Aromatase Deficiency Causes Altered Expression of Molecules Critical for Calcium Reabsorption in the Kidneys of Female Mice*

Orhan K Öz,[1,2] Asghar Hajibeigi,[1,2] Kevin Howard,[1] Carolyn L Cummins,[3] Monique van Abel,[4] Rene JM Bindels,[4] R Ann Word,[5] Makoto Kuro-o,[6] Charles YC Pak,[2,7] and Joseph E Zerwekh[2,7]

ABSTRACT: Kidney stones increase after menopause, suggesting a role for estrogen deficiency. ArKO mice have hypercalciuria and lower levels of calcium transport proteins, whereas levels of the klotho protein are elevated. Thus, estrogen deficiency is sufficient to cause altered renal calcium handling.

Introduction: The incidence of renal stones increases in women after menopause, implicating a possible role for estrogen deficiency. We used the aromatase deficient (ArKO) mouse, a model of estrogen deficiency, to test the hypothesis that estrogen deficiency would increase urinary calcium excretion and alter the expression of molecular regulators of renal calcium reabsorption.

Materials and Methods: Adult female wildtype (WT), ArKO, and estradiol-treated ArKO mice ($n = 5–12$/group) were used to measure urinary calcium in the fed and fasting states, relative expression level of some genes involved in calcium reabsorption in the distal convoluted tubule by real-time PCR, and protein expression by Western blotting or immunohistochemistry. Plasma membrane calcium ATPase (PMCA) activity was measured in kidney membrane preparations. ANOVA was used to test for differences between groups followed by posthoc analysis with Dunnett's test.

Results: Compared with WT, urinary Ca:Cr ratios were elevated in ArKO mice, renal mRNA levels of transient receptor potential cation channel vallinoid subfamily member 5 (*TRPV5*), *TRPV6, calbindin-D$_{28k}$,* the Na+/Ca+ exchanger (*NCX1*), and the *PMCA1b* were significantly decreased, and klotho mRNA and protein levels were elevated. Estradiol treatment of ArKO mice normalized urinary calcium excretion, renal mRNA levels of *TRPV5, calbindin-D$_{28k}$, PMCA1b,* and *klotho,* as well as protein levels of calbindin-D$_{28k}$ and Klotho. ArKO mice treated with estradiol had significantly greater PMCA activity than either untreated ArKO mice or WT mice.

Conclusions: Estrogen deficiency caused by aromatase inactivation is sufficient for renal calcium loss. Changes in estradiol levels are associated with coordinated changes in expression of many proteins involved in distal tubule calcium reabsorption. Estradiol seems to act at the genomic level by increasing or decreasing (klotho) protein expression and nongenomically by increasing PMCA activity. PMCA, not NCX1, is likely responsible for extruding calcium in response to in vivo estradiol hormonal challenge. These data provide potential mechanisms for regulation of renal calcium handling in response to changes in serum estrogen levels.
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Key words: aromatase, calcium transport proteins, estrogen, kidney, klotho

INTRODUCTION

THE INCIDENCE OF kidney stones in women is lower than in men throughout adult life. Premenopausal women have one half the prevalence of calcium stone disease than men of comparable age.[1,2] However, this sex difference disappears rapidly after the onset of menopause.[3–5] By 55–60 yr of age, the prevalence of calcium stone disease is equal in men and women and remains equal henceforth.[5] Several studies have provided evidence that urinary calcium excretion increases at menopause,[6–8] thereby increasing the risk for calcium-containing stones. However, additional changes in urinary composition (e.g., hypocitraturia, hyperoxaluria, hyperuricosuria) could also contribute

[1]Department of Radiology, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [2]Center for Mineral Metabolism and Clinical Research, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [3]Howard Hughes Medical Institute, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [4]Department of Physiology, University Medical Centre Nijmegen, Nijmegen, The Netherlands; [5]Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [6]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [7]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

to an increased risk for renal stones. We recently undertook a retrospective analysis of the database from our large kidney stone patient registry and identified a major role for the increased urinary calcium excretion in postmenopausal women with kidney stones.[6]

Nordin and Polley[7] compared urinary calcium excretion in matched pairs of normal pre- and postmenopausal women who had not received hormone replacement therapy. Urine calcium/creatinine was significantly greater in the postmenopausal women. This rise in calcium excretion at the menopause could not be accounted for by the associated rise in plasma calcium or its fractions because it remained significant even when pre- and postmenopausal women were matched for total, ultrafilterable, ionized, or complexed calcium. The authors suggested that estrogens promote tubular reabsorption of calcium, an observation that was subsequently confirmed in a second study by the same investigators.[8] McKane et al.[9] also examined the effect of estrogen replacement therapy (ERT) on renal tubular calcium reabsorption before and after 6 mo of therapy in early postmenopausal women (median age, 51 yr). Their study is significant in that they directly measured serum ultrafilterable calcium and tubular reabsorption of calcium before and during PTH clamp. Before PTH administration, ERT had no effect on serum ultrafilterable calcium. ERT increased the tubular reabsorption of calcium, an effect ascribed to a commensurate increase in PTH and consistent with an effect of ERT in reducing bone resorption. However, after PTH clamp, the tubular reabsorption of calcium was greater after ERT than at baseline, suggesting a direct effect of ERT in promoting increased tubular reabsorption of calcium independent of PTH's action. Taken together, these observations are consistent with the hypothesis that impaired tubular reabsorption of calcium and renal calcium loss from estrogen deficiency could be one factor contributing to increased stone formation in postmenopausal women.

The mechanism by which estrogens might regulate calcium reabsorption is incompletely understood. The major part of renal tubular reabsorption of calcium occurs along the proximal tubule and thick ascending limb by a passive paracellular flux. The remaining calcium reabsorption occurs in the distal part of the nephron and is hormonally regulated. This process can be envisaged as a three-step process consisting of facilitated entry across the apical membrane mediated by an epithelial calcium channel (TRPV5), cytosolic diffusion of calcium bound to calcium binding proteins such as calbindin-D_{28k}, and active extrusion of calcium across the basolateral membrane mediated by PMCA and NCX1.[10] Thus, decreased expression or function of any of these components could impair calcium reabsorption.

The aromatase-deficient mouse is a model of estrogen deficiency caused by inactivation of the gene responsible for biosynthesis of estrogens.[11] In this study, we used the ArKO mouse model to determine whether estrogen deficiency, as opposed to complete gonadal failure of ovariectomy models or surgically induced menopause, is sufficient to alter renal calcium reabsorption and investigate potential underlying mechanisms.

MATERIALS AND METHODS

Animals

The inactivated aromatase gene was maintained on the hybrid 129SvEV/C57Bl6. Female ArKO mice were produced by breeding heterozygotes. In some studies, 10-wk-old animals were treated three times per week with 20 μg estradiol/mouse, or sesame oil only (placebo) for 3 wk. All procedures were approved by the UT Southwestern IACUC.

Analytical studies

Urine was collected from wildtype (WT) and ArKO age-matched mice (10–13 wk old) as single spontaneous voids between 1:00 p.m. and 3:00 p.m. or in metabolic cages during an 18-h fast. Urinary calcium was determined by atomic absorption spectroscopy. Urinary creatinine and phosphorous were determined on an automated clinical system, the Roche Mira System. Blood was harvested by closed cardiac puncture at the time of death. Serum 1,25-dihydroxyvitamin D