Cardiovascular, Pulmonary, and Renal Pathology

A Murine Model of Phosphate Nephropathy

Philipp Eller,^{*,†} Kathrin Eller,^{‡§}
Alexander H. Kirsch,[‡] Josef J. Patsch,^{*}
Anna M. Wolf,[¶] Andrea Tagwerker,[‡]
Ursula Stanzl,^{*} Reinhard Kaindl,^{||}
Volker Kahlenberg,^{||} Gert Mayer,[‡]
Josef R. Patsch,^{*} and Alexander R. Rosenkranz^{‡§}

From the Departments of Internal Medicine I and Internal Medicine IV,[‡] and the Tyrolean Cancer Research Institute,[¶] Innsbruck Medical University, Innsbruck; the Institute of Mineralogy and Petrography,^{||} Innsbruck University, Innsbruck; and the Divisions of Angiology,[†] and Nephrology and Hemodialysis,[§] Department of Internal Medicine, Graz Medical University, Graz, Austria*

We established a murine model of phosphate nephropathy with secondary hyperparathyroidism. db/db mice, which develop obesity and type 2 diabetes mellitus, were uninephrectomized at the age of 6 weeks and were fed either standard chow or a phosphorus-rich diet during the next 8 weeks. Thereafter, renal cryosections showed abundant tubular casts with a strong histochemical von Kossa reaction in all db/db mice on the phosphorus-rich diet but none in the controls. X-ray diffraction and Raman spectroscopy proved that these tubular casts consist mostly of hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$. These intraluminal precipitations were located in distal tubuli and collecting ducts and were associated with degenerative tubular changes and peritubular infiltration of T cells and macrophages. In line, kidneys of db/db mice on the phosphorus-rich diet displayed significantly increased mRNA expression of the T_H1 cytokines interferon γ , IL-6, and tumor necrosis factor α . In addition, mice developed signs of secondary hyperparathyroidism as shown by elevated serum phosphate, decreased serum calcium, and increased parathyroid hormone, osteopontin, and fibroblast growth factor 23 levels. db/db mice on the phosphorus-rich diet also presented with significantly lower body weight, lower homeostasis model assessment of insulin resistance index, and hypertrophic cardiomyopathy. Thus, we provide a murine model of phosphate nephropathy and secondary hyperparathyroidism, which can be used for future pharmacologic and pathophysiologic studies to analyze the effect of hyperphosphatemia on renal, metabolic, and cardiovas-

cular phenotypes. (Am J Pathol 2011, 178:1999–2006; DOI: 10.1016/j.ajpath.2011.01.024)

Inorganic phosphorus is essential for multiple biological functions, such as intracellular signal transduction, the production and function of cell membranes, and energy exchange. Although more than 80% of total body phosphorus is stored in bone and teeth, phosphorus is also found in the intracellular compartment and in serum (primarily in the form of anions such as H_2PO_4^- and HPO_4^{2-} , which are commonly referred to as phosphate). In the steady state, serum phosphate levels are maintained within the physiological range by regulation of dietary absorption, bone formation, bone resorption, and by renal excretion. In the presence of a normal kidney function, renal excretion of excess phosphate is primarily responsible for maintaining phosphate balance. This regulation relies on the inhibition of the tubular sodium phosphate cotransporters and on increased levels of fibroblast growth factor 23 (FGF23) and parathyroid hormone.^{1–4}

Hyperphosphatemia occurs whenever the amount of phosphate in the extracellular space exceeds the renal capacity for excretion. Hyperphosphatemia is, thus, a nearly universal complication of advanced renal failure. Large observational studies have also shown a graded association between levels of serum phosphate and all-cause mortality in patients with chronic renal failure. The positive phosphate balance seems to cause an accelerated progression of vascular calcification.^{5–8}

An acute phosphate load that overwhelms renal capacity for excretion can be derived from endogenous and exogenous sources. Large amounts of endogenous phosphates are released from intracellular stores in tumor lysis syndrome, rhabdomyolysis, and lactic acidosis. Acute hyperphosphatemia can also result from ingestion of excessive amounts of phosphate-containing laxatives and enemas administered for colonoscopy preparation.⁹ Both endogenous and exogenous phosphate loads can

Supported by Austrian Research Funds (P21402 to A.R.R.).

P.E. and K.E. contributed equally to the manuscript.

Accepted for publication January 7, 2011.

