Effect of 2′-phosphophloretin on renal function in chronic renal failure rats

B. E. Peerce, L. Weaver, and R. D. Clarke

1Department of Physiology and Biophysics and 2Animal Resource Center, The University of Texas Medical Branch, Galveston, Texas 77555-0641

Submitted 13 October 2003; accepted in final form 2 February 2004

Peerce, B. E., L. Weaver, and R. D. Clarke. Effect of 2′-phosphophloretin on renal function in chronic renal failure rats. Am J Physiol Renal Physiol 287: F48–F56, 2004.—Hyperphosphatemia and secondary hyperparathyroidism are common and severe complications of chronic renal failure. Therapies to reduce serum phosphate have been shown to reduce serum parathyroid hormone (PTH) and slow the progression of renal failure. The effect of the inhibitor of intestinal phosphate absorption, 2′-phosphophloretin (2′-PP), on serum and urine chemistry, renal histology, and cardiac structure in the uremic rat model of renal failure, 5/6 nephrectomy (5/6 NX), was examined. The effect of 2′-PP on serum phosphate, serum PTH, serum total Ca\(^{2+}\), and ionized Ca\(^{2+}\), Ca\(^{2+}\) × Pi product, urine protein, urine osmolality, and creatinine clearance in 5/6 NX rats was examined. Uremic rats in chronic renal failure were gavaged daily with 25 μM 2′-PP. Over the course of a 5-wk experiment, serum chemistry in untreated uremic rats, 2′-PP-treated uremic rats, and age-matched control rats with normal renal function was determined twice a week. Urine creatinine, urine osmolality, urine phosphate, and urine protein were determined once a week from 24-h collections. 2′-PP reduced serum phosphate 40 ± 3% compared with a 17% increase in untreated uremic control rats. 2′-PP did not alter total serum Ca\(^{2+}\). During 5-wk experiments, serum PTH increased 65 ± 25% in untreated uremic rats and decreased 70 ± 7% in uremic rats treated with 25 μM 2′-PP. Creatinine clearance decreased 20% in untreated uremic rats compared with a 100% increase in 2′-PP-treated uremic rats. Urine protein decreased and urine osmolality increased in uremic rats treated with 2′-PP. The mechanism of the effect of 2′-PP on serum phosphate was inhibition of intestinal phosphate absorption. 2′-PP decreased intestinal phosphate absorption 50% without altering dietary protein absorption or intestinal Ca\(^{2+}\) absorption. Over the course of the 5-wk treatment with 2′-PP, uremic animals treated with 2′-PP had a 2–4% weight gain/wk, similar to the weight gain seen in age-matched control rats with normal renal function.

sodium-phosphate cotransport; uremia

Hyperphosphatemia and secondary hyperparathyroidism are common and severe complications of chronic renal failure (4, 8, 43, 44, 46). Elevated serum Ca\(^{2+}\) × Pi product has been implicated in CaHPO\(_4\) deposition in soft tissue and in arterial and cardiac complications (4, 25, 41). Elevated serum phosphate may also contribute to parathyroid gland hyperplasia and hypertrophy (1, 17, 29). Therapies designed to reduce serum phosphate and serum parathyroid hormone (PTH) have been effective in slowing the progression of renal failure (5, 12, 25, 30, 49) and, in some clinical trials, have been shown to reverse the loss of renal function (3, 4, 27, 29).

The kidney, small intestine, and bone are the major organs involved in phosphate homeostasis. The intestinal brush-border membrane Na\(^+-\)phosphate cotransporter (NaPi-IIb) absorbs up to 70% of dietary phosphate. In the serum, phosphate exists as a free ion and calcium salt. The kidney proximal tubule reabsors 70% of the filtered phosphate, and the distal kidney reabsorbs ~20% (43, 49).

In addition to NaPi-IIb, the NaPi family of proteins includes two renal Na\(^+-\)phosphate cotransporters, NaPi-IIa and NaPi-Ia, and the ubiquitous Na\(^+-\)phosphate cotransporters Pit-1 and Pit-2. NaPi-IIa and NaPi-Ia are involved in renal tubule phosphate reabsorption. PTH regulation of proximal tubule phosphate reabsorption is well documented. PTH downregulates NaPi-IIa expression in the proximal tubule brush-border membrane, inducing phosphaturia (41). In renal failure, PTH receptors become unresponsive to PTH by elevated serum PTH concentrations (7, 31, 50, 51). The mechanism of proximal tubule PTH insensitivity in chronic renal failure is not completely understood.

Phosphophloretins have been shown to be effective inhibitors of small intestine brush-border membrane (37, 38) and renal brush-border membrane (39) Na\(^+-\)phosphate cotransport in isolated brush-border membrane vesicles (BBMV) in vitro. The water-soluble phosphophloretin derivative 2′-phosphophloretin (2′-PP) inhibited Na\(^+-\)-dependent phosphate uptake into intestinal BBMV with an IC\(_{50}\) of 40 nM. 2′-PP inhibited NaPi-Ia transport of phosphate with an IC\(_{50}\) of 50 nM in renal cortex BBMV and distal tubule-enriched apical membrane vesicles (39). An alkylated phosphophloretin (2′-phospho-4′, 4,6 trimethoxyphloretin) inhibited NaPi-IIa-mediated, Na\(^+-\)dependent phosphate uptake into renal cortex BBMV and proximal tubule-enriched BBMV with an IC\(_{50}\) of 23 nM (39). In vivo, 2′-PP reduced serum phosphate in adult rats with an IC\(_{50}\) of 10 μM.

