Direct micropuncture evidence that matrix extracellular phosphoglycoprotein inhibits proximal tubular phosphate reabsorption

David G. Shirley, Nuno J.R. Faria, Robert J. Unwin and Hamish Dobbie

Centre for Nephrology, University College London Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK

Correspondence and offprint requests to: David G. Shirley; E-mail: david.shirley@ucl.ac.uk

Abstract

Background. Matrix extracellular phosphoglycoprotein (MEPE) is a putative phosphatonin that we have shown in previous studies to be phosphaturic in rats. Its site of action in the nephron remains to be confirmed.

Methods. We made micropuncture collections from late proximal convoluted tubules in anaesthetized rats to assess...
directly the effect of MEPE on phosphate reabsorption in the proximal tubule.

**Results.** MEPE had no effect on glomerular filtration rate or single-nephron filtration rate, but it increased phosphate excretion significantly. In animals infused with vehicle alone (time controls), no significant change was seen in either the proximal tubular fluid:plasma phosphate concentration ratio (TF/Pₚ) or the fraction of filtered phosphate reaching the late proximal convoluted tubule (FDPi), whereas in rats infused with MEPE, TF/Pₚ increased from 0.49 ± 0.07 to 0.68 ± 0.04 (n = 22; P = 0.01) and FDPi increased from 0.20 ± 0.03 to 0.33 ± 0.03 (n = 22; P < 0.01).

**Conclusions.** The results confirm the phosphaturic effect of MEPE and indicate that much, if not all, of this effect is a result of reduced reabsorption of phosphate in the proximal convoluted tubule. This is consistent with the recent finding of MEPE-induced reductions in apically located NaPT2a in the proximal tubule.

**Keywords:** kidney; matrix extracellular phosphoglycoprotein; micropuncture; phosphatonin; proximal convoluted tubule

**Introduction**

In addition to the well-known regulatory factors parathyroid hormone (PTH) and calcitriol, it is now thought that phosphate balance might be controlled or influenced by a group of circulating peptides, collectively known as ‘phosphatonin’, that can induce renal phosphate wasting [1,2]. The putative phosphatonin, identified from studies of tumour-induced osteomalacia or of genetic disorders associated with hypophosphataemia, include fibroblast growth factor-23 (FGF-23), secreted frizzled-related protein-4, fibroblast growth factor-7 and matrix extracellular phosphoglycoprotein (MEPE).

MEPE, which was first isolated from tumour-derived tissue from a patient with oncogenic osteomalacia [3], has been found to have important effects on bone mineralization [4], but in a recent renal clearance study we showed that MEPE also causes a dose-dependent phosphaturia as a consequence of reduced renal phosphate reabsorption [5]. In the present investigation, in order to identify the tubular site of action of MEPE, we have used micropuncture to assess directly phosphate reabsorption in the proximal tubule in vivo.

**Materials and methods**

Adult male Sprague–Dawley rats (body weight 240–260 g) were anaesthetized with intraperitoneal sodium thiopentone (100 mg/kg body weight; Link Pharmaceuticals, Horsham, Sussex, UK) and prepared surgically for micropuncture studies as described previously [6]. Briefly, cannulae were inserted in a jugular vein and femoral artery, a tracheotomy was performed, the bladder was catheterized and the left kidney was exposed via a flank incision. Perirenal fat was removed, and the kidney was placed in a specially designed Perspex dish, with a gap for entry and exit of the renal artery and vein, and the ureter; the dish was clamped to the operating table to minimize movement of the exposed kidney during respiration, and the left ureter was catheterized. The kidney was bathed continuously in mineral oil heated to 37°C. Animals were infused with 0.9% NaCl solution at 4 mL/h throughout.

