlusion. In modified stop-flow experiments, adenosine appeared in the urine in a peak that corresponded most closely with proximal tubule fluid. 5'-Nucleotidase, the enzyme which catalyzes the dephosphorylation of 5'-AMP or 5'-IMP to adenosine, inosine, and hypoxanthine, respectively, was found to be located primarily on the external membranes and mitochondria of proximal tubule cells, but not in distal tubule or collecting duct cells. Since adenosine has been demonstrated to elicit renal vasoconstriction and is produced by the ischemic kidney, it is suggested that adenosine may be involved in the mediation of postocclusion renal ischemia.

ADENOSINE has been proposed as a mediator in the control of blood flow in heart muscle, skeletal muscle, and brain and has been shown to be formed by the dephosphorylation of AMP, a reaction catalyzed by the enzyme 5'-nucleotidase. 5'-Nucleotidase activity has been demonstrated in a variety of tissues, including isolated membranes from rat kidney, and indirect evidence suggests that adenosine may play a role in the regulation of renal blood flow. For example, in heart and skeletal muscle, adenine nucleotides and adenosine produce vasodilation; however, in the dog kidney, ATP and ADP elicit vasodilation, whereas AMP and adenosine elicit vasoconstriction. Furthermore, adenosine-induced renal vasoconstriction in the rat was observed after salt restriction and, hence, activation of the renin-angiotensin system, but not after salt loading. Considering these findings, it is of interest that Scott et al. observed that renal venous blood collected after release of occlusion of the renal artery produced dilation of the vessels of the perfused dog hindlimb but constriction of the vessels of the contralateral kidney. Also of interest are the observations that reactive ischemia occurred following a 2-minute period of renal artery occlusion in 52% of experiments completed on dogs and that AMP was found in the renal venous blood of ischemic kidneys. In addition, dipyridamole, a compound that inhibits adenosine uptake by red cells and tissues, greatly potentiated the vasoconstrictor response to bolus injections of adenosine and AMP, and also enhanced reactive ischemia in the dog kidney. Dipyridamole also has been shown to restore the renal autoregulatory response after spontaneous renal autoregulatory failure. Thus, it may be postulated that adenosine accumulation is partially responsible for the phenomenon of postocclusive renal reactive ischemia and that adenosine
may be involved in the vascular adjustments to changes in renal perfusion pressure. Preliminary results from experiments on in situ dog kidney preparations indicate adenosine formation with ischemia; however, no correlation could be found between renal artery perfusion pressure (80-175 mm Hg) and tissue or urine adenosine levels. The lack of correlation in these perfusion experiments does not, however, preclude a role for adenosine in mediating the vasoconstrictor response to renal artery occlusion, since concomitant release of a vasoconstrictor substance, such as one of the prostaglandins, could mask an adenosine-induced vasoconstriction.

Whereas considerable work has been done to evaluate renal function and hemodynamics subsequent to adenosine infusion, further investigation is needed to evaluate the endogenous formation and release of adenosine by the kidney. The present study was undertaken to examine quantitatively adenosine production in the dog kidney. Stimulation of adenine nucleotide catabolism by brief periods of renal ischemia was used to determine whether adenosine levels in tissue, venous blood, and urine would increase with hypoxia as predicted by a hypothesis for postocclusive renal vascular constriction. Basic information also was sought as to the site(s) of production and release of adenosine in the kidney. A cortical or medullary site of release would reflect a possible influence on blood flow distribution within the kidney.