Five-sixth nephrectomy rats (5/6 NX) are a well-established and -documented renal failure model system. As a function of time postsurgery and dependent on the postsurgery Ca\(^{2+}\) and phosphorus dietary content, 5/6 NX rats develop renal failure. Many of the serum and systemic complications of chronic renal failure in humans are also seen in the 5/6 NX rat model, including hyperphosphatemia and secondary hyperparathyroidism. We have examined the effect of 2′-PP on serum phosphate, serum PTH, and multiple measures of renal function in 5/6 NX rats with chronic renal failure. In 5-wk experiments, 2′-PP reduced serum phosphate and serum PTH and increased creatinine clearance to levels consistent with moderate to early renal failure. The results indicate that inhibition of intestinal phosphate absorption reduced serum phosphate and serum PTH without altering dietary protein absorption, that 2′-PP treatment improved renal function and reduced serum phosphate, and that reduced serum PTH does not reverse...
renal hypertrophy but may slow the progressive loss of renal function in the remnant kidney model.

MATERIALS AND METHODS

Materials. Phosphate, creatinine, and calcium assay kits were purchased from EQual Diagnostics (Exton, PA). Phosphate and calcium standards were purchased from Sigma (St. Louis, MO). An I-PTH assay kit was purchased from Immutops (San Clemente, CA). Reagents and chemicals, which were used in the synthesis of 2'-PP, were purchased from Aldrich (Milwaukee, WI). All other chemicals were purchased from Fisher Scientific (Houston, TX) and were reagent grade or better.

Animals. Eight-week-old male Sprague-Dawley 5/6 NX and age-matched control rats with normal renal function were purchased from Charles River Laboratories. Briefly, rats were anesthetized with ketamine/diazepam, and the right kidney was removed. One week later, two-thirds of the left kidney were removed. The animals were allowed to recover for 1 wk. The second week post-surgery, animals were randomly divided into three groups of eight rats, marked, weighed, and housed individually. During a 2-wk acclimation period, animals were placed on a 3-h feeding window and a 12:12-h light-dark cycle. The animals were fed normal rat chow (Teklab 7201) containing 19% protein, 0.67 g/0.1 kg phosphorus, and 0.97 g/0.1 kg calcium and had unlimited access to water.

The handling, treatment, and experiments involving animals were submitted for approval to, examined by, and approved by the UTMB Institutional Animal Care and Use Committee and were in compliance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals.

Rats were gavaged daily with vehicle (150 mM NaCl and 5 mM citrate buffer, pH 6) or vehicle plus 25 μM 2'-PP. Blood was withdrawn from the saphenous vein twice a week (14). Collected blood was allowed to clot, and serum was collected by centrifugation for 20 min in a microfuge. Sera were carefully removed and stored on ice. Once a week, rats were placed in metabolic cages for 24-h fecal and urine collection. The animals were weighed once a week immediately before blood was drawn. Each experimental treatment with 2'-PP was 5 wk long. During the course of these studies, none of the rats died during the acclimation and experimental treatment period (2-mo total experimental period).

At the beginning of the treatment period, 5/6 NX rat weights were not significantly different from the weights of age-matched control rats: 326 ± 17 g (n = 48) for 5/6 NX rats and 324 ± 8 g (n = 8) for age-matched control rats. At the end of the 5-wk experiment, untreated 5/6 NX rats weighed 301 ± 13 g (n = 16), 2'-PP-treated rats weighed 360 ± 14 g (n = 26), and age-matched control rats weighed 372 ± 16 g (n = 8).

Serum and urine chemistry. Serum and urine phosphate concentrations were determined spectrophotometrically at 340 nm using clinical phosphorus kits. Standard curves for phosphate were generated using 1, 5, and 10 mg/dl phosphorus standards. Serum and urine calcium were determined spectrophotometrically using the arsenazo dye method (32). Calcium at 5, 10, and 15 mg/dl were used as standards. Serum and urine creatinine were determined by the method of Jaffe (13). Serum protein was determined by the method of Lowry (40) and enrichments of the brush-border membrane alkaline phosphatase (9) and y-glutamyl transpeptidase (36), were determined. After isolation, BBMV were resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, and stored as aliquots in liquid nitrogen until needed. During the course of these studies, rat intestinal BBMV were 18- to 20-fold enriched in the brush-border membrane markers alkaline phosphatase and γ-glutamyl transpeptidase compared with the total homogenate.

Na+-dependent phosphate uptake into intestinal BBMV was performed using a rapid mixing/rapid sampling procedure (37–39, 48). Na+-dependent uptake was defined as uptake in the presence of 100 mM NaCl, 100 mM mannitol, 10 mM HEPES/Tris, pH 7.5, and 100 μM [32P]phosphate minus uptake in the presence of 100 mM KCl, 100 mM mannitol, 10 mM HEPES/Tris, pH 7.5, and 100 μM [32P]phosphate. Uptakes were performed for 5 s at 23°C.

In some experiments, the effect of 2'-PP or phloretin on Na+-dependent phosphate uptake into intestinal BBMV was determined. In these experiments, 2'-PP concentration was varied between 5 and 500 nM, and phloretin concentration was varied between 100 nM and 10 μM.

