Phosphate Excretion and Phosphate Transporter Messenger RNA in Uremic Rats Treated with Phosphonoformic Acid

DAVID P. BROOKS, SHUJATH M. ALI, LISA C. CONTINO, ELWOOD STACK, TODD A. FREDRICKSON, JOHN FEILD and RICHARD M. EDWARDS

Departments of Renal Pharmacology (D.P.B., S.M.A., L.C.C., E.S., T.A.F., R.M.E.) and Molecular Genetics (J.F.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

Accepted for publication February 3, 1997

ABSTRACT

The prevention of phosphate retention in chronic renal disease may reduce both renal osteodystrophy and disease progression. We evaluated the expression of the sodium-dependent phosphate transporter, NaPi-2, and the response to phosphonoformic acid (PFA) in rats with 5/6 nephrectomy-induced renal failure. Partial nephrectomy resulted in a significant proteinuria and reduced renal function. In addition, there was an ~50% reduction in the expression of NaPi-2 mRNA. Treatment of rats for 48 hr with PFA (0.6% in glucose drinking fluid) had no effect on NaPi-2 mRNA; however, PFA resulted in a significant increase in fractional phosphate excretion in both normal (7 ± 0.5% vs. 3 ± 0.2%) and uremic (60 ± 4% vs. 36 ± 4%) rats. Plasma phosphate concentration was higher in uremic rats (2.5 ± 0.1 mM) compared with normal rats (1.9 ± 0.04 mM) but not in uremic rats treated with PFA (2.1 ± 0.04 mM). These data suggest that PFA can increase renal phosphate excretion independent of changes in phosphate transporter expression and prevent phosphate retention.

Phosphate absorption from the intestine and reabsorption from the renal tubule play important roles in the control of inorganic phosphate metabolism. Both processes involve sodium-dependent phosphate transport. Studies using brush border membrane vesicles indicate that this transporter is regulated by a number of factors, including dietary phosphate intake (Loghman-Adham, 1993). Thus, increasing or decreasing dietary phosphate intake results in decreased and increased transport, respectively. Such alterations in dietary phosphate intake result in changes in the $B_{\text{max}}$ value for the sodium-dependent phosphate transporter, with no change in the apparent $K_m$ value, suggesting a change in the number of transporters. In situations of reduced glomerular filtration, phosphate retention occurs, and this may contribute to the subsequent progression of renal disease (Loghman-Adham, 1993). As observed with a high-phosphate diet, uremia can also result in a reduction in the maximum rate of sodium-dependent phosphate transport in renal brush border membrane vesicles, consistent with a reduction in the number of transporters (Motock et al., 1991).

Studies in both animal models of renal failure and patients suggest that a reduction in phosphate intake may be beneficial. Such therapies primarily involve reducing intestinal phosphate absorption using low-phosphate diets or phosphate binders, both of which have limitations. In the past few years, the genes encoding sodium-dependent phosphate transporters from a number of different species have been cloned (Biber and Murer, 1994), raising the specter of novel therapeutics that could alter phosphate transport. PFA is an antiviral agent that has been shown to inhibit sodium-dependent phosphate transport specifically (Szepanska-Konkel et al., 1986); however, long-term use of this agent may be compromised because of potential renal toxicities. This agent, however, provides an important tool with which to evaluate whether inhibition of sodium-dependent phosphate transport can enhance phosphate excretion under conditions in which there is an apparent reduction in the number of transporters. In the present study, we evaluated the effect of induction of renal failure on the expression of the sodium-dependent phosphate transporter in the rat (NaPi-2; Magagnin et al., 1993) and the response to administration of PFA.

Methods

**Experimental animals.** Male Sprague-Dawley rats with initial body weights of ~250 g were used. Rats were housed individually and provided food and water ad libitum. The food contained 0.7% inorganic phosphate. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and using aseptic techniques, a 5/6 nephrectomy was performed. A midline abdominal incision was made, the right kidney was removed and approximately two thirds of the left kidney was performed. A midline abdominal incision was made, the right kidney was removed and approximately two thirds of the left kidney was performed. A midline abdominal incision was made, the right kidney was removed and approximately two thirds of the left kidney was performed.

**Reagents.** Phosphonoformic acid (PFA) was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sodium phosphate (NaPi), sodium glucose cotransporter (SGT), polymerase chain reaction (PCR), and antisense ribonucleic acid (RNA) probes were purchased from Promega (Madison, WI). Rabbit anti-NaPi-2 antibodies were kindly provided by Dr. R. Murer (Basel, Switzerland). The polymerase chain reaction primers were obtained from Integrated DNA Technologies (Coralville, IA).

**Methods.**

Received for publication August 26, 1996.