Address reprint requests to Philipp Eller, M.D., Department of Internal Medicine I, Innsbruck Medical University, Anichstrasse 35, 6020 Innsbruck, Austria. E-mail: philipp.eller@i-med.ac.at.

lead to phosphate nephropathy.^{10–15} Acute phosphate nephropathy is a clinical pathologic entity characterized by acute and subsequent chronic renal failure due to tubular precipitation of calcium phosphate deposits forming crystals of hydroxyapatite.¹⁶ It often followed exposure to oral sodium phosphate bowel purgatives, which were withdrawn from the market in December 2008 after a respective warning from the US Food and Drug Administration. Inadequate hydration and chronic kidney disease are established risk factors for acute phosphate nephropathy.¹⁶ Most of our present information on phosphate nephropathy derives from case series and retrospective epidemiologic analyses.^{11,14,17–19} Moreover, there is no specific therapy for established phosphate nephropathy.

To study hyperphosphatemia and its renal, metabolic, and cardiovascular complications in more detail, we established and present herein a new animal model for phosphate nephropathy with established secondary hyperparathyroidism.

Materials and Methods

Study Design

Five-week-old, male, db/db, leptin receptor-deficient mice (BKS.Cg-Dock7^m +/+ *Lepr^{db}*/J) and male C57BL/6J control mice were obtained from Charles River (Sulzfeld, Germany). The animals were maintained in a virus/antibody-free central animal facility of the Innsbruck Medical University, underwent an uninephrectomy at 6 weeks of age, and were afterward fed either standard chow or a phosphorus-rich diet (Altromin, Lage, Germany), respectively. The phosphorus-rich diet contained 20.46 g/kg of phosphorus. The db/db mice ate on average 1.6 g of chow per day and therefore ingested 1 g/kg of phosphorus per day. The oral daily dosage of phosphorus in our experimental design was similarly potent to Fleet's phospho-soda used for bowel cleansing in humans (ie, 0.5 g/kg of phosphate per day). The phosphorus-rich diet also contained 500 IU/kg of vitamin D₃, 11.23 g/kg of calcium, and 175.88 g/kg of crude protein. Body weight, fasting glucose levels, and urinary albumin excretion were measured regularly once a week. The 24-hour urine samples were collected in metabolic cages. Animals were sacrificed at the age of 15 weeks. All animal experiments were approved by Austrian veterinary authorities.

X-Ray Diffraction and Raman Spectroscopy

X-ray diffraction data of the polycrystalline material were collected on a Siemens D5000 powder diffractometer in Bragg-Brentano geometry in the range of 3° to 70°2 θ (Cu-K α radiation, parallel beam optics, scintillation counter, generator settings: 40 kV/40 mA, step size: 0.01°2 θ , counting time per step: 3 seconds). Raman spectra of the aggregate were obtained in the spectral range of 100 to 1200 cm⁻¹ by a Horiba LabRam-HR 800 confocal Raman microspectrometer. The sample was excited by the 532-nm emission line of a 30-mW neodymium:yttrium-aluminum-garnet laser under a 100 $\times$ objective with a

numerical aperture of 0.9. The size and power of the laser spot on the sample were approximately 1 μ m and 0.5 mW, respectively. The scattered light was dispersed by a grating with 1800-mm⁻¹ lines and collected by a 1024 $\times$ 256-pixel open electrode charged-coupled device detector. The spectral resolution, determined by measuring the Rayleigh line, was approximately 1.4 cm⁻¹. Background was subtracted by fitting a polynomial function of third order to the spectrum. The wave number accuracy of approximately 0.5 cm⁻¹ was achieved by adjusting the zero-order position of the grating and checked by a neon spectral calibration lamp. The measured spectra were loaded into the software Spectral-ID by Galactic and compared with database spectra supplied by Horiba.

Metabolic Studies

Blood glucose levels were measured using OneTouch Ultra2 (Lifescan, Vienna, Austria), urine creatinine was quantified spectrophotometrically using a picric acid-based method (Sigma, St. Louis, MO), and urinary albumin was determined by a double-sandwich enzyme-linked immunosorbent assay (Abcam, Cambridge, MA) as reported previously.²⁰ Serum urea, serum calcium, and serum phosphate levels were measured with standard laboratory techniques. The glucose tolerance test was performed at the age of 7 weeks and the insulin tolerance test at the age of 15 weeks. For the glucose tolerance test, we administered glucose (1.0 g/kg) by intraperitoneal injection after an overnight fast and measured blood glucose levels before and 15, 30, 60, and 120 minutes after glucose application. For the insulin tolerance test, we injected 0.75 U/kg of human insulin aspart (Novo Nordisk, Sorgenfri, Denmark) and measured the blood glucose level before and 15, 30, 45, 60, 75, and 90 minutes after insulin application. At the age of 15 weeks, we also measured fasting insulin levels (Crystal Chem, Downers Grove, IL), serum FGF23 levels (Kainos Laboratories Inc., Tokyo, Japan), serum osteopontin levels (R&D Systems, Minneapolis, MN), and intact parathyroid hormone levels (ALPCO, Salem, NH) by enzyme-linked immunosorbent assay. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by use of the glucose-insulin product divided by the constant 22.5.^{21–22} Furthermore, we measured the fat cell diameter in subcutaneous and visceral (epididymal) adipose tissue at a magnification of 100 using a ProgRes C12 camera and the ProgRes Capture Pro 2.5 software with the straight line tool (Jenoptik GmbH, Jena, Germany). At least 200 adipocytes from both subcutaneous and visceral adipose tissue were evaluated for each mouse in a blinded fashion.