In some experiments, the effect of phosphate concentration on Na+-dependent phosphate uptake and on 2'-PP inhibition of Na+-dependent phosphate uptake into intestinal BBMV was examined. In these experiments, Na+-dependent phosphate uptake was determined as described above. 2'-PP concentration was varied between 5 nM and 1 μM. Phosphate concentration was varied between 10 and 500 μM.

Heart and kidney histology. At the end of the 5-wk experiment, 2'-PP-treated and untreated uremic control rats were killed. Hearts were perfused with 250 mM sucrose, 1 mM EDTA, and 10 mM Tris·HCl, pH 7, and weighed. The left ventricle was removed and weighed. Tissue was fixed in 10% buffered formalin. Cardiac sections were examined by a pathologist who was not aware of which sections corresponded to treatment and control.

Remnant kidneys were removed and weighed. The remnant kidneys were added to 10 ml of 10% buffered formalin and stored at 4°C for 12 h. The formalin was then removed and replaced with fresh 10% buffered formalin. Paraffin sections (4 μm) were stained with hematoxylin and eosin or periodic acid-Schiff (PAS). For each experimental group, multiple sections (12–20 sections/animal) from two animals were selected randomly. Renal sections were coded and then examined by a pathologist who was blinded to which sections corresponded to which group.

Synthesis of 2'-PP. 2'-PP was synthesized from phosphoridin and dibenzylphosphite in N,N-dimethylacetamide (37). 2'-PP was purified...
by chromatography and recrystallization from ethylacetate (37): melting point 171-172°C, 1H-NMR (d6-DMSO) δ 13.0 [singlet (s), 1H], 10.7 [broad singlet (brs), 1H], 9.2 (brs, 1H), 7.03 [doublet (d), J = 8.6 Hz, 2H], 6.64 (d, J = 8.4 Hz, 2H), 6.63 (dd, J = 1.2, 2.1 Hz, 1H), 2.77 (d, J = 7.6 Hz, 2H). 31P-NMR in D2O yielded a single peak at ~4 parts per million comprising 98% of the phosphorus signal. 31P-NMR in DMSO yielded a single peak at ~4.3 pulses/min.

Statistical analysis. Results are presented as means ± SE for all rats in the experimental group. Numbers of rats used are shown in the figures or described in RESULTS. Comparisons were made between uremic rats and age-matched controls. Serum phosphate values from age-matched control rats with normal renal function are means ± SE of 3 determinations and 3 experiments; uremia results are from 42 rats, and age-matched control results are from 8 rats. 2'-PP, 2'-phosphophloretin; PTH, parathyroid hormone.

RESULTS

Renal function of rats at the onset of treatment. Table 1 summarizes the serum chemistry and renal function assays before the start of treatment with 2'-PP. Serum phosphate concentration and serum PTH concentration were markedly elevated compared with age-matched controls with normal renal function. Plasma Ca2+ was not significantly different compared with age-matched controls. Serum creatinine was elevated, and creatinine clearance was 30% of that in age-matched controls. The magnitude of hyperparathyroidism (serum PTH 6 times age-matched controls), hyperphosphatemia (20% increase), elevated serum creatinine, reduced creatinine clearance (30% of normal controls), low urine osmolality (33% of age-matched controls), and elevated Ca2+ × PO4 product (30% higher than age-matched controls) is consistent with chronic renal failure (5, 20, 42).

Effect of 2'-PP on serum phosphate and intestinal phosphate absorption in chronic renal failure rats. The effect of 25 μM 2'-PP on serum phosphate is shown in Fig. 1. Compared with age-matched control rats with normal renal function (D), uremic rats gavaged with vehicle (●, dashed line) were hyperphosphatemic. Serum phosphate in untreated uremic rats (5/6 NX rats gavaged with vehicle) was 23% higher than in age-matched control rats at the start of treatment and increased as a function of time during the 5-wk experiment. Serum phosphate in untreated uremic rats increased an additional 12 ± 1% (n = 16 rats) during the course of the experiment. Serum phosphate in 5/6 NX rats gavaged with 2'-PP (■, solid line) decreased as a function of time from 9.8 ± 0.6 mg/dl (n = 24 rats) at the start of treatment with 2'-PP to 6.5 ± 0.3 mg/dl (n = 24 rats) after 5 wk of treatment with 2'-PP.

The effect of 2'-PP on intestinal phosphate absorption was determined from the phosphorus ingested in the diet minus fecal phosphorus. At the beginning of the experiment, intestinal phosphate absorption in uremic rats was 70 ± 6% (n = 24 rats) of ingested phosphorus. Treatment with 25 μM 2'-PP reduced intestinal phosphate absorption to 32 ± 5% (n = 16 rats) after 1 wk of treatment and 28 ± 4% after 4 wk of treatment with 2'-PP. Intestinal phosphate absorption in untreated uremic rats was 70 ± 4% (n = 8 rats) and 67 ± 5% (n = 8 rats) at 1 and 4 wk, respectively.

The effect of gavage on intestinal protein absorption and intestinal Ca2+ absorption was also examined to determine the specificity of 2'-PP for intestinal phosphate absorption and as a control for nonspecific intestinal malabsorption. Before the start of gavage, intestinal protein absorption was 92 ± 3% (n = 8 rats) in uremic rats and 94 ± 2% (n = 4 rats) in age-matched control rats with normal renal function. After 4 wk of gavage, protein absorption was 92 ± 3% in untreated uremic rats and 90 ± 4% in 2'-PP-treated rats. Intestinal Ca2+ absorption in uremic rats was 80 ± 6% (n = 24 rats) before the start of the experiment. Intestinal Ca2+ absorption in untreated uremic rats was 81 ± 5% (n = 8 rats) after 4 wk of treatment. Intestinal Ca2+ absorption in uremic rats treated with 2'-PP was 77 ± 5% (n = 16 rats) after 4 wk of treatment with 2'-PP.