ABBREVIATIONS: PFA, phosphonoformic acid; NaPi, sodium phosphate; SGT, sodium glucose cotransporter; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
was infarcted by ligating two or three branches of the left renal artery. The incision was closed using standard procedures. In control animals, sham surgery was performed by making a midline abdominal incision, maneuvering the intestines to expose the kidneys and then closing the incision.

**Clearance studies.** 5 to 6 weeks after 5/6 nephrectomy or sham surgery, rats were placed on 1.5% glucose water in place of tap water. 24 hr later, rats were placed in metabolic cages, and 24-hr urine samples were collected. After an additional 24 hr, a group of rats that had underwent sham surgery or 5/6 nephrectomy had PFA (0.6%) included in their glucose drinking solution. Animals received PFA for 48 hr. At the end of this period, animals were anesthetized with pentobarbital, a blood sample was taken and the kidneys were removed. Blood was centrifuged, and plasma was taken for assay. Remnant healthy portions of renal tissue were dissected, frozen over dry ice and stored at −80°C for subsequent evaluation of NaPi-2 mRNA.

**Analyses.** Plasma and urinary sodium creatinine and urea nitrogen concentrations were analyzed using a clinical analyzer (Synchron AS/8, Beckman Instruments, Brea, CA). Urinary protein concentration was analyzed according to the sulfosalicylic acid method (Davidsohn and Henry, 1969). Plasma and urinary inorganic phosphorus was analyzed according to a quantitative colorimetric method (Sigma Diagnostics, St. Louis, MO).

Expression of NaPi-2 and sodium glucose cotransporter mRNA was determined using total RNA (20 μg), which was denatured according to the formaldehyde or glyoxal denaturation method and electrophoresed on 1% agarose gel. The fractionated RNA was immobilized on NYTRAN nylon membrane and UV cross-linked. The quality of the RNA was examined by staining the blot with methylene blue.

An 895-bp PCR product encompassing residues 975 to 1870 of the human phosphate transporter (NaPi-3; Magagnin et al., 1993), which has high homology to the rat transporter (NaPi-2; Magagnin et al., 1993), was random-prime-labeled with 32P-dATP and used as a probe for NaPi-2. A 980-bp EcoRV/NspI PCR product specific for the type 1 phosphate transporter (NaPi-1) was similarly labeled to use as a probe for NaPi-1. In addition, we measured mRNA levels for the renal SGT-1. Forward (5′-ACT-GTT-GGA-GGC-TTC-TTC-CT-3′) and reverse (5′-GTA-ACT-GGT-GAT-GGA-CTG-GA-3′) primers to the published sequence of the human SGT cDNA (Hediger et al., 1989) at sites of homology to the human and rat SGT cDNAs (GenBank M24847 and D16101) were selected and synthesized in-house. After reverse transcription of rat kidney total RNA, this primer pair was used to make an ~1207-bp cDNA fragment in the first-cycle PCR. Because SGT-1 is not a very prevalent message, a second-cycle PCR was performed using internal (nested) forward (5′-ATA-TTC-ATC-AAT-CTG-GGC-TTC-3′) and reverse (5′-TAG-ATG-TCC-ATG-GTG-AAG-AG-3′) primers to obtain a 730-bp fragment. This fragment was subcloned into the PCR II vector (Invitrogen, San Diego, CA) and sequenced to establish homology to the rat SGT sequence.
The cDNA fragment was gel-purified and radiolabeled to use as probe.

To investigate mRNA expression for the less abundant sodium phosphate transporter (NaPi-1), an RNA dot blot with 100 μg of total RNA was prepared. A similar blot was prepared with 20 μg of total RNA to probe for NaPi-2. The dot blots were UV cross-linked before hybridization. Prehybridization was carried out for 4 hr at 42°C in 50% formamide, 5× SSPE, 5× Denhardt’s solution (0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.5% SDS and 100 μg/ml salmon sperm DNA. Hybridization was performed overnight at 42°C after the addition of the radiolabeled probe in the prehybridization buffer containing 10% dextran sulfate. The next day, the filter was washed three times for 10 min each in 1× SSPE/0.1% SDS at room temperature followed by two final 30-min washes in 0.5× SSPE/0.1% SDS at 65°C. The blots were exposed to the X-ray film with an intensifying screen.

To ensure that equal quantities of total RNA were loaded onto the gel, the blot was reprobed with a radioactively labeled rat GAPDH cDNA (bases 550-1004 of the rat mRNA sequence, 453-bp fragment). mRNA intensity was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the ratio of NaPi-2 mRNA/GAPDH mRNA was calculated.

**Data analyses.** Data are presented as mean ± S.E.M. Clearances of creatinine and phosphate and fractional excretions of sodium and phosphate were calculated using standard formulas. Statistical analysis was performed using an analysis of variance followed by paired or unpaired t-tests, as appropriate.