**Methods**

**Preparations**

**Tissue, Blood, and Urine Adenosine Levels following Renal Artery Occlusion**

Rats (albino Sprague-Dawley), cats, and dogs were anesthetized with sodium pentobarbital (rats, 50 mg/kg, ip; dogs and cats, 30 mg/kg, iv). In 12 rats, the kidneys were approached via a midline abdominal incision. One kidney, either right or left, was frozen in situ in aluminum tongs precooled in liquid nitrogen. The contralateral kidney was made ischemic for a period of 1 or 3 minutes by clamping the renal artery and vein. At the end of the ischemic period, the ischemic kidney was also frozen in situ. In six dogs and three cats, the left or right kidney was approached via a flank incision and a ligature was placed around the renal artery. A control tissue sample was frozen in situ with a rongeur precooled in liquid nitrogen. Immediately after the control sample was obtained, the renal artery was occluded and the kidney was left in situ for a period of 3-10 minutes. Additional tissue samples were obtained in the cat after 1.5 and 3.0 minutes of ischemia and in the dog after 5 and 10 minutes of ischemia. These tissue samples were stored in liquid nitrogen.

Dog arterial blood, renal venous blood, and urine were collected for purine analysis. The renal artery, the renal vein, and the ureter of the left kidney were cannulated, and the dog was given heparin, 1000 U/kg, with supplemental doses given each hour. Blood from the femoral artery supplied the left kidney. The renal venous blood was returned to the general circulation via a cannula in the left external jugular vein. A soft, blunt cannula was placed in the left ureter with its tip positioned 3 cm from the renal pelvis. Venous and arterial blood was collected from side-tubes in the venous and arterial circuits, respectively. Renal artery flow was monitored with an electromagnetic flow meter, and perfusion and systemic pressures were also measured throughout the experiment.

An infusion of isotonic mannitol (0.30 M) at a rate of approximately 3.3 ml/min was administered to produce a urine flow of 1-2 ml/min. After cannulation of the renal artery and vein, an equilibration period of 45-60 minutes elapsed. A control sample of 10 ml of urine was collected and the time for its collection was recorded. Approximately 20 ml of renal arterial and renal venous blood were collected simultaneously. All blood samples were collected into an equal volume of 0.9% saline at 0°C in centrifuge tubes immersed in ice. Plasma and cells were separated by centrifugation and the plasma processed for purine analysis as previously described. Percent recoveries from whole blood were determined by adding known amounts of adenosine, inosine, and hypoxanthine to normal blood samples which were processed simultaneously with experimental samples. After collection of the control urine and blood samples, a series of three occlusions of the renal artery for 1, 2, and 3 minutes was performed. Immediately after each occlusion (0.25 minute), 10 ml of urine and 20 ml of venous blood were collected. A recovery period of 20-30 minutes elapsed after each occlusion. After recovery from the first and second occlusion periods, control urine and arterial and renal venous blood samples were collected. The total volume of blood collected from each dog was 180 ml.

**Localization of Adenosine Release in the Kidney**

**Experiments Employing Ureteral Occlusion and Renal Artery Occlusion.** The stop-flow technique as described by Malvin et al. was used to locate the segments where adenosine entered the nephron. To stimulate adenosine production, the renal artery was occluded after ureter occlusion. Thus, adenosine and its degradative products would appear only in those segments of the nephron where they are produced or secreted. Sodium ferrocyanide and p-aminohippurate (PAH) were administered via the femoral vein before release of the occlusions. Their detection in the urine was used to mark the beginning of filtration at the glomerulus [Na4Fe(CN)6 + PAH] and the proximal tubular fluid present prior to ureter occlusion release (PAH alone).

In four dogs, the left ureter was cannulated and...
the renal artery was fitted with a noncannulating electromagnetic flow probe. The dogs were also given approximately 250 ml of hypotonic saline (0.45%) during the surgery to assure adequate hydration. After approximately 1 hour, when urine flow had stabilized, the experiment was begun.

After collection of two control urine and arterial blood samples, the ureter was occluded thereby stopping most filtration. PAH (100 mg) was injected intravenously 15 seconds prior to the renal artery occlusion. The renal artery was then occluded for 3 minutes. Fifteen seconds before release of the renal arterial occlusion and 30 seconds before release of the ureteral occlusion, sodium ferrocyanide (1 g) was injected intravenously. The total time of the ureteral occlusion was 5-8 minutes. After release of the ureteral occlusion, serial urine samples of 0.5 or 1 ml were collected.