The effect of uremia and 2'-PP on fractional excretion of phosphate (FEp) was also examined. At the start of the experiment, FEp of 5/6 NX rats was 18 ± 0.9% (n = 24 rats). FEp of untreated 5/6 NX rats increased to 20.9 ± 1.9% (n = 8 rats) at the end of the 5-wk experiment (not significant compared with 5/6 NX rats at the beginning of the experiment).

Table 1. Serum chemistry and renal function assay before treatment with 2'-PP

<table>
<thead>
<tr>
<th>Serum PO4, mg/dl</th>
<th>Serum Ca2+, mg/dl</th>
<th>Serum PTH, ng/ml</th>
<th>Creatinine Clearance, ml·min⁻¹·kg⁻¹</th>
<th>Serum Creatinine, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uremia</td>
<td>9.8 ± 0.6</td>
<td>9.5 ± 0.5</td>
<td>176 ± 15</td>
<td>2.8 ± 0.16</td>
</tr>
<tr>
<td>Age-matched controls</td>
<td>8.0 ± 0.3</td>
<td>9.4 ± 0.4</td>
<td>32 ± 5</td>
<td>7.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 determinations and 3 experiments; uremia results are from 42 rats, and age-matched control results are from 8 rats. 2'-PP, 2'-phosphophloretin; PTH, parathyroid hormone.

Fig. 1. Effect of uremia and 2'-phosphophloretin (2'-PP) on serum phosphate. Five-sixth nephrectomy rats were gavaged daily with vehicle (●, dashed line) or vehicle and 25 μM 2'-PP (■, solid line) as described in MATERIALS AND METHODS. On the indicated day, blood was withdrawn from the saphenous vein and phosphate was assayed as described in MATERIALS AND METHODS. Results are means ± SE of 16 determinations (8 rats/group). Results shown are from a single experiment and are representative of 3 separate experiments. Serum phosphate values from age-matched control rats with normal renal function (○, solid line) are shown for comparison. P < 0.01, untreated uremic rats vs. 2'-PP-treated uremic rats. P < 0.01, untreated uremic rats on day 0 vs. day 35.
FE_\text{Pi} of 2'-PP-treated rats decreased to 7.8 ± 1.4% (n = 16 rats) at the end of the 5-wk experiment (P < 0.01).

Effect of 2'-PP on plasma Ca^{2+}. The effect of 25 μM 2'-PP on serum Ca^{2+} is shown in Fig. 2. 2'-PP did not significantly alter serum Ca^{2+}. Serum Ca^{2+} in 5/6 NX rats was 10 mg/dl at the start of the experiment before treatment with 2'-PP. Serum Ca^{2+} in uremic rats treated with 2'-PP (○, dashed line) did not significantly change (10.2 mg/dl) during the treatment with 2'-PP. Untreated uremic rats (●, solid line) had a 4.7% increase in serum Ca^{2+} during the experiment and were slightly hypercalcemic (serum Ca^{2+} of 11 vs. 10.2 mg/dl for 2'-PP-treated uremic rats and 10 mg/dl for age-matched control rats with normal renal function; □, solid line) at the end of the experiment.

Ionizable Ca^{2+} in untreated uremic rats decreased 6 ± 3% (n = 8 rats). The change in ionizable Ca^{2+} in untreated uremic control rats was not statistically significant. Ionizable Ca^{2+} increased 11 ± 2% in 2'-PP-treated uremic rats (n = 16 rats) over the 5-wk experiment and 19 ± 3% compared with untreated uremic control rats. Compared with ionizable Ca^{2+} at the start of treatment with 2'-PP and compared with untreated uremic control rats, the effect of 2'-PP on ionizable Ca^{2+} was significant (P < 0.1).

The effect of 2'-PP on the Ca^{2+} × HPO_4 product is shown in Fig. 3. The Ca^{2+} × HPO_4 product in 2'-PP-treated rats (●, solid line) decreased 28% over the first 2 wk of treatment and remained stable at ~70% of the starting value during the remainder of the experiment. The Ca^{2+} × HPO_4 product in untreated uremic control rats (○, dashed line) increased slowly over the course of the first 3 wk of the experiment. Shown for comparison are results from age-matched control rats (□, dashed line).

Effect of 2'-PP on creatinine clearance and serum i-PTH. Figure 4 shows the development of secondary hyperparathyroidism in 5/6 NX rats. Serum i-PTH in untreated uremic rats (○, solid line) increased 50% during the experiment. Serum i-PTH in uremic rats treated with 2'-PP (●, dashed line) decreased from 178 pg/ml at the start of the experiment to 50 pg/ml 3 wk after initiation of treatment and 42 pg/ml at the end of the experiment.