Experiments were begun after a 2-h equilibration period; after the first hour of the equilibration period, a bolus of [³H]inulin (60 μCi; Perkin Elmer, Cambridge, UK) was given, followed by an infusion of [³H]inulin at a rate of 60 μCi/h. During an initial 1-h control period, urine was collected from the ureter, and 3–4 micropuncture samples were collected from late proximal convoluted tubules as described previously [7]. Samples were obtained using micropipettes, tip internal diameter 9–10 μm, inserted into candidate proximal tubular loops. Microdroplets of Sudan black-stained oil were then injected into the tubules; multiple reappearances of the droplet indicated placement too proximally, in which case the pipette was withdrawn and a new nephron was selected for a further attempt, whereas no reappearance of the droplet or only one reappearance was taken to indicate that the puncture site was a late proximal convolution. Once a late proximal tubule segment had been entered, a column of oil 4–5 tubular diameters in length was injected and allowed to flow just distal to the puncture site. Gentle aspiration was then applied to the pipette so that tubular fluid arriving at the collection site was drawn into the micropipette. Tubular fluid was collected for 6–12 mins; once each collection was completed, the pipette was withdrawn, and the tubular sample was deposited under oil onto a watch glass. After each puncture, the next puncture site was selected following a systematic pattern that ensured that no nephron was sampled more than once. At the end of each collection, Microfil (Flow-Tec, Carver, MA, USA) was injected into the nephron through another micropipette, filling the nephron lumen. Microfil hardens into a cast of the nephron which is then examined at the end of the experiment. Examination of these Microfil casts confirmed the late proximal location of all the tubular fluid collections.

Immediately after the control period, the intravenous infusion was changed to recombinant full-length human MEPE (raised in insect Spodoptera frugiperda cells; a gift from Acoligix, Hayward, CA, USA) at a dose of 100 μg/h in seven animals or continued as saline alone in another seven animals, for the next 2 h. This dose of MEPE had previously been shown to have a maximal effect on phosphate excretion [5]. During the second of the 2 h (the experimental period), urine and late proximal tubular micropuncture collections were resumed. Arterial blood samples (~100 μL) were collected hourly.

In a separate group of seven animals, parathyroid hormone 1–34 (Sigma) was infused at a dose of 20 μg/h, instead of MEPE; these experiments were used primarily as a positive control, in order to check the sensitivity of the micropuncture assessment of proximal tubular phosphate reabsorption.

**Analyses**

Urine, plasma and tubular fluid [³H]inulin activities were determined using β-emission spectroscopy (Packard TracerModel 2900TR, Pong-borough, Berkshire, UK) by means of a Packard Radio-flow system (Perkin Elmer, Cambridge, UK). Plasma [³H]inulin at any given time (for comparison with corresponding tubular fluid samples) was calculated assuming that counts altered linearly between successive hourly determinations. Using previously calibrated constriction pipettes, duplicate samples (62 nL) of tubular fluid were deposited in 200 volumes of ultrapure water in microvials for subsequent analysis using capillary electrophoresis [8], and duplicate samples (38 nL) were deposited in scintillation vials for [³H]inulin determination. Urine samples were diluted 1:200 in ultrapure water, and plasma samples were diluted 1:100 in ultrapure water, before being frozen for subsequent capillary electrophoresis analysis.

**Calculations and statistics**

Glomerular filtration rate (GFR) was measured as the clearance of [³H]inulin (Cᵢ), calculated using the standard formula. Single-nephron GFR (SNGFR) was calculated using the formula:

\[
\text{SNGFR} = \frac{\text{TF} \times \text{VT}_{\text{TF}}}{\text{F}_{\text{TN}}} \]

where

\[
\text{TF} = \text{tubular fluid [³H]inulin count,}
\]

\[
\text{VT}_{\text{TF}} = \text{tubular fluid flow rate, and}
\]

\[
\text{F}_{\text{TN}} = \text{plasma [³H]inulin count.}
\]

The fractional delivery of phosphate to the late proximal convoluted tubule (FDPi) was calculated as the tubular fluid/plasma concentration ratio for phosphate (TF/Pₚ) divided by that for inulin (TF/Pᵢ):

\[
\text{FDPi} = \frac{\text{TF/P}_{\text{TF}}}{\text{P}_{\text{TF}}/\text{P}_{\text{In}}}
\]
Results are presented as means ± SEM. Comparisons between values obtained during the control period and the experimental period in each group were made using Student’s unpaired or paired t-test as appropriate. A P-value of <0.05 was considered statistically significant.

