Maleic Acid–Induced Proximal Tubulopathy: Na:K Pump Inhibition

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(J. Am. Soc. Nephrol. 1993; 4:142–147)

ABSTRACT

Maleic acid (MA) administration to experimental animals induces a rapid, reversible, complex dysfunction of the renal tubule resembling Fanconi’s syndrome. The intent of this work was to characterize the changes in the Na:K pump along the nephron during the development and recovery from MA injury to better define the site of damage and to correlate the observed changes in Na:K pump function with alterations in metabolic function. Male Sprague-Dawley rats were studied before and 2 and 24 h after the injection of MA (100 mg/kg iv). MA induced an early and reversible decline in Na:K pump activity in the proximal convoluted tubule (PCT) from 2,324 ± 61 to 1,446 ± 55 pmol/mm·h (P < 0.001). This decrement was transient because enzyme activity returned to near baseline by 24 h after MA administration. The changes in Na:K pump activity were restricted to the PCT because no change in pars rectae, in medullary thick ascending limb, or in medullary collecting tubules was observed. PCT obtained from MA-treated rats 2 h after drug injection showed a decline in 14CO2 formation from radiolabeled glutamine, implying impaired oxidation of the carbon skeleton of the amino acid. This decline was transient with recovery of oxidative rates to normal 24 h after MA administration. It was concluded that a reversible, segment-specific impairment in PCT Na:K pump occurs early after the administration of MA. The decline in PCT Na:K pump activity is paralleled by a decrement in oxidative metabolism and may underlie the many consequences of this model of proximal tubulopathy that are reflections of impairment in sodium-dependent transport processes.

Key Words: Na:K pump, glutamine decarboxylation, Fanconi’s syndrome

Maleic acid (MA) administration to experimental animals induces a rapid, reversible, complex dysfunction of the renal tubule resembling Fanconi’s syndrome (1–20). This dysfunction consists of glycosuria (11,18), aminoaciduria (10,11,17), bicarbonaturia (1,2,6,7), and phosphaturia (1,2,11), along with a diuresis. Although this array is readily equated with damage to the proximal tubule (1,2), several investigators (3,15) have suggested that nephronal dysfunction distal to the proximal convoluted tubule (PCT) may contribute to the abnormalities observed. These claims have been challenged by structural (4,16,21) and functional studies (1,2), but no segment-specific evaluation of biochemical alterations has heretofore been described.

The majority of the transport abnormalities induced by MA and inferred to reflect a reabsorptive dysfunction in the proximal nephron are Na-dependent processes (Na cotransport for glucose, amino acids, and phosphate and countertransport for H ions). It is likely, therefore, that a common pathway of impaired Na handling may underlie the multitude of observed defects and, specifically, that an impairment in basolateral Na:K pump function may mediate the manifestation of the syndrome. This effect may be primary by direct inhibition of the pump or secondary either to impairment in mitochondrial oxidative metabolism and reduction in pump activity by energy-substrate depletion or, alternatively, to reduction in Na entry at the luminal site.

The intent of this work was to: (1) characterize the changes in the Na:K pump along the nephron during the development and recovery from MA injury to better define the site of damage; and (2) correlate the observed changes in Na:K pump function with alterations in metabolic function. Because large doses of MA can cause tubular necrosis and therefore render functional studies of little import, we selected a dose of MA that induces reversible functional defects but no frank necrosis.
METHODS

Male Sprague Dawley rats (200 to 250 g) were studied before and 2 and 24 h after injection of MA (100 mg/kg iv; pH adjusted to 7.4 by NaOH).

Tubule Microdissection

The animals were euthanized by exsanguination from the abdominal aorta under general anesthesia, and the left kidney was perfused with a collagenase-containing solution. Tubules were dissected in the cold under stereomicroscopic observation in a medium containing (millimolar): NaCl, 137; KCl, 5; MgCl₂, 1; CaCl₂, 0.25; Tris-HCl, 10; pH 7.4. The tubules were individually transferred to concave bacteriologic slides and were photographed to determine their length.