Renal Staining

Renal cryosections were targeted with 1% silver nitrate solution (Sigma). The argentaffin reaction described by von Kossa is photochemical in nature where the activation energy is supplied by UV light.²³ The calcium ions were reduced by the UV light and replaced by silver ions reacting with the phosphate deposits. Thus, these phos-

Figure 1. Exploratory laparotomy in mice with phosphate nephropathy. Multiple white granular deposits beneath the renal fascia were evident by macroscopic inspection of the kidneys in mice on the phosphorus-rich diet. The **black arrow** indicates the position of the single kidney in the abdomen.

phosphate deposits are visualized by the metallic silver. The cryosections were counterstained with nuclear fast red (Sigma). Formalin-fixed renal tissue was embedded in paraffin, cut in 4- μ m sections, and stained with acid fuchsin and orange G using standard staining techniques. To determine the nephron segments that contained the inorganic precipitations, the renal specimens were stained with lectins of *Arachis hypogaea* and *Dolichos biflorus* (Sigma), which are markers of distal tubuli and collecting ducts, as shown by Markowitz et al.¹³ Briefly, deparaffinized tissue sections were treated by autoclave for 7 minutes at 121°C in target retrieval solution (Dako, Glostrup, Denmark), quenched in 0.3% H₂O₂ in methanol, blocked with biotin/avidin blocking kit (Vector Laboratories Inc., Burlingame, CA), and then overlaid with biotin-conjugated *A. hypogaea* and *D. biflorus*, each at a concentration of 0.01 mg/mL, for 50 minutes at room temperature. Color reactions were developed with aminoethylcarbazole after incubation with extravidin-peroxidase (Sigma).

The three-layer immunoperoxidase technique on frozen tissue sections (4 μ m) was used for the detection of macrophages and T cells in the kidney sections. Macrophages were stained using the rat anti-mouse F4/80 and CD68 antibody (Serotec, Oxford, England). A semiquantitative scoring system was performed as follows: 0, 0 to 4 cells stained positive; 1+, 5 to 10 cells; 2+, 10 to 50 cells; 3+, 50 to 200 cells; and 4+, more than 200 cells stained positive per low-power field. For the detection of CD4⁺ and CD8⁺ T cells, rat anti-mouse CD4 and CD8 α monoclonal antibodies (Serotec) were used, respectively. Biotin-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody, followed by incubation with an avidin-biotin complex and subsequent development with 0.4% 3-amino-9-ethylcarbazole for 6 minutes

and counterstaining with Gill's Hematoxylin No. 3 (Polysciences, Warrington, PA). Quantification of T cells was performed by counting the number of cells in six adjacent high-power fields of renal cortex and medulla.

Cardiovascular Studies

After sacrifice, mice were perfused transcardially with phosphate-buffered saline. Transverse sections of the perfused hearts were next fixed in formalin and scanned by Epson 3170 Photo (Seiko Epson, Nagano, Japan). The left ventricular dimension, posterior wall thickness, and septal wall thickness were measured in a blinded fashion according to the American Society of Echocardiography guidelines.²⁴

Figure 2. Histologic verification of phosphate nephropathy. There was not a single deposit with a positive von Kossa stain in the control db/db mice fed standard chow (A), whereas renal cryosections revealed abundant tubular calcium phosphate deposits with a positive histochemical von Kossa reaction in db/db mice fed the phosphorus-rich diet (B). Kidney-sections of db/db mice fed the phosphorus-rich diet were also stained with acid fuchsin and orange G and showed degenerative tubular changes with concomitant fibrosis (D), which were absent in db/db mice fed standard chow (C). Lectin staining with *A. hypogaea* (E and F) and *D. biflorus* (G and H) showed no calcifications in distal tubuli (E) and collecting ducts (G) of controls, whereas calcifications (asterisks) were located to the distal tubuli (F) and collecting ducts (H) of db/db mice on the phosphorus-rich diet. Representative pictures are shown with a magnification of 100 $\times$ (A–D) and 200 $\times$ (E–H), respectively.

Figure 3. Raman spectroscopy of renal precipitations in phosphate nephropathy. The Raman spectrum of the murine tubular aggregates (**A**) showed an intense, sharp band at 961 cm^{-1} and several other weaker bands, which are typical for submicrocrystalline hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ in biological tissues. At the bottom, there is the reference Raman spectrum of human tooth (**B**).