Results

Whole-kidney GFRs (left kidney only) are shown in Figure 1A. (There were no significant differences between values for GFR in the micropunctured and contralateral kidneys.) GFR was similar in the two groups during both the control and experimental periods. In both groups, there was a modest fall between control and experimental periods, but this was not statistically significant. Values for SNGFR (Figure 1B) were also very similar in the two groups during the control and experimental periods. As with the whole-kidney GFR, SNGFR fell slightly (but not significantly) between control and experimental periods.

During the control period, plasma phosphate concentrations were $2.05 \pm 0.08$ mmol/L in the vehicle-infused group and $2.15 \pm 0.12$ mmol/L in the MEPE-infused group. In the experimental period, the corresponding values were $1.93 \pm 0.12$ and $2.03 \pm 0.06$ mmol/L. There were no significant differences between the groups during either period, and no significant change in either group between control and experimental periods.

Phosphate excretion (left kidney only) is shown in Figure 1C. There was a wide variation in baseline urine phosphate excretion in individual rats, but closely comparable means in the two groups. There was no significant change in phosphate excretion between the control and experimental periods in vehicle-infused rats, but a marked increase was seen in rats infused with MEPE, confirming previous findings in clearance studies [5].

Figure 2A shows values (mean ± SEM) for TF/P$_{Pi}$ derived from individual collections from the late proximal convoluted tubule (PCT) in the two groups of rats. There was no significant change in TF/P$_{Pi}$ between the control and experimental periods in vehicle-infused animals, but an increase was observed during MEPE infusion. In order to take account of any differences in water reabsorption, these data were factored for TF/P$_{In}$, thereby providing values for fractional phosphate delivery (FD$_{Pi}$; TF/P$_{Pi}$/In) to the late PCT (Figure 2B). Again, in vehicle-infused animals, there was no change between the control and experimental periods, whereas FD$_{Pi}$ increased in rats infused with MEPE. Individual values for FD$_{Pi}$ varied substantially not only between animals but also between tubules from the same rat (and during a given period). To reduce the scatter, individual values during each period ($n = 3$–$4$) were averaged for each rat, thereby allowing paired comparisons to be made; the results are presented in Figure 3. Considerable variations between rats were still evident, but in every animal infused with MEPE, there was an increase in FD$_{Pi}$, whereas vehicle-infused rats showed no overall change.

Effect of PTH

Infusion of PTH had no effect on GFR or SNGFR, but caused an increase in phosphate excretion from $7 \pm 4$ μmol/h during the control period to $29 \pm 7$ μmol/h ($P < 0.05; n = 7$) during the experimental period. At the same time, the fractional delivery of phosphate to the late proximal convoluted tubule increased from $0.22 \pm 0.05$ to $0.35 \pm 0.02$ ($P = 0.01; n = 7$, derived from three or four collections per rat in each period).

Discussion

The present study confirmed that MEPE is phosphaturic in rats, an effect that could not be attributed to an increase in the filtered load of phosphate and was therefore a consequence of reduced phosphate reabsorption. This effect of
MEPE is not dependent on changes in the plasma concentrations of PTH, calcitriol or FGF-23 [9]. Given the fact that most phosphate reabsorption occurs in the proximal tubule, this nephron segment was a strong candidate for MEPE’s site of action. Circumstantial support came from reports of MEPE immunoreactivity and mRNA in the proximal tubule [10] and reduced phosphate uptake by cultured proximal tubular cells incubated with MEPE [4]. Nevertheless, the present investigation provides the first direct evidence that MEPE does indeed inhibit proximal tubular phosphate reabsorption. In contrast to vehicle-infused animals, rats given MEPE exhibit an increase in TF/P\(_{Pi}\) at the late PCT, as well as an increase in the fractional delivery of phosphate (FD\(_{Pi}\); calculated as TF/P\(_{Pi}\)/In\(_{Pi}\)).