### Results

5 weeks after 5/6 nephrectomy, there was a significant decrease in phosphate transporter mRNA (fig. 1). The ratio of NaPi-2 mRNA/GAPDH mRNA indicated that there was an ~50% reduction in NaPi-2 mRNA (fig. 1). In addition to the 2.6-kb band corresponding to NaPi-2, a higher-molecular-weight band (9.0 kb) was observed; this was also reduced in rats with renal failure. Treatment of rats for 48 hr with PFA had no effect on NaPi-2 mRNA in either control or uremic rats. Comparison of the expression of NaPi-2 and the type 1 sodium-dependent phosphate transporter by dot blot confirmed the reduced expression of NaPi-2 but indicated that the expression of NaPi-1 was unchanged (fig. 2). To confirm that the NaPi-1 probe could indeed detect NaPi-1 mRNA, we evaluated 4 μg of poly A (+) RNA from human kidney and were able to detect 2.3- and 1.8-kb mRNA bands (data not shown). Northern blot analysis of the sodium-glucose co-transporter demonstrated no change of expression in rats with renal failure or rats treated with PFA (fig. 3).

The degree of renal failure was highlighted by a significant proteinuria and elevated plasma creatinine and urea nitrogen concentrations compared with control animals (table 1). Treatment with PFA for 2 days had no effect on body weight, urinary protein excretion or plasma creatinine and urea nitrogen concentrations in either control rats or rats with uremia (table 1). There was no difference in urinary phosphate excretion between normal rats and rats with renal failure; however, treatment with PFA resulted in a significant increase in both groups (fig. 4). Plasma phosphate concentration was significantly elevated in uremic rats receiving vehicle (fig. 4), but in uremic rats treated with PFA, plasma phosphate concentration was not significantly different from that of control animals (fig. 4). In addition to increasing total phosphate excretion, PFA treatment resulted in an increase in both phosphate clearance (fig. 5) and fractional phosphate excretion (fig. 6). The effect of PFA on these parameters was selective for phosphate because PFA had no effect on either creatinine clearance (fig. 5) or the fractional excretion of sodium (fig. 6). Creatinine clearance was significantly reduced (fig. 5) and phosphate clearance and the fractional excretion of sodium and phosphate were significantly increased in uremic rats (figs. 5 and 6). These changes did not alter the responsiveness to PFA (figs. 5 and 6).

**TABLE 1** Body weight (BWT), urinary protein excretion (UPROTV), plasma creatinine (PCR) and plasma urea nitrogen (PUN) in control rats and 5/6 nephrectomy-induced uremic rats receiving as drinking fluid either glucose (1.5%) or glucose containing PFA (0.6%).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Renal failure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWT (g)</td>
<td>481 ± 7</td>
<td>486 ± 7</td>
</tr>
<tr>
<td>UPROTV (mg/dl)</td>
<td>8 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>PCR (mg/dl)</td>
<td>0.40 ± 0.02</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>PUN (mg/dl)</td>
<td>14 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td><strong>PFA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWT (g)</td>
<td>401 ± 11</td>
<td>395 ± 10</td>
</tr>
<tr>
<td>UPROTV (mg/dl)</td>
<td>102 ± 5</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>PCR (mg/dl)</td>
<td>1.3 ± 0.07</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>PUN (mg/dl)</td>
<td>56 ± 5</td>
<td>38 ± 2</td>
</tr>
</tbody>
</table>

* n = 10 or 11 rats/group.

* P < .05 vs. control.
Discussion

In the present study, we observed that the rat sodium-dependent phosphate transporter (NaPi-2) mRNA was significantly reduced in rats with 5/6 nephrectomy-induced renal disease. This observation is consistent with a report that in renal brush border membrane vesicles from uremic rats, there was a reduction in the rate for sodium-dependent phosphate transport (Hruska et al., 1982). Northern analysis demonstrated the presence of a 2.6-kb message, corresponding to NaPi-2, similar to that observed previously (Custer et al., 1994). In addition, we observed a 9.0-kb message that has not been reported previously. In the mouse, however, Collins and Ghishan (1994) observed three bands (10.0, 4.6 and 2.6 kb) and speculated that these may represent alternately spliced or processed forms of the transcript. The reduction in NaPi-2 expression was specific inasmuch as the expressions of the sodium-glucose transporter and the type 1 phosphate transporter, NaPi-1, were unchanged. The Northern blot analysis of the sodium-glucose transporter revealed two bands, consistent with previous reports describing splice variants (Hediger et al., 1989; Yet et al., 1994).