Reverse Transcription and Real-Time PCR

Total RNA was isolated from kidneys using Trizol (Sigma) according to a standard protocol. Thereafter, $2\text{ }\mu\text{g}$ of total RNA was reverse transcribed using a Superscript III Transcription Kit (Nitrogen, Carlsbad, CA) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). For linear amplification of FoxP3 and tumor necrosis factor α (TNF- α), SYBR Green Master Mix (Nitrogen) and the following primers were used: FoxP3 forward 5'-TCTTGCCAAGCTGGAAGACT-3' and reverse 5'-AGCTGATGCATGAAGTGTGG-3' and TNF- α forward 5'-GAAGTGGCAGAAGAGGCACT-3' and reverse 5'-AGGGTCTGGGCCATAGAACT-3'.²⁵ For quantification of IL-6, IFN- γ , Gata-3, IL-10, and IL-17, Taqman Master mix (Applied Biosystems) and the gene expression assays Mm00446190_m1, Mm00901778_m1, Mm00484683_m1, Mm00439616_m1, and Mm00439619_m1 (Applied Biosystems) were used. 18S served as the reference gene.

Statistical Analysis

Results are presented as mean $\pm$ SEM. Normal distribution of data was assessed by the Kolmogorov-Smirnov test with Lilliefors correction. Both groups were compared by either nonparametric Mann-Whitney *U*-test or unpaired *t*-test as appropriate, depending on the distribution of the tested variable. A two-tailed $P < 0.05$ was

considered statistically significant. All statistical analyses were performed with SPSS statistical software version 15.0 for Windows (SPSS Inc., Chicago, IL).

Results

The Model

For our animal model, we subjected db/db, leptin receptor-deficient mice (BKS.Cg-*Dock7*tm +/+ *Lepr*^{db}/J) to uninephrectomy to simulate chronic kidney disease at the age of 6 weeks and fed them either a phosphorus-rich diet or standard chow, respectively, for the next 8 weeks. db/db mice have hyperphagia, obesity, type 2 diabetes mellitus, polyuria, and, thus, volume depletion, which is another established risk factor for phosphate nephropathy.¹⁶

Renal Phenotype

All seven db/db mice on the phosphorus-rich diet and none of the eight controls fed standard chow developed

Figure 4. Leukocyte infiltration in phosphate nephropathy. Kidney infiltration of CD4⁺ T cells (**A** and **B**), CD8⁺ T cells (**C** and **D**), CD68⁺ cells (**E** and **F**), and F4/80⁺ cells (**G** and **H**) in controls (**A**, **C**, **E**, and **G**) and mice with phosphate nephropathy (**B**, **D**, **F**, and **H**) are shown. Representative pictures were taken at a magnification 400 $\times$ for T cells (**A**–**D**) and at a magnification of 200 $\times$ for macrophages (**E**–**H**).

Figure 5. Peritubular infiltration and renal cytokine expression in phosphate nephropathy. Peritubular infiltration of CD4⁺ T cells, CD8⁺ T cells (A), and macrophages (B) in phosphate nephropathy (white bars; *n* = 7) compared with controls (black bars; *n* = 8). Kidneys of db/db on phosphorus-rich diet (white bars; *n* = 7) displayed significantly increased mRNA expression of the T_H1 cytokines IFN-γ, IL-6, and TNF-α, whereas the master regulator of T_H2 cells, Gata-3, was comparable to the levels of kidneys of db/db mice fed standard chow (black bar; *n* = 8) (C). IL-10 and FoxP3 were found to be significantly increased in kidneys of db/db mice fed the phosphorus-rich diet. **P* < 0.05.

phosphate nephropathy. Exploratory laparotomy of mice fed the phosphorus-rich diet showed multiple white granular deposits beneath the renal fascia by macroscopic inspection (Figure 1). There were no such visible deposits in control db/db mice fed standard chow. To verify