ATPase Assay

Adenosine triphosphatase (ATPase) activity was measured (22) after osmotic permeabilization and cryopermeabilization by the incubation of tubule segments for 15 min at 37°C in a 2-μL droplet of the following solutions. For the determination of total ATPase activity in the eluate was counted in a liquid scintillation spectrophotometer. For the determination of Mg-dependent ATPase activity, NaCl and KCl were omitted. Tris-HCl was 150 mM, and 1 mM ouabain was added. Phosphate liberated by the hydrolysis of [γ-32P]ATP was separated by filtration through a Millipore filter (Millipore Continental Water Systems, Bedford, MA) after adsorption of the unhydrolyzed nucleotide on activated charcoal, and the radioactivity in the eluate was counted in a liquid scintillation spectrophotometer. For the in vitro effects of MA, the tubules were incubated with the chemical for 15 min before permeabilization.

Total and Mg-dependent ATPase activities were each determined on five replicate samples (3- to 5-mm tubule each) from individuals animals and were expressed as picomoles of CO₂ formed per centimeter of tubule per hour. Sodium- and potassium-dependent, ouabain-inhibitable ATPase was taken as the difference between the means of each group of measurements and thus represents a single datum point in each animal; the results show mean values of these determinations in all animals within a given experimental group. To minimize the variability between experiments, one appropriate control and one experimental animal were studied simultaneously.

Glutamine Oxidation

The method used for the determination of metabolic 14CO₂ production from uniformly 14C-labeled glutamine by isolated tubules has been previously described (23). PCT segments were dissected in a modified Eagle's medium containing (in micromoles per liter): NaCl, 137; KCl, 5; MgCl₂, 1; Na₂HPO₄, 0.44; CaCl₂, 0.25; glucose, 5.5; amino acids; vitamins; N-hydroxyethylpiperazine-N'-(2-ethanesulfonic acid (HEPES), 20; and pH adjusted to 7.4. Tubules were then transferred with 1 μL of solution onto a small disc of dry BSA in the hollow of a bacteriologic slide and were photographed to determine their length. The microdissection solution was aspirated and replaced by 2 μL of cold incubation medium (same composition with the addition of [U-14C]glutamine purchased from Du Pont, NEN Research Products). The tubule slide was immediately covered with a second opposing glass slide containing a 4-μL KOH droplet placed on an aluminum foil disc in the center of the concavity. The two slides were sealed together by interposed Vaseline. We then started the incubation by immersing the glass slides into a water bath maintained at 37°C for 20 min. At the end of the incubation, the glass slides were put on ice and all KOH droplets on the aluminum foil were rapidly transferred into counting vials containing 4 mL of Aquasol (Amersham Co., Arlington Heights, IL). Each vial was counted for 5 min.

CO₂ production was determined in each animal in five replicate samples, each sample consisting of 5 to 12 μm of tubule. The radioactivity of the samples was corrected for background and spontaneous substrate degradation. Metabolic carbon dioxide production formed from the labeled substrate was calculated by dividing the corrected counting rate of each sample by the specific radioactivity per carbon of glutamine. Results are expressed as picomoles of CO₂ formed per centimeter of tubule per hour.

Metabolic Balance Studies

Metabolic balance studies were obtained starting 2 days before MA administration and continuing for 2 more days thereafter. The purpose of this extended evaluation was to profile the effect of MA sequentially. Animals were placed individually in metabolic cages (Nalge, Rochester, NY) for one period of 24 h before the prescribed metabolic balance study days and were allowed to acclimate to the cage; the urinary excretion of water and ammonium was determined daily. Ammonium was measured by the titrimetric method of Cunaro and Weiner (24) used previously (25).

Statistical Analysis

Results were compared by analysis of variance followed by unpaired t test. When more than two groups were compared, such as in the biochemical studies, the Bonferroni adjustment for the value of P deemed significant was used, with significance being
accepted for $P < 0.05/N$, where $N$ is the number of comparisons. For this study, all $P$ values less than 0.01 satisfy this condition.

**RESULTS**

**Na:K Pump**

MA induced an early and reversible decline in Na:K pump activity in the PCT (Figure 1). Na-K-ATPase activity decreased from 2,324 ± 61 to 1,446 ± 55 pmol/mm·h ($P < 0.001$) 2 h after MA administration. This decrement was transient because enzyme activity returned to near baseline by 24 h after MA administration (to 2,288 ± 87 pmol/mm·h). The changes in Na:K pump activity were restricted to the PCT because no change in pars recta (PR) (Figure 1) or in any medullary segment (medullary thick ascending limb [MAL] or medullary collecting duct [MCT]) was observed (Figure 2). This in vivo segment-specific decline was in contrast to the in vitro effect of MA, which is a generalized inhibition of the Na:K pump. In the PCT, the incubation of tubules with 1 mM MA in vitro for 15 min leads to a decline in activity similar to that observed in vivo (from 2,370 ± 64 to 1,245 ± 62 pmol/mm·h; $P < 0.001$). In the MAL, where in vivo activity is unaffected, the incubation of tubules with MA in vitro leads to a decline in Na:K pump activity from 4,413 ± 105 to 1,732 ± 61 pmol/mm·h ($P < 0.001$).