To date, two different types of Na\(^+\)/Pi transporters have been identified by expression or homology cloning. NaPi-1 (type I) was the first to be cloned; it was isolated from a rabbit kidney cortex cDNA library (Werner et al., 1991). It is present primarily in the renal brush border membrane and, when expressed in *Xenopus* oocytes, demonstrates activity similar to that observed with the Na\(^+\)/Pi cotransporter in rabbit brush border membrane. Sequences similar to NaPi-1 have also been identified in the human and mouse kidney (Chong et al., 1993, 1995); these type I transporters do not appear to be regulated (Biber and Murer, 1994). Our present data demonstrating little change in NaPi-1 expression is consistent with the lack of regulation of this transporter (Biber et al., 1993; Verri et al., 1995). In addition, this transporter is in much lower abundance than NaPi-2, accounting for the requirement of a dot blot rather than a Northern blot analysis to evaluate expression. Additional transporters have been cloned from the rat (NaPi-2; Magagnin et al., 1993), human (NaPi-3; Magagnin et al., 1993), rabbit (NaPi-6; Verri et al., 1995) and bovine (NBL-1; Helps et al., 1995) renal epithelial cells. These transporters have been designated type II and do appear to be regulated. Thus, evidence suggests that the two classes of transporters respond differently to dietary changes; rabbits fed a low-phosphate diet demonstrated an increase in NaPi-6 mRNA (type II) but no change in NaPi-1 mRNA (type I) (Verri et al., 1995). Our observation that NaPi-2 expression was reduced in uremic rats is consistent with the type II transporter being regulated.

The reduction in NaPi-2 expression that we observed in the present study in uremic animals is an adaptive response to

**Fig. 4.** Urinary phosphate excretion (top) and plasma phosphate concentration (bottom) in control rats and 5/6 nephrectomy-induced uremic rats receiving as drinking fluid either glucose (1.5%) or glucose containing PFA (0.6%). n = 10 or 11 rats/group. *P < .05 vs. control. †P < .05 vs. vehicle.

**Fig. 5.** Creatinine clearance (top) and phosphate clearance (bottom) in control rats and 5/6 nephrectomy-induced uremic rats receiving as drinking fluid either glucose (1.5%) or glucose containing PFA (0.6%). n = 10 or 11 rats/group. *P < .05 vs. control. †P < .05 vs. vehicle.
decreased nephron number and thus the ability to excrete inorganic phosphate. In the present study, 5/6 nephrectomy resulted in a significant reduction in glomerular filtration rate, as indicated by reduced creatinine clearance and an accompanying increase in fractional excretion of phosphate and sodium. The increased fractional excretion in phosphate, however, was not sufficient to maintain phosphate homeostasis in uremic rats, which demonstrated significant hyperphosphatemia. The mechanisms involved in this adaptive response of reduced expression of phosphate transporter are unclear; however, one possible mediator is parathyroid hormone, which is increased when renal function deteriorates (Bricker 1972; Goldman and Bassett, 1954; Reiss et al., 1969). Parathyroid hormone is known to increase phosphate reabsorption and abrogate phosphate retention. Treatment with a selective phosphate transporter inhibitor, however, increased phosphate excretion and abrogated the phosphate retention.

In summary, our data indicate that renal failure induced by 5/6 nephrectomy results in a reduction in the expression of the sodium-dependent phosphate transporter and phosphate reabsorption. The authors are grateful to Sue Tirri for expert secretarial assistance.

References


Kempson, R.: Glucose and sodium transport in renal proximal tubules (Kempson et al., 1987; Szepanska-Konkel et al., 1986). Furthermore, PFA can result in a marked increase in the fractional excretion of phosphate in thyroparathyroidectomized rats without changes in urinary cAMP, suggesting that parathyroid hormone plays no role in the response to PFA (Van Scy et al., 1988). PFA may be competing with inorganic phosphate for transport because feeding rats a low-phosphate diet leads to a regulatory increase in phosphate absorption and an increase in the bioavailability of PFA (Loghman-Adham et al., 1994).

Our observation that PFA can increase phosphate excretion in uremic rats, despite the apparent reduction in transporter expression, suggests that blockade of either renal or intestinal phosphate transport might prevent the phosphate retention associated with progressive renal disease. There is good evidence that phosphate retention plays a role in both the progressive loss of renal function and the secondary hyperparathyroidism and subsequent renal osteodystrophy.

In summary, our data indicate that renal failure induced by 5/6 nephrectomy results in a reduction in the expression of the sodium-dependent phosphate transporter and phosphate retention. Treatment with a selective phosphate transporter inhibitor, however, increased phosphate excretion and abrogated the phosphate retention.


Send reprint requests to: David Brooks, Ph.D., SmithKline Beecham, Department of Renal Pharmacology, UW2521, P.O. Box 1539, King of Prussia, PA 19406-0939.