The arterial blood samples were collected into 15-ml tubes containing heparin. The plasma was separated by centrifugation at 0°C for 10 minutes at 2000 g and frozen. Control urine samples and one-half of the serial urine samples (every other sample) were frozen for later analysis for PAH and sodium ferrocyanide. The remaining samples were processed for purine analysis.

Histochemistry. A rigid cannula was placed in the left common iliac artery with its tip positioned slightly below the left renal artery, and a ligature was placed and tightened around the aorta just above the left renal artery. The kidney was fixed with an infusion of 250 ml of 0.75% glutaraldehyde-buffered solution (pH 7.2). After fixation, the kidney was rinsed by perfusion with a buffered solution of 0.3 M glucose and 3% dextran (pH 7.3). A second rinse consisted of 0.1 M Tris-maleate buffer (pH 7.2) containing 0.2 M sucrose and 0.4 M dithiothreitol. Samples of cortical and medullary tissue were stored overnight at 4°C in the second rinsing solution.

In order to determine the cellular sites of 5'-nucleotidase activity, frozen sections of kidney tissue (50 µm thick) were incubated for 1 hour at 37°C in a medium containing 0.1 M Tris-maleate, 0.2 M sucrose, 10 mM CaCl₂, and 4 mM Pb(NO₃)₂. Substrates used were the sodium salts of 5'-AMP, 5'-IMP, and 2',3'-AMP in 1 mM concentrations; substrate was excluded from some incubation experiments with lead nitrate to check for nonenzymatic lead precipitation. 2',3'-AMP, a substrate for a variety of phosphatases, was also incubated with some tissue sections to differentiate between the release of phosphate by 5'-nucleotidase and that by other phosphatases. Tissue incubated with a mononucleotide substrate in the presence of 5'-nucleotidase and lead nitrate show an electron opaque precipitate which results when the released inorganic phosphate is trapped by the lead at the site of enzymatic activity. This precipitate is detectable with electron microscopy. The final steps of tissue processing for electron microscopy were those described previously.30

Analytical Procedures

Sample Processing

Kidney Tissue. The frozen samples of kidney tissue were pulverized, treated with perchloric acid, and the soluble fraction was neutralized as previously described.2 An aliquot was adsorbed on 100 m of charcoal by shaking at 4°C for 4 hours. In some experiments, the acid extracts were adsorbed on the charcoal without prior neutralization. The purines were then eluted from the charcoal and analyzed.2

Urine. Urine samples were immediately placed in a centrifuge tube containing 100 mg of charcoal and the tubes were immersed in ice. Samples from the ureteral occlusion studies were diluted with 5 ml of glass-distilled water to assure adequate adsorption because of their small volume (0.5-1 ml). Adsorption on and elution from the charcoal were accomplished by the same procedures described for the tissue samples. The residue from the charcoal eluates was reconstituted in 1 ml of glass-distilled water. Percent purine recovery from urine was determined in the same manner as described previously for blood.

Chemical Analyses

AMP, adenosine, inosine, and hypoxanthine were measured by the spectrophotometric enzymatic assay previously described.3 Sodium ferrocyanide was analyzed in appropriately diluted urine samples by the method of Berliner et al.31 using a spectral absorption peak at 420 nm. PAH was determined by the Bratton-Marshall reaction.32

Results

The Effect of Renal Artery Occlusion on Kidney Tissue, Urine, and Blood Levels of Purine Nucleotides, Nucleosides, and Bases

In all three species (dog, cat, and rat), adenosine, inosine, and hypoxanthine accumulated in the tissue during renal artery occlusion; hypoxanthine accumulated to the greatest extent (Table 1). In dogs with renal artery occlusion, tissue adenosine levels were significantly increased at 10 minutes and inosine and hypoxanthine at 5 and 10 minutes.