**Glutamine Oxidation**

To further characterize the defect in proximal tubule function, we examined the ability of this segment to metabolize the carbon skeleton of glutamine. PCT obtained from MA-treated rats 2 h after drug injection showed a decline in $^{14}$CO$_2$ formation from radiolabeled glutamine from 303 ± 19 to 171 ± 13 pmol/cm·h ($P < 0.01$) (Figure 3), implying impaired oxidation of the carbon skeleton of the amino acid. This decline was transient, with recovery of oxidative rates to normal 24 h after MA administration. The addition of MA (1 mM) to the incubation medium with normal PCT led to almost complete obliteration of $^{14}$CO$_2$ formation from radiolabeled glutamine in most experiments.

In contrast to its effects on glutamine oxidation, MA led to an increase in 24-h urinary volume and urinary ammonium excretion from 712 ± 89 to 1,254 ± 216 µEq/day ($P < 0.01$) that was also limited to the day of the injection, with rates returning to baseline values subsequently (Figure 4).
rats were studied sequentially.

Discrepancies in renal ammonium excretion before (Cl, C2, control days) and after MA administration (M, day of injection; R, recovery day). Seven rats were studied sequentially.

**DISCUSSION**

The findings of this study can be summarized as follows: (1) MA led to a decline in Na:K pump activity in PCT without any changes in subsequent segments (PR, MAL, MCT); (2) this decline in PCT Na:K pump occurred early after drug administration and was of a transient nature, with enzyme activity returning to baseline within 24 h; (3) the decline in Na:K pump activity in the PCT was paralleled by an impairment in glutamine oxidation in this same segment, but with enhanced urinary ammonium excretion.

A limited number of studies with renal homogenates have examined the changes in Na-K-ATPase activity pursuant to MA administration. A decline in cortical Na-K-ATPase activity has been reported by several investigators (5,12), without any change in medullary enzyme activity (12). The cause of the decrease in PCT Na:K pump activity is likely multifactorial, in view of the multiplicity of the changes that MA induces. The decline in proximal Na:K pump activity could be related to three separate, but possibly coexisting mechanisms: (1) a primary inhibition of the pump; (2) a reduction in mitochondrial metabolism that restricts energy-requiring Na:K pump activity; or (3) a luminal impairment of Na-coupled entry processes. MA does not appear to directly affect renal tubular reabsorption at the luminal membrane (15,19), and hence, the latter mechanism is unlikely. MA impairs mitochondrial oxidative metabolism, leading to a reduction in renal arteriovenous oxygen differences (6) and a decrement in renal cortical concentrations of ATP (12,18,20). It is conceivable that a decline in ATP generation may lead to a decrease in Na:K pump. The latter finding could be underlying the decline in Na:K pump activity. Kramer and Gonick (12) observed in the rat a parallel decline in cortical ATP and Na:K pump activity.

MA has been previously shown to be a potent inhibitor of Na-K-ATPase activity *in vitro* (12). This *in vitro* inhibition occurs at levels of the drug smaller than those required to inhibit Krebs cycle system enzymes (12,26). The effect of MA is not a generalized, nonspecific toxic effect because several renal enzymes remain unaffected, such as succinate dehydrogenase and acid phosphatase (12). These observations support a role for a direct inhibition of the pump as contributing to the decline in activity, acting in consort with the reduction in substrate availability. A direct inhibition of the Na-K-pump as contributing to the effect of maleate is favored by the observation that the transport defect due to maleate can be attenuated without a correction of the decline in cortical ATP content (20). Further, agents that lower ATP to similar levels, such as lithium and arsenate, have different consequences on renal function (7,20).

Our findings of a normal Na:K pump activity in several nephron segments beyond the PCT are consistent with the observations of Al-Bander *et al.* (1,2), based on elegant clearance studies that the postproximal nephron retains its normal capacity to reabsorb Na and Cl, particularly our finding of normal MAL Na:K pump activity. Kramer and Gonick (12) proposed that MA induced a reduction in Na reabsorption in the proximal tubule as its primary effect. The absence of a chlorouresis that would have followed such a defect led others (8,9) to suggest that the primary reduction was in net reabsorption of bicarbonate and, in consequence, a reduction in Na reabsorption. Al-Bander *et al.* (1,2), however, have found that MA induced a substantial increase in the rate at which Na and Cl were delivered out of the proximal tubule and reclaimed in the postproximal nephron.