phosphate nephropathy, we next performed histologic analyses looking for tubular hydroxyapatite crystals, which are the pathognomonic hallmark of phosphate nephropathy. The renal cryosections revealed abundant intraluminal tubular deposits of these crystals with a strong histochemical reaction in the von Kossa stain in db/db mice on the phosphorus-rich diet (Figure 2B). There was not a single deposit with a positive von Kossa reaction in the db/db controls fed standard chow (Figure 2A). Next, we performed X-ray diffraction and Raman spectroscopy of the inorganic extracts from kidneys to analyze the mineral composition of the tubular precipitations. Despite the low crystallinity of the sample, a qualitative phase analysis based on a comparison between the experimental X-ray diffraction data and the entries of the Powder Diffraction File 4 database allowed the identification of an apatite phase [Ca₅(PO₄)₃(OH,F)] as the main constituent. Additional lines may point to the simultaneous presence of monetite (CaHPO₄) as a second phase. These results were in good agreement with the outcome of the spectroscopic investigations. The Raman spectrum of the aggregate showed an intense, sharp band at 961 cm⁻¹, a weaker band at 1010 cm⁻¹, and several broad, weak bands around 420, 600, 780, and 1080 cm⁻¹ (Figure 3). Intensity, full width at half maximum, and wave number of most of these bands, with the exception of the one at 1010 cm⁻¹, are typical for submicrocrystalline hydroxyapatite in biological tissues. The weak band at 1010 cm⁻¹ might be related to subordinately occurring material of an unidentified phase. Thus, X-ray diffraction and Raman spectroscopy showed without doubt that the tubular precipitations in our animal model consisted mostly of hydroxyapatite Ca₅(PO₄)₃(OH) crystals.

These multiple intraluminal deposits of hydroxyapatite were associated with degenerative tubular changes, such as tubular ectasia and simplification, with desquamated apoptotic epithelial cells, diffuse interstitial edema, and fibrosis (Figure 2, C and D). Glomeruli appeared unremarkable. The hydroxyapatite crystals were located in distal tubuli (Figure 2, E and F) and collecting ducts (Figure 2, G and H), as shown by lectin staining using *A. hypogaea* and *D. biflorus*, which are specific markers for

Table 1. Biochemical Parameters in Phosphate Nephropathy

	Controls (<i>n</i> = 8)	Phosphorus (<i>n</i> = 7)	<i>P</i> value
Serum phosphate, mmol/L	2.80 ± 0.12	3.77 ± 0.77	0.021
Serum calcium, mmol/L	2.31 ± 0.05	1.97 ± 0.14	0.050
Serum urea, mmol/L	10.9 ± 0.3	14.1 ± 1.6	0.092
Urinary albumin:creatinine ratio	0.14 ± 0.02	0.20 ± 0.06	0.42
Body weight, g	52.3 ± 4.2	44.5 ± 2.1	0.001
Kidney weight, g	0.261 ± 0.010	0.223 ± 0.004	0.026
Glomerular diameter, μm	69.0 ± 0.7	64.4 ± 0.8	<0.0001
Fasting blood glucose, mmol/L	19.1 ± 2.9	4.9 ± 0.4	0.001
Fasting insulin, pmol/L	625 ± 107	1006 ± 198	0.054*
HOMA insulin resistance	64 ± 7	33 ± 9	0.021
Visceral adipocyte diameter, μm	114.7 ± 0.7	102.2 ± 0.7	< 0.0001
Subcutaneous adipocyte diameter	103.7 ± 0.7	102.8 ± 0.7	0.33
Septal wall thickness, mm	1.27 ± 0.06	1.52 ± 0.02	0.014
Posterior wall thickness, mm	1.34 ± 0.07	1.64 ± 0.09	0.029
Left ventricular diameter, mm	5.01 ± 0.24	5.19 ± 0.04	0.55

*By Mann-Whitney *U*-test.

Figure 6. Secondary hyperparathyroidism and FGF23 excess in phosphate nephropathy. db/db mice fed the phosphorus-rich diet (white bars; $n = 7$) had significantly higher serum parathyroid hormone, higher serum FGF23, and higher serum osteopontin levels when compared with db/db mice fed standard chow (black bars; $n = 8$). * $P < 0.05$; ** $P < 0.005$.

the respective nephron segments. These apatitic precipitations were associated with a prominent renal infiltration of leukocytes (Figures 4 and 5). CD4⁺ and CD8⁺ T cells were found to infiltrate kidneys of db/db mice on the phosphate-rich diet throughout the interstitium (Figure 4, A-D, and Figure 5A). In contrast, most macrophages, stained with CD68 and F4/80, infiltrated into the peritubular regions in close proximity to the tubular hydroxyapatite crystals (Figure 4, E-H, and Figure 5B). In line, kidneys of db/db mice on the phosphorus-rich diet displayed significantly increased mRNA expression of the T_H1 cytokines IFN- γ , IL-6, and TNF- α , whereas the master regulator of T_H2 cells, Gata-3, was comparable to the levels of kidneys of db/db mice fed standard chow (Figure 5C). IL-10 and FoxP3 were found to be significantly increased in kidneys of db/db mice on the phosphorus-rich diet (Figure 5C), whereas IL-17 was not detectable (data not shown). Blood sample analyses showed a significant increase of serum phosphate levels and a significant decrease of serum calcium levels in db/db mice on the phosphorus-rich diet when compared with db/db control mice fed standard chow (Table 1).