The levels of urinary adenosine, inosine, and hypoxanthine are presented in Table 2. Urinary nucleosides and bases are expressed as nanomoles per milliliter of urine, and since urine flow varied somewhat among the dogs, urinary values are also expressed as nanomoles per minute released into the urine. Under control conditions, small but measurable quantities of adenosine, inosine, and hypoxanthine were present in the urine. The urinary levels of these compounds increased significantly after 2 and 3 minutes of renal ischemia. Urine recoveries

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Footnotes:
30 Electron microscopy were those described previously.
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for adenosine, inosine, and hypoxanthine were 75.5 ± 4.9%, 78.5 ± 5.6%, and 69.0 ± 8.9% (mean ± SE; n = 8), respectively. Results were corrected for recoveries.

Venous plasma adenosine levels increased slightly but not significantly over control levels. This is an understandable result since adenosine is known to be rapidly deaminated and incorporated into red cells and, hence, the loss of adenosine in blood between the level of the arterioles and site of collection would be quite large. This loss is clearly reflected in the percent recovery values observed. Recoveries of adenosine, inosine, and hypoxanthine from whole blood were 11.3 ± 3.6%, 33.4 ± 3.4% and 27.5 ± 3.9% (n = 5), respectively.

Localization of the Site of Adenosine Entry into Kidney Tubules

With establishment of renal artery occlusion after ureteral occlusion, nucleosides and bases could enter the renal tubules only across the luminal surface of the tubules near their sites of production and through the glomeruli only after release of both occlusions. Furthermore, the sodium ferrocyanide that was administered intravenously just before the release of ureteral occlusion could enter the urine only in glomerular filtrate formed after the release of both arterial and ureteral occlusions. The first appearance of the sodium ferrocyanide marks the appearance of postocclusion filtrate in the shortest

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Adenosine, Inosine, and Hypoxanthine in nmol/g Wet Weight ± SE in Normal Cat, Rat, and Dog Kidney after Renal Artery Occlusion</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>(n = 3)</td>
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<tr>
<td></td>
<td>4.1 ± 0.5 P &lt; 0.001</td>
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<tr>
<td>Adenosine</td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>7.6 ± 0.9 P &lt; 0.001</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>16.2 ± 1.2 P &lt; 0.001</td>
</tr>
<tr>
<td>Rat</td>
<td>(n = 12)</td>
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<tr>
<td>Adenosine</td>
<td>6.7 ± 0.2 P &lt; 0.001</td>
</tr>
<tr>
<td>Inosine</td>
<td>4.7 ± 0.7 P &lt; 0.02</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>4.1 ± 0.7 P &lt; 0.001</td>
</tr>
<tr>
<td>Dog</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>7.6 ± 3.4 25.9 ± 8.1</td>
</tr>
<tr>
<td>Inosine</td>
<td>4.3 ± 1.9 84.5 ± 28.7</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>11.0 ± 6.0 75.3 ± 21.6</td>
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<tr>
<th>Table 2</th>
<th>Adenosine, Inosine, and Hypoxanthine Release and Concentration in Dog Urine after Renal Artery Occlusion</th>
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<tr>
<td></td>
<td>Duration of occlusion</td>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
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<tr>
<td>Adenosine</td>
<td>0.99 ± 0.32</td>
</tr>
<tr>
<td>Inosine</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.98 ± 0.70</td>
</tr>
</tbody>
</table>