The bulk of proximal tubular phosphate reabsorption is mediated by the apical sodium–phosphate co-transporter NPT2a [11]; downregulation of phosphate transport is achieved by internalization of NPT2a transporters via endocytosis and subsequent degradation in lysosomes. Our results are therefore consistent with those of a recent study demonstrating a reduction in the apical expression of NPT2a in the renal cortex of MEPE-infused rats [9]. However, they are less easy to reconcile with a report of upregulated NPT2a in transgenic mice chronically overexpressing MEPE protein [12], a situation in which compensatory mechanisms may come into play.

During the control period in both groups and during the experimental period in saline-infused animals, fractional delivery of phosphate to the end of the PCT was found to average 20–25%, whereas overall fractional excretion of phosphate was only ~6%. Although at first sight this difference seems considerable, it indicates that up to 80% of filtered phosphate is taken up by the PCT and only ~14% in the pars recta and/or more distally. It is likely that most of the difference is accounted for by reabsorption in the pars recta, although an element of phosphate reabsorption in the distal nephron cannot be ruled out [13]. An additional potential explanation is nephron heterogeneity, with functional differences between the (accessible) superficial nephrons and the deep nephrons.

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It was notable in this study that the phosphate content of tubular fluid varied considerably from nephron to nephron, even when the sampling was only a few minutes apart. In this context, immunostaining work by Murer’s group [14] found major differences between even directly adjacent nephrons in the amount of NPT2a present in the brush-border membrane, while functional data from experiments on isolated proximal tubules in vitro also indicated that phosphate transport varied greatly between tubules [15]. The large variations in tubular phosphate we found between spatially and temporally proximate collections are therefore perhaps not surprising.
Changes in 11β-HSD2 expression in a low-protein rat model of IUGR

Finally, our findings with PTH confirmed that the hormone increases phosphate excretion, largely or wholly as a consequence of reduced fractional phosphate reabsorption in the proximal tubule, an effect that has been linked to a PTH-induced reduction in apical NPT2a expression [11]. In these respects, clear parallels are evident between the renal actions of PTH and MEPE.

In summary, the present study has provided the first direct, definitive evidence that MEPE inhibits phosphate reabsorption in the PCT. As with PTH, it is likely that this effect results from reduced apical expression of the NaPT2a co-transporter, though the details of the mechanism remain to be defined.

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Conflict of interest statement. R.U.J.U. is currently receiving financial support from Acologix towards a PhD studentship.

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Changes in 11β-hydroxysteroid dehydrogenase type 2 expression in a low-protein rat model of intrauterine growth restriction

Iris Östreicher1, Jorge Reis Almeida2,3, Valentina Campean3, Manfred Rauh1, Christian Plank1, Kerstin Amann3,* and Jörg Dötsch1,4,*

1Department of Pediatrics and Adolescent Medicine, University Erlangen-Nuremberg, Erlangen, Germany, 2Nephrology Unit and Laboratory of Morphometry and Cardiovascular Morphology, State University of Rio de Janeiro, Rio de Janeiro, Brazil, 3Department of Pathology, University Erlangen-Nuremberg, Erlangen, Germany, and 4Present address: Department of Pediatrics and Adolescent Medicine, University of Cologne, Cologne, Germany

Correspondence and offprint requests to: Jörg Dötsch; E-mail: joerg.doetsch@uk-koeln.de

Both authors contributed equally to this work.

Abstract

Background. Intrauterine growth restriction (IUGR) is associated with systemic hypertension of the offspring later in life. The exact mechanisms are still incompletely understood. 11β-Hydroxysteroid dehydrogenase 2 (11β-HSD2) in the distal renal tubule protects the mineralocorticoid receptor from cortisol. As we did not find a suppression of 11β-HSD2 in total kidney of IUGR animals, our objective

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