These db/db mice with phosphate nephropathy displayed also a secondary hyperparathyroidism with significantly elevated serum parathyroid hormone levels that were associated with a secondary FGF23 excess and elevated serum osteopontin levels (Figure 6). At the end of the experiment, both the mean kidney weight and the mean glomerular diameter were significantly higher in the db/db control mice when compared with the db/db mice on the phosphorus-rich diet (Table 1). There was no significant difference in albuminuria between the two treatment groups (Table 1). Of note, uninephrectomized C57BL/6 mice fed the phosphorus-rich diet did not develop phosphate nephropathy (data not shown).

Metabolic Phenotype

Mice with phosphate nephropathy had a restrained insulin resistance. db/db mice on the phosphorus-rich diet had less body weight (Figure 7A), a significantly lower fasting blood glucose level (Figure 7C), a lower HOMA-IR index, and most importantly smaller adipocyte diameters in the visceral adipose tissue (Table 1). Moreover, the mice on the phosphorus-rich diet also performed better on the glucose tolerance test (Figure 7B) and on the insulin tolerance test when compared with controls (Figure 7D). There was no significant difference in the size of adipocytes in subcutaneous adipose tissue.

Cardiovascular Phenotype

After 8 weeks of treatment on the phosphorus-rich diet, there was no detectable vascular calcification in the aorta or in the renal vessels in db/db mice, which have a C57BL/6 background and are known to be resistant to vascular calcification.²⁶ db/db mice on the phosphorus-rich diet, however, displayed significantly thicker septal and posterior walls of the left ventricle, which are remi-

Figure 7. Insulin resistance is restrained by phosphate nephropathy. db/db mice fed the phosphorus-rich diet (white bars; $n = 7$) gained less body weight (A), had lower fasting blood glucose levels (C), and showed a better performance on the glucose tolerance test (B) and the insulin tolerance test (D) when compared with control db/db mice (black bars; $n = 8$). Significant difference of * P value < 0.05 between the db/db mice fed standard chow (black bars/diamonds) and the db/db mice fed the phosphorus-rich diet (white bars/squares). Significant difference of ** $P < 0.05$ between the db/db mice fed standard chow (black bars/diamonds) and the C57BL/6 control mice fed the phosphorus-rich diet (gray bars/triangles; $n = 3$).

niscent of hypertrophic cardiomyopathy in end-stage renal failure (Table 1).

Discussion

This is, to our knowledge, the first report of an animal model of phosphate nephropathy. Up to date, there is no specific treatment for phosphate nephropathy. Therefore, this murine model may serve as a tool to test pharmacologic interventions to counteract and to treat the precipitation of hydroxyapatite crystals in the renal tubuli. The murine model of phosphate nephropathy displayed plenty of tubular aggregates with strong histochemical reaction in the von Kossa stain, which were located to the distal tubuli and collecting ducts. X-ray diffraction and Raman spectroscopy proved these precipitations to be submicrocrystalline hydroxyapatite crystals. The associated tubular degenerative changes and the peritubular infiltration of lymphocytes and macrophages exactly mirror the pathology of biopsy-proven acute phosphate nephropathy in humans.^{11,14,17,27–33}

Moreover, the presented animal model has manifold facets of overt hyperphosphatemia and of secondary hyperparathyroidism. Uninephrectomized db/db mice on the phosphorus-rich diet displayed not only elevated serum phosphorus and decreased serum calcium levels but also a strong secondary up-regulation of serum FGF23,³ serum parathyroid hormone, and serum osteopontin levels. A similar secondary FGF23 excess and secondary hyperparathyroidism were also described in the animal model by El-Abbadi et al,²⁶ where uremia was generated first by a two-step surgical procedure for partial renal ablation before application of an oral phosphate overload. In contrast to the model of El-Abbadi et al, our animals did not develop arterial medial calcification in the 8 weeks on the phosphorus-rich diet. This difference is probably due to the different genetic background of the mice used. DBA/2 mice are prone to develop medial calcifications, whereas C57BL/6 mice are resistant to vascular calcifications. Another important difference to the model of El-Abbadi et al is the fact that our mice were not uremic and can thereby serve to study early changes in calcium and phosphorus metabolism in chronic kidney disease. Furthermore, we were able to evaluate the morphologic changes in the kidney due to high oral phosphate load, which is not possible in the renal ablation model of Gallimore et al³⁴ that is induced by sequential electrocoagulation of the right renal cortex and left nephrectomy. A limitation of our model is, in contrast to the typical histologic changes in humans with phosphate nephropathy, the lack of renal fibrosis in our db/db mice treated with the phosphorus-rich diet at the evaluated time point.