Figure 5 shows the effect of 2'-PP on creatinine clearance. Creatinine clearance was 2.8 ml·min^{-1}·kg body wt^{-1} at the
start of the experiment. Creatinine clearance in untreated uremic rats (□, dashed line) continued to decrease slowly to 2.3 ml·min⁻¹·kg body wt⁻¹ after 4 wk. Creatinine clearance in 2'-PP-treated 5/6 NX rats (●, solid line) increased biphasically to 6.2 ml·min⁻¹·kg body wt⁻¹ (6.25 ± 0.3 ml·min⁻¹·kg body wt⁻¹, n = 3 experiments in 8 rats/group).

Urine osmolality. The effect of 5/6 NX and 2'-PP on urine osmolality is shown in Fig. 6. Urine osmolality of untreated 5/6 NX rats (○, dashed line) was below 500 mosmol/kgH₂O 3 wk after surgery at the start of the experiment and decreased a further 10 ± 5% (n = 8 rats) during the course of treatment. 2'-PP-treated uremic rats (●, solid line) had an immediate 30 ± 5% increase in urine osmolality, which continued for the first 7–14 days of treatment with 2'-PP. Urine osmolality of 2'-PP-treated uremic rats increased to 1,100 ± 80 mosmol/kgH₂O (n = 16 rats). Urine osmolality of 2'-PP-treated uremic rats was 80% of that in age-matched control rats with normal renal function. Urine osmolality in age-matched control rats with normal renal function was 1,320 ± 120 mosmol/kgH₂O (n = 8 rats), consistent with previous results (21–23).

Urine volume/24 h in untreated uremic rat groups was 40 ± 10 ml (n = 8 rats). Urine volume/24 h in 2'-PP-treated uremic rats was 20 ± 4 ml (n = 16 rats).

Urine protein. Urine protein increased in untreated uremic rats from 40 mg/24 h (40 ± 4 mg/24 h) at the start of the experiment to 80 mg/24 h (80 ± 10 mg/24 h) at the end of the experiment. Urine protein in 2'-PP-treated uremic rats decreased 56% over the course of the 5-wk experiment (17.5 ± 2.5 mg/24 h).

Effect of 2'-PP treatment on BBMV Na⁺-dependent phosphate uptake and 2'-PP inhibition of phosphate uptake. The effect of treatment with 2'-PP on small intestinal BBMV Na⁺-dependent phosphate uptake was examined to determine whether treatment with 2'-PP altered Na⁺-phosphate cotransporter activity or sensitivity to 2'-PP. Treatment with 2'-PP did not alter the IC₅₀ for 2'-PP inhibition of Na⁺-dependent phosphate uptake into intestinal BBMV isolated from uremic rats (Table 2). The IC₅₀ values for 2'-PP inhibition of Na⁺-dependent phosphate uptake into intestinal BBMV isolated from uremic rats were similar in 2'-PP-treated rats, untreated uremic rats, and age-matched control rats with normal renal function.

The effect of uremia and treatment with 2'-PP on the kinetics of Na⁺-dependent phosphate uptake was also examined. Compared with age-matched control rats with normal renal function, Na⁺-dependent phosphate uptake into intestinal BBMV in uremic rats was reduced 44% (Table 2). Treatment of uremic rats with 2'-PP had a slight effect on Na⁺-dependent phosphate uptake compared with untreated uremic control rats, reducing the apparent Vₘₐₓ for 2'-PP-treated rats, which was 20 ± 1.9 pmol·mg⁻¹·s⁻¹ (n = 3) for 2'-PP-treated rats compared with 14 ± 1 pmol·mg⁻¹·s⁻¹ (n = 3) for untreated uremic rats. The apparent Kₘ for phosphate was unaffected by treatment or uremia.

Table 2. Effect of uremia and 2'-PP on Na⁺-dependent phosphate uptake into intestinal BBMV.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>2'-PP (IC₅₀), nM</th>
<th>Na⁺-Dependent Phosphate Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vₘₐₓ, pmol·mg⁻¹·s⁻¹</td>
<td>Kₘ, µM</td>
</tr>
<tr>
<td>Age-matched control rats</td>
<td>40 ± 4</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Uremia untreated</td>
<td>40 ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>2'-PP-treated</td>
<td>42 ± 3</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 determinations performed in triplicate. IC₅₀ values for 2'-PP and Kₘ values for phosphate were not statistically different between the 3 groups. Vₘₐₓ of Na⁺-dependent phosphate uptake was not statistically different in a comparison of 2'-PP-treated uremic rats and age-matched control rats. Significant difference in Vₘₐₓ for age-matched control rats vs. untreated uremic rats, P < 0.01.

Fig. 5. Effect of uremia and 2'-PP on creatinine clearance. Five-sixth nephrectomy rats were gavaged daily with vehicle (□, dashed line) or vehicle and 25 µM 2'-PP (●, solid line) as described in MATERIALS AND METHODS. On the indicated day, animals were placed in metabolic cages for 24-h urine collections. On the following day, blood was withdrawn from the saphenous vein. Urine and serum creatinine were determined as described in MATERIALS AND METHODS. Results are means ± SE of 16 determinations (8 rats/group). Results shown are from a single experiment and are representative of 3 separate experiments. P < 0.01, untreated uremic rats vs. 2'-PP-treated uremic rats.