|         | nmol/ml ± SE                                                                                                   | nmol/min ± SE              |
| Adenosine | 1.64 ± 0.48                                                                                                   | 10.75 ± 5.31 P < 0.04      | 10.33 ± 2.90 P < 0.01 |
| Inosine  | 0.40 ± 0.13                                                                                                   | 3.99 ± 1.41 P < 0.01       | 4.67 ± 0.57 P < 0.01  |
| Hypoxanthine | 2.89 ± 0.70                                                                                                  | 16.43 ± 9.94 P < 0.05      | 18.14 ± 2.94 P < 0.05 |
nephrons with increasing concentrations reflecting the contribution of the longer nephrons. The shape of the sodium ferrocyanide concentration curve thus is an indication of the variability in the appearance of this filtrate in the urine and, therefore, an indication of nephron heterogeneity. Similarly, PAH, which is secreted into proximal tubules, can be used as an indicator of tubular fluid present after filtration is stopped. By comparing the urinary concentrations of adenosine, inosine, and hypoxanthine in a given fraction to the appearance of PAH and sodium ferrocyanide in the series of fractions, it was possible to localize the approximate site of entry of these compounds into the urine. Results of one such experiment are shown in Figure 1. After a 3-minute renal artery occlusion, adenosine, inosine, and hypoxanthine appeared in the urine in relatively sharp peaks. The highest levels of these compounds were obtained when PAH and sodium ferrocyanide had just increased, and, in all dogs, these peak concentrations occurred sooner than did the peaks of PAH (two dogs) and sodium ferrocyanide (six dogs). Since it was not possible to measure all five compounds in each sample, and because the measurement of adenosine required separate urine samples (0.5 ml), the first fraction of postocclusion glomerular filtrate could not be precisely determined. However, the consecutive appearance of the peak increases in adenosine and its metabolites, and that of PAH indicates that adenosine was detectable in the highest concentration only in the tubular fluid that was formed immediately prior to arterial occlusion. The sample fractions in which adenosine appeared at maximal concentration showed PAH at 58 ± 2.5% of its maximum concentration and sodium ferrocyanide at 15 ± 2.3% of its maximum concentrations. In those filtrates that appeared after the reestablishment of the circulation, the adenosine concentration gradually decreased. In fact, when the sodium ferrocyanide curve reached a maximum, the adenosine concentration had decreased to near control levels. In contrast to adenosine, inosine and

![Figure 1](http://circres.ahajournals.org/)

**Figure 1** Adenosine, inosine, hypoxanthine, sodium ferrocyanide, and PAH in dog urine in one experiment after 8 minutes of ureteral occlusion and 3 minutes of renal artery occlusion.
FIGURE 2  Electron micrographs of dog kidney proximal tubule cells (A) and distal tubule (DT) cells (B) incubated with 5'-AMP. In panel A, precipitate is seen in the brush border (bb) and the cristae of mitochondria (m). In panel B, no precipitate is present at the cell margins (arrows); N = nucleus. Panels A and B are sections from the same dog preparation.

hypoxanthine tended to decrease more gradually and paralleled the curves of sodium ferrocyanide and PAH.

To locate the sites of adenosine production in the dog kidney, histochemical staining for 5'-nucleotidase in renal cortical and medullary sections was carried out in four kidneys. The number of cortical or medullary sections stained ranged from 5 to 14 per kidney. The results are shown in electron micrographs (Figs. 2 and 3). In the tissue sections incubated with 5'-AMP or 5'-IMP, the precipitate was associated with external membranes, mitochondria of the proximal tubule cells, and the brush border (Fig. 2). It also was associated with fibroblasts or interstitial cells and endothelial cells. No activity was observed in glomerular cells or distal tubule cells (Fig. 3). Sections incubated with 2',3'-AMP revealed nonspecific phosphatase activity associated only with the brush borders of proximal tubule cells and endothelial cells of medullary capillaries. Thus, the hydrolysis of phosphomonoesters in these two latter structures may be attributed either to nonspecific phosphatase activity or to the combination of 5'-nucleotidase and nonspecific phosphatase enzymes. Nonenzymatic precipitation of lead was not observed in tissue sections incubated only with lead nitrate.