The uninephrectomized db/db mice on the phosphorus-rich diet also had a restrained insulin resistance with a lower HOMA-IR index and smaller visceral adipocytes and developed a concentric left ventricular cardiac hypertrophy. It is not yet clear whether these metabolic and cardiac phenotypes are primary or secondary effects of hyperphosphatemia, secondary hyperparathyroidism, or

blood pressure elevation. A limitation of this study may be that the food intake of db/db mice on the phosphorus-rich diet on the one hand and standard chow on the other hand was not controlled and normalized. db/db mice on the phosphorus-rich diet had a lower body weight, which may be due to less food intake and may explain at least in part the better insulin resistance when compared with mice on a standard diet. However, the reduced weight gain could also be a consequence of impaired kidney function and cardiac hypertrophy. A big advantage of this model of phosphate nephropathy is the fact that it does not rely on genetic defects in the renal phosphate metabolism to produce the renal phenotype, as holds true for mice homozygous for the disrupted type IIa Na/Pi cotransporter gene *Npt2*, for instance.³⁵

Interestingly, kidneys of db/db mice on the phosphorus-rich diet displayed a decreased kidney size compared with controls. This difference in kidney size may be due to the different glycemic control and the resulting diabetic nephropathy of the control mice. Alternatively, the different kidney weight could also result from a beginning chronic kidney disease in db/db mice with phosphate nephropathy, as indicated by the trend to higher urea levels in these mice on the phosphorus-rich diet.

In conclusion, the present study opens the door for future pharmacologic and pathophysiologic studies that analyze, on the one hand, phosphate nephropathy and, on the other hand, the effect of hyperphosphatemia and secondary hyperparathyroidism on renal, metabolic, and cardiovascular phenotypes. This animal model may, thus, aid the study of the phosphate homeostasis in more detail because it is still associated with adverse clinical outcomes in both acute phosphate nephropathy and chronic renal failure.^{5,7,8,36–38}

References

1. Murer H, Lotscher M, Kaissling B, Levi M, Kempson SA, Biber J: Renal brush border membrane Na/Pi-cotransport: molecular aspects in PTH-dependent and dietary regulation. *Kidney Int* 1996, 49:1769–1773
2. Prie D, Friedlander G: Genetic disorders of renal phosphate transport. *N Engl J Med* 2010, 362:2399–2409
3. Wolf M: Forging forward with 10 burning questions on FGF23 in kidney disease. *J Am Soc Nephrol* 2010, 21:1427–1435
4. Prie D, Urena Torres P, Friedlander G: Latest findings in phosphate homeostasis. *Kidney Int* 2009, 75:882–889
5. Foley RN, Collins AJ, Herzog CA, Ishani A, Kalra PA: Serum phosphorus levels associate with coronary atherosclerosis in young adults. *J Am Soc Nephrol* 2009, 20:397–404
6. Maizel J, Six I, Slama M, Tribouilloy C, Sevestre H, Poirat S, Giummelly P, Atkinson J, Choukroun G, Andrejak M, Kamel S, Maziere JC, Massy ZA: Mechanisms of aortic and cardiac dysfunction in uremic mice with aortic calcification. *Circulation* 2009, 119:306–313
7. Dhingra R, Sullivan LM, Fox CS, Wang TJ, D'Agostino RB, Sr., Gaziano JM, Vasan RS: Relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. *Arch Intern Med* 2007, 167:879–885
8. Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G: Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005, 112:2627–2633
9. Eckstein J, Savic S, Eugster T, Pargger H, Gurke L, Pechula M, Steiger J, Mayr M: Extensive calcifications induced by hyperphosphatemia caused by phosphate-based enema in a patient after kidney transplantation. *Nephrol Dial Transplant* 2006, 21:2013–2016