Fig. 6. Effect of uremia and 2'-PP on urine osmolality. Five-sixth nephrectomy rats were gavaged daily with vehicle (○, dashed line) or vehicle and 25 µM 2'-PP (●, solid line) as described in MATERIALS AND METHODS. On the indicated day, animals were placed in metabolic cages for 24-h urine collections. Urine osmolality was determined as described in MATERIALS AND METHODS. Results are means ± SE of 16 determinations (8 rats/group). Results shown are from a single experiment and are representative of 3 separate experiments. P < 0.01, untreated uremic rats vs. 2'-PP-treated uremic rats.
The effect of phosphate concentration on 2'-PP inhibition of Na\(^+\)-dependent phosphate uptake into intestinal BBMV isolated from 2'-PP-treated rats is shown in Fig. 7. Figure 7 is a Wolff-Augustinin-Hofstee plot of the effect of phosphate concentration on 2'-PP inhibition of Na\(^+\)-dependent phosphate uptake into intestinal BBMV isolated from 2'-PP-treated rats at 25 nM 2'-PP (□, dashed line) and 100 nM 2'-PP (○, solid line). Phosphate uptake in the absence of 2'-PP (●, solid line) is shown for comparison. As a function of 2'-PP concentration, the lines were shifted to the left, consistent with competition between 2'-PP and phosphate on the Na\(^+\)-phosphate cotransporter. The calculated \(V_{\text{max}}\) (y-intercept) was unaffected by 2'-PP. The results of the effect of 2'-PP treatment on intestinal BBMV Na\(^+\)-phosphate cotransporter activity and sensitivity to 2'-PP were consistent with previous results with rat intestinal BBMV (37). These results also indicate that 2'-PP did not alter Na\(^+\)-phosphate cotransporter kinetics and suggest that the effect of 2'-PP on serum phosphate was not due to a nonspecific intestinal toxicity of 2'-PP.

**Kidney histology.** Light microscopy of sections from the remnant kidneys of uremic control rats (Fig. 8) and 2'-PP-treated uremic rats (Fig. 9) was similar. Sections from untreated uremic rats revealed slight (<20% of the proximal tubules examined) distension of the proximal tubules and decreased tubule lumens. Bowman’s space was slightly distended (arrow in Fig. 8). PAS-positive material in the lumens appeared to be proteinacious and plaquelike (results not shown). In the parenchyma, there were a few scattered areas of mild fibrosis with no obvious monocyte or lymphocyte infiltration. PAS staining revealed slight mesangial cell expansion and a slight thickening of the basement membrane. There were no glomerular adhesions, and capillary membranes were within normal limits. Sections from 2'-PP-treated uremic rats revealed less distension and proteinacious plaquelike material in the proximal tubules (<10% of the tubules). Fibroblast and monocyte infiltration was not observed. PAS staining revealed slight and very mild segmental mesangial cell expansion and a slight thickening of the basement membrane. Both 2'-PP-treated and untreated uremic rats had similar degrees of renal hypertrophy. Untreated uremic rats had a larger number of proximal tubules containing PAS-positive material in the tubule lumen.

![Fig. 7. Effect of 2'-PP and phosphate concentration on Na\(^+\)-dependent phosphate uptake into intestinal brush-border membrane vesicles (BBMV) of isolated uremic rats treated with 2'-PP. Small intestinal BBMV were isolated from uremic rats treated with 2'-PP for 5 wk as described in MATERIALS AND METHODS. Na\(^+\)-dependent \[^{32}\text{P}\]phosphate uptake was determined as a function of phosphate concentration between 10 and 250 \mu M in the absence of inhibitor (●, solid line), in the presence of 25 nM 2'-PP (□, broken line), and in the presence of 100 nM 2'-PP (○, solid line). Results were plotted as a Wolff-Augustinin-Hofstee plot and fitted by least squares analysis using Enzfitter. Results are means ± SE of triplicate determinations and representative of 3 experiments.](image1)

![Fig. 8. Renal histology of remnant kidneys from untreated uremic rats. Light microscopic sections from remnant kidneys from untreated uremic control rats at 8 wk postsurgery and 5 wk of treatment are shown. Remnant kidneys were prepared as described in MATERIALS AND METHODS and stained with hematoxylin and eosin. Single thin arrow (left) shows expansion of Bowman’s capsule, and double arrow (right) shows early stage of segmentation. Note spaces between structures (US).](image2)

![Fig. 9. Renal histology of remnant kidneys from 2'-PP-treated uremic rats. Light microscopic sections from remnant kidneys from 2'-PP-treated uremic rats 8 wk postsurgery and 5 wk of treatment with 2'-PP. Sections were stained with hematoxylin and eosin. Glomeruli remain rounded, and there is no expansion of Bowman’s space. Note that there are no spaces between structures.](image3)
Cardiac histology. Sagittal sections of the hearts from uremic rats and 2'-PP-treated uremic rats were very similar. Cardiomyocytes were within normal limits, and there was no hypertrophy. The cardiac interstitium appeared normal, and there was no fibrosis or inflammation. Coronary vessels and the aorta appeared to be within normal limits.

Left ventricular (LV) wall thickness was slightly greater in untreated uremic rats than in 2'-PP-treated uremic rats (3.25 ± 0.25 mm in untreated uremic rats vs. 4.25 ± 0.2 mm in 2'-PP-treated uremic rats). Left ventricular hypertrophy (LVH), expressed as LV grams per kilogram body weight, was also elevated in untreated uremic rats. Values were 1.7 ± 0.05 LV g/kg body wt (n = 8) for untreated uremic rats and 1.49 ± 0.06 LV g/kg body wt (n = 8) for 2'-PP-treated uremic rats.