Discussion
Degradative Metabolism of Adenine Nucleotides in Mammalian Kidney

The results of earlier studies by other investigators in which nucleosides were measured indicated that adenosine was not found in large concentrations in homogenates of ischemic kidney tissue. Assay sensitivity, however, did not permit detection of adenosine, inosine, or hypoxanthine in concentrations of less than 100 nmol/g wet weight. In contrast, recent studies and the studies described in the present report used more sensitive assays and indicate that, under control conditions, adenosine is present in kidney tissue. Also, under conditions of ischemia, the tissue adenosine concentrations increased several-fold.

The measurements of urinary adenosine, inosine, and hypoxanthine indicate that there is an efflux of these compounds under control conditions. With restoration of urine flow after renal artery occlusion, there was an increase in the urinary concentrations of these compounds. Salvage of adenosine, inosine, and hypoxanthine by tubule cells, however, might limit the amounts of these compounds that appear in the urine; also, dilution resulting from water entering the tubules could influence the apparent concentrations observed. This could be expected to occur to some extent, since the urine flow in these experiments was about 1-2 ml/min and the nucleosides and bases were in contact with the tubule cells for an extended period of time.

Adenosine is produced by the kidneys in small but measurable quantities under control conditions, and production increases markedly during ischemia. It is possible that the accumulation of a large tissue concentration of adenosine is prevented by further degradation of adenosine to inosine and hypoxanthine in the tissue. Also, insofar as cortico-
medullary distinctions in adenosine production are concerned, it is possible that local changes in adenosine levels with ischemia were obscured by the presence of medullary tissue in the samples analyzed in this study. Differences may, therefore, have been masked by the sampling technique.

Data have been reported which indicate that the direct dephosphorylation of 5'-AMP to adenosine does occur under control conditions in the kidney, although some deamination of 5'-AMP to 5'-IMP may also occur by adenylic acid deaminase. The stop-flow studies following a period of renal artery occlusion showed, however, that adenosine levels in the newly formed urine rose more sharply and reached higher levels than did those of inosine (Fig. 1); this finding supports preferential dephosphorylation of 5'-AMP by 5'-nucleotidase.

For adenosine to be responsible for the phenomenon of reactive ischemia, it is necessary that adenosine concentrations at the arteriolar level be such that renal vascular resistance can be readily affected. The studies of Tagama and Vander indicate that the renal vasculature is extremely sensitive to exogenous adenosine. Furthermore, since the exogenous amounts of adenosine necessary to produce renal vasoconstriction and to decrease glomerular filtration rate are of the same order of magnitude as those measured in the present study after renal arterial occlusion, it appears that sufficient adenosine can be produced by the kidney to play a role as an endogenous vasoconstrictor. However, the concentration of adenosine at the level of the arterioles remains a critical unknown factor.

**Sites of Production and Release of Adenosine**

Support for a proximal tubule cell origin of the adenosine is found in the histochemical studies. The proximal tubule cells demonstrated prominent 5'-nucleotidase activity on the lateral and basal external membranes. This is in agreement with the distribution of the enzyme in cardiac and skeletal muscle. Some activity was present in the cristae of mitochondria of these cells and in the brush border. Other cortical nephron cell types including glomerular cells and distal tubule cells did not demonstrate 5'-nucleotidase activity. Other than capillary endothelial cells, medullary tissue did not show 5'-nucleotidase activity. Thus, these data indicate that proximal tubule cells are capable of the dephosphorylation of AMP and IMP,
but this capability is not shared by cells of the more distal portions of the renal tubules or by the cells of the glomeruli or medulla.

Results of the stop-flow experiments in which adenosine production was stimulated by renal artery occlusion show that adenosine, inosine, and hypoxanthine first appear in the urine in a peak corresponding most closely with the appearance of postocclusion proximal tubule fluid ahead of the glomerular filtrate marker. Thus, the histochemical and filtration data indicate that the production and renal tubule entry of adenosine occur primarily in the cortex, perhaps selectively at the level of the proximal tubule cells.

Acknowledgments

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