The restricted proximal effect of MA can be deduced from a variety of observations, including the resultant lysozymuria (5), aminoaciduria (10,11,17), glucosuria (11,18), and bicarbonaturia (1,2,6,7). The aminoaciduria has been found by micropuncture and microperfusion to be due to an inhibition of the saturable (Na-dependent) component of reabsorption in passive permeability and to be restricted to the proximal tubule (10). These findings are in contrast to the observations of Bergeron *et al.* (3), who suggest that MA-induced Fanconi syndrome is mainly caused by modification of membrane permeability with increased efflux of amino acids into the lumen of the distal nephron rather than by altered transport and reduced influx at proximal sites.

Ultrastructural studies uniformly localize the effects of MA to the proximal nephron (4,16,21), with no changes in the glomerulus or distal segments of the nephron. In the proximal nephron, both PCT and
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PR have been reported to be injured by doses of MA (16,21) larger than the ones we used, which may explain the intactness of the Na,K pump in the PR in our study.

The selective toxicity sites of MA may result from the selective permeability of renal cortical cell membrane to maleate and its high accumulation in this tissue (17,20). Further, the kidneys are the major tissue for the metabolism of maleate (17,20) by both maleate hydratase (20) and succinyl coenzyme A transferase pathways (20). This renal metabolism may contribute to the transient character of the defect. Thus, although MA is capable of inhibiting the Na,K pump uniformly in vitro, the discrete in vivo defect is likely related to the way MA is handled by the filtering nephron, with high rates of accumulation of the compound proximally and minimal distal delivery at the doses used here. With larger doses of MA, more-distal segments may be affected, but this is usually with proximal nephron necrosis, which obviates the usefulness of this model for functional studies.

The effects of maleate on the Na,K pump are not restricted to the kidney. Wapnir et al. (27) observed a decline in mucosal intestinal Na-K-ATPase activity in the absence of any impairment of glucose or amino acid absorption. The divergence in the latter effect from the observation in the kidney may be related to the independence of intestinal amino acid and glucose transport from sodium.

The parallel decrease in glutamine decarboxylation and the increase in ammonia excretion may appear paradoxical. Increased ammonia production after MA has been described in intact dogs (9), isolated dog renal cortical tubules (8), and rat renal cortical slices (13). Maleate, however, inhibits coenzyme A–dependent intramitochondrial oxidation of α-ketoglutarate, presumably by coenzyme A depletion (14); because α-ketoglutarate is the entry site of the glutamine carbon skeleton into the Krebs cycle, a reduction in renal ammoniagenesis by interfering with the renal oxidation of glutamine would have been expected. This paradox has been interpreted to imply a stimulation of brush border membrane maleate-sensitive glutaminase activity of δ-glutamyl transpeptidase (phosphate independent) (13).

The findings, however, can be reconciled by an alternate interpretation more consistent with the observation of increased renal accumulation and urinary excretion of α-ketoglutarate after maleate administration (9). Gougoux et al. (9) have provided evidence in the dog that maleate markedly increased renal glutamine utilization and ammonia production, but that the kidney metabolized glutamine into α-ketoglutarate, indicating only a metabolic block at the α-ketoglutarate dehydrogenase step. Thus, the deamidation of glutamine to glutamate and the deamination of the latter to α-ketoglutarate are stimulated by maleate, resulting in both increased ammonia generation and α-ketoglutarate accumulation.

In summary, our results show that the impairment in the PCT Na,K pump occurs early after the administration of MA and reverses within 24 h. This decline is restricted to the PCT with no changes in pump activity in distal nephron segments. The decline in PCT Na,K pump activity is paralleled by an impairment in oxidative metabolism in this segment. The latter may contribute to the decline in Na,K pump activity and to the glucosuria because, at high MA concentrations in vitro, the efflux of glucose across the luminal membrane has been found to be increased (28,29). The predicted consequence of the decline in the Na,K pump is a decrement in Na-dependent transport. This mechanism may underlie the many consequences of this model of proximal tubulopathy, which are reflections of impairment in sodium-dependent transport processes.

ACKNOWLEDGMENTS

This study was performed with the technical assistance of Yong Chen.

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