DISCUSSION

A correlation between the severity of secondary hyperparathyroidism and serum phosphate has been suggested by clinical and animal trials employing reduced phosphorus diets (2, 3, 5, 24–26, 29, 30) by the effect of high-phosphate media on parathyroid glands in vitro (1, 19, 42), the effect of phosphate binders on the progression of chronic renal failure (6, 10), and the effect of phosphate on PTH secretion in vitro (20, 44). Animal studies and clinical trials have shown that reductions in dietary phosphate sufficient to reduce serum phosphate delay or reverse the progression of chronic renal failure (6, 10), and parathyroid glands in vitro (1, 19, 42), the effect of phosphate binders on the progression of chronic renal failure (6, 10), and the effect of phosphate on PTH secretion in vitro (20, 44).

Animal studies and clinical trials have shown that reductions in dietary phosphate sufficiently to reduce serum phosphate delay or reverse the progression of chronic renal failure to end-stage renal failure. The use of phosphate binders to reduce dietary phosphorus absorption has been shown to significantly reduce serum phosphate and the Ca\(^{2+}\) x Pi product (6, 10). Because the major phosphate uptake pathway in the small intestine is the Na\(^+\)-phosphate cotransporter NaPi-IIb (12), inhibition of this pathway by a specific reagent would be a significant advance in the treatment of phosphate retention in renal failure. A specific inhibitor of NaPi-IIb offers a major advantage compared with phosphate binders because reductions in dietary phosphate sufficient to reduce serum phosphate severely reduce dietary protein.

A phosphorylated derivative of phloretin, 2'-PP, has been shown to be a specific inhibitor of intestinal phosphate absorption in vitro (37–39) and in vivo (37). Previous studies using intestinal (37, 38) and renal (39) BBMV and aged adult rats (37) indicated that 2'-PP is a specific inhibitor of NaPi-IIb with IC\(_{50}\) values of 40 nM in vitro and 12 μM in vivo. 2'-PP inhibition of renal phosphate reabsorption appears to be limited to inhibition of NaPi-Ia (39). These studies have been extended to the uremic rat.

The effect of 25 μM 2'-PP on renal function and the progression of renal failure over a 2-mo experimental and a 5-wk treatment period were examined. At the beginning of treatment with 2'-PP, rats were in moderately severe chronic renal failure (Table 1). This assignment was based on a comparison with literature values and the average of three separate determinations of serum PTH, serum phosphate, serum creatinine, urine osmolality, and creatinine clearance (5, 20, 43).

The effect of 2'-PP on serum phosphate is shown in Fig. 1. Daily gavage with 25 μM 2'-PP decreased serum phosphate in uremic rats 42 ± 1.6%. In comparison, serum phosphate in untreated uremic rats continued to increase during the 5-wk experimental treatment 17 ± 2%.

2'-PP did not alter total serum Ca\(^{2+}\) during the 5-wk experiment (Fig. 2). 2'-PP did increase ionizable Ca\(^{2+}\) 19% over the 5-wk experiment, which could account for some of the observed decrease in serum PTH. Decreased serum phosphate yielded a 35% decrease in the Ca\(^{2+}\) x Pi product (Fig. 3) over the 5-wk experiment.

Consistent with previous studies with low-phosphorus diets (2–5, 8, 16, 18, 24–27, 44–47) and 5/6 NX rats, reduced serum phosphate decreased serum PTH. Serum PTH in uremic rats treated with 2'-PP decreased from 180 to 42 pg/ml over the course of the 5-wk experiment. In the absence of 2'-PP, uremic rat serum PTH approximately doubled over the 5-wk experiment.

The effect of 2'-PP on the glomerular filtration rate was examined using creatinine clearance. In 2'-PP-treated 5/6 NX rats, creatinine clearance increased 58 ± 6%, and serum creatinine decreased to 0.34 mg/ml during the 5-wk experiment. Creatinine clearance in untreated uremic rats fell slightly to 2.3 ml/min 1.7 kg body wt\(^{-1}\).

The use of creatinine clearances in the examination of treatment efficacy has been questioned (22). Creatinine is not an ideal substance for the determination of renal function due to creatinine renal creatinine secretion. To confirm the effect of 2'-PP on creatinine clearance, urine osmolality, urine protein, renal morphology, and cardiac morphology were also examined.

Renal failure is associated with reduced urine osmolality, increased urine volume, and increased urine protein. Urine osmolality increased 120% in 2'-PP-treated uremic rats over the course of treatment. The effect of 2'-PP treatment on urine osmolality of uremic rats is consistent with a minimal change in water reabsorption and a minimal decrease in distal tubule and collecting duct function. Urine protein decreased ~50% in 2'-PP-treated uremic rats during the 5-wk experiment. These results are consistent with recent studies showing reversal of protein-induced tubulointerstitial damage early in the development of proteinuria (19). There does not appear to be a causal relationship between phosphate and urine protein. There is a correlation between the severity of renal failure and the degree of proteinuria (16) and the severity of renal failure and the severity of hyperphosphatemia.

Renal failure is also associated with tubule and glomerular hypertrophy, inflammation, and fibrosis. Cardiomyocyte apoptosis, cardiac ischemia, and hypertrophy of the left ventricle wall are associated with the later stages of renal failure (11, 28). Figure 8 demonstrates slight renal hypertrophy of the remnant kidney in untreated uremic rats. There was slight epithelial cell expansion and slight expansion of Bowman’s space around the glomerulus. Approximately 20% of the tubules contained PAS-positive material in the lumen. Fibrosis was found only around the poles where renal tissue was removed during surgery. 2'-PP-treated uremic rats had similar epithelial cell expansion and little expansion of Bowman’s space. Fewer than 10% of the tubules contained PAS-positive material.