10. Beloosesky Y, Grinblat J, Weiss A, Grosman B, Gaftor U, Chagnac A: Electrolyte disorders following oral sodium phosphate administration for bowel cleansing in elderly patients. *Arch Intern Med* 2003, 163:803–808
11. Desmeules S, Bergeron MJ, Isenring P: Acute phosphate nephropathy and renal failure. *N Engl J Med* 2003, 349:1006–1007
12. Grossman RA, Hamilton RW, Morse BM, Penn AS, Goldberg M: Nontraumatic rhabdomyolysis and acute renal failure. *N Engl J Med* 1974, 291:807–811
13. Markowitz GS, Nasr SH, Klein P, Anderson H, Stack JI, Alterman L, Price B, Radhakrishnan J, D'Agati VD: Renal failure due to acute nephrocalcinosis following oral sodium phosphate bowel cleansing. *Hum Pathol* 2004, 35:675–684
14. Markowitz GS, Stokes MB, Radhakrishnan J, D'Agati VD: Acute phosphate nephropathy following oral sodium phosphate bowel purgative: an underrecognized cause of chronic renal failure. *J Am Soc Nephrol* 2005, 16:3389–3396
15. O'Connor LR, Klein KL, Bethune JE: Hyperphosphatemia in lactic acidosis. *N Engl J Med* 1977, 297:707–709
16. Markowitz GS, Perazella MA: Acute phosphate nephropathy. *Kidney Int* 2009, 76:1027–1034
17. Ori Y, Herman M, Tobar A, Chernin G, Gaftor U, Chagnac A, Izhak OB, Korzets A: Acute phosphate nephropathy—an emerging threat. *Am J Med Sci* 2008, 336:309–314
18. Brunelli SM, Lewis JD, Gupta M, Latif SM, Weiner MG, Feldman HI: Risk of kidney injury following oral phosphosoda bowel preparations. *J Am Soc Nephrol* 2007, 18:3199–3205
19. Hurst FP, Bohlen EM, Osgard EM, Oliver DK, Das NP, Gao SW, Abbott KC: Association of oral sodium phosphate purgative use with acute kidney injury. *J Am Soc Nephrol* 2007, 18:3192–3198
20. Rosenkranz AR, Mendrick DL, Cotran RS, Mayadas TN: P-selectin deficiency exacerbates experimental glomerulonephritis: a protective role for endothelial P-selectin in inflammation. *J Clin Invest* 1999, 103:649–659
21. Levy JC, Matthews DR, Hermans MP: Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 1998, 21:2191–2192
22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985, 28:412–419
23. von Kossa J: Ueber die im Organismus kuenstlich erzeugbaren Verkalkungen. *Beitr Pathol Anat* 1901, 29:163–202
24. Sahn DJ, DeMaria A, Kisslo J, Weyman A: Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. *Circulation* 1978, 58:1072–1083
25. Eller P, Eller K, Wolf AM, Reinstadler SJ, Tagwerker A, Patsch JR, Mayer G, Rosenkranz AR: Atorvastatin attenuates murine anti-glomerular basement membrane glomerulonephritis. *Kidney Int* 2010, 77:428–435
26. El-Abbadi MM, Pai AS, Leaf EM, Yang HY, Bartley BA, Quan KK, Ingalls CM, Liao HW, Giachelli CM: Phosphate feeding induces arterial medial calcification in uremic mice: role of serum phosphorus, fibroblast growth factor-23, and osteopontin. *Kidney Int* 2009, 75:1297–1307
27. Aasebo W, Scott H, Ganss R: Kidney biopsies taken before and after oral sodium phosphate bowel cleansing. *Nephrol Dial Transplant* 2007, 22:920–922
28. Beyea A, Block C, Schned A: Acute phosphate nephropathy following oral sodium phosphate solution to cleanse the bowel for colonoscopy. *Am J Kidney Dis* 2007, 50:151–154
29. Connor A, Sykes L, Roberts IS, Weston CE: Acute phosphate nephropathy after sodium phosphate preparations. *BMJ* 2008, 337:a182
30. Gonlusen G, Akgun H, Ertan A, Olivero J, Truong LD: Renal failure and nephrocalcinosis associated with oral sodium phosphate bowel cleansing: clinical patterns and renal biopsy findings. *Arch Pathol Lab Med* 2006, 130:101–106
31. Manley P, Somerfield J, Simpson I, Barber A, Zwi J: Bilateral uraemic optic neuritis complicating acute nephrocalcinosis. *Nephrol Dial Transplant* 2006, 21:2957–2958
32. Rocuts AK, Waikar SS, Alexander MP, Rennke HG, Singh AK: Acute phosphate nephropathy. *Kidney Int* 2009, 75:987–991
33. Steinman TI, Samir AE, Cornell LD: Case records of the Massachusetts General Hospital: case 27–2008: a 64-year-old man with abdominal pain, nausea, and an elevated level of serum creatinine. *N Engl J Med* 2008, 359:951–960
34. Gallimore B, Gagnon RF, Richards GK: Role of an intraperitoneal catheter implant in the pathogenesis of experimental *Staphylococcus epidermidis* peritoneal infection in renal failure mice. *Am J Nephrol* 1988, 8:334–343
35. Chau H, El-Maadawy S, McKee MD, Tenenhouse HS: Renal calcification in mice homozygous for the disrupted type IIa Na/Pi cotransporter gene *Npt2*. *J Bone Miner Res* 2003, 18:644–657
36. Tonelli M, Pannu N, Manns B: Oral phosphate binders in patients with kidney failure. *N Engl J Med* 2010, 362:1312–1324
37. Zaritsky JJ, Salusky IB: Ironing out the phosphorus problem. *Kidney Int* 2010, 77:845–847
38. Block GA, Brillhart SL, Persky MS, Am A, Slade AJ: Efficacy and safety of SBR759, a new iron-based phosphate binder. *Kidney Int* 2010, 77:897–903