The effect of 2'-PP treatment on renal histology is consistent with a delay in the progression of renal failure and not a reversal of renal hypertrophy. Before the start of 2'-PP treatment, rat remnant kidneys had increased in size ~40%. 2'-PP treatment did not decrease kidney size but did appear to decrease plaque formation in tubule lumens and decrease the
rate of extracellular matrix deposition and mesangial cell expansion. Based on the size of proximal tubule cells, 2'-PP treatment did not reverse tubule cell expansion. The absence of gross changes in rat kidney morphology is consistent with previous studies (34, 35).

Cardiac sections from untreated and 2'-PP-treated uremic rats were similar. There was no inflammation or evidence of ischemic damage. LV wall diameter was larger in untreated uremic rats than in 2'-PP-treated uremic rats. Expressed per kilogram rat body weight, LHV was 1.7 g/kg body wt in untreated uremic rats compared with 1.4 g/kg body wt in 2'-PP-treated uremic rats, suggesting moderate LVH in untreated uremic rats.

The time course of the change in serum phosphate correlated with the decrease in serum PTH and the decrease in Ca\(^{2+}\) \(\times\) P product. A plot of the change in serum PTH vs. the change in serum phosphate was linear, with a correlation coefficient of 0.983. The excellent correlation between serum phosphate and serum PTH suggests that the 2'-PP-mediated decrease in serum phosphate resulted in a similar decrease in serum PTH. A similar plot of the change in creatinine clearance vs. the change in serum phosphate was also linear, with a correlation coefficient of 0.91. These results are consistent with previous studies examining the effect of reduced dietary phosphorus on serum phosphate and renal function (4).

The mechanism responsible for the effect of reduced serum phosphate on serum PTH remains unclear. The effect of 2'-PP on ionized Ca\(^{2+}\), the parathyroid gland regulator, was 11%. It is unlikely that the 11% increase in ionizable Ca\(^{2+}\) was responsible for the reduced serum PTH. Reduced parathyroid gland responsiveness to serum Ca\(^{2+}\) is a hallmark of chronic renal failure. An effect of serum phosphate on parathyroid gland growth and PTH synthesis has been suggested (1, 20, 25, 33, 45, 46). The mechanism responsible for the effect of reduced serum phosphate on creatinine clearance and urine osmolality may be related to the decrease in urine protein (16), reduced Ca\(^{2+}\) retention in the proximal tubules, and tubule proteinase activity (42).

The mechanism responsible for reduced serum PTH and serum phosphate in uremic rats treated with 2'-PP appeared to be the result of inhibition of intestinal phosphate absorption at the intestinal Na\(^{+}\)-phosphate cotransporter. Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV isolated from uremic rats, uremic rats treated with 2'-PP, and age-matched control rats with normal renal function was similar. The three study groups had similar IC\(_{50}\) values for 2'-PP inhibition of Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV and similar \(K_m\) values for phosphate (Table 2). The absence of an effect of 2'-PP treatment on intestinal Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV indicates that the effect of 2'-PP on serum phosphate was not the result of nonspecific intestinal toxicity reducing serum phosphate.

Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV isolated from 2'-PP-treated uremic rats retained 2'-PP sensitivity and phosphate dependence similar to that seen with intestinal BBMV isolated from adult rats (37). 2'-PP inhibition of Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV isolated from 2'-PP-treated uremic rats at variable phosphate concentrations (Fig. 7) indicates that 2'-PP and phosphate compete for the Na\(^{+}\)-phosphate cotransporter. These results are similar to results from isolated rat, rabbit, and human BBMV and suggest that the mechanism of 2'-PP inhibition of intestinal phosphate absorption was inhibition of the intestinal Na\(^{+}\)-phosphate cotransporter and was similar to the effect of 2'-PP on Na\(^{+}\)-dependent phosphate uptake into intestinal BBMV in vitro.

The results indicate that 2'-PP is an effective treatment for hyperphosphatemia and secondary hyperparathyroidism in the remnant kidney rat model of chronic renal failure. Based on the increase in creatinine clearance, increased urine osmolality, and decreased urine protein, 2'-PP treatment of uremic rats appeared to improve renal function over the course of the 5-wk experimental treatment. Inhibition of intestinal phosphate absorption was as effective a method of reducing serum phosphate as very-low-phosphorus diets (0.02 g phosphorus/100 g rat chow).

ACKNOWLEDGMENTS

The authors thank Lisa Brown for assistance in the care of the rats used in this study. The authors thank Dr. Karen Vargas and Dr. Randall Ruble, Animal Resource Center, University of Texas Medical Branch (UTMB; Galveston, TX) for assistance with tissue specimens, and Dr. Greg Hall, Eli Lilly (Indianapolis, IN), for interpretation of cardiac and renal histology. The authors gratefully acknowledge the assistance of Dr. Srinivasan Rajaraman (Dept. of Nephrology, UTMB) for assistance in the renal histological studies. The authors also gratefully acknowledge the expert assistance of Ed Ezell (Sealy Ctr., UTMB) in the analysis of 2'-PP preparations.

REFERENCES

41. Slusarski D, Ebert J, and Frøkiær J. Chronic metabolic acidosis in azotemic rats on a high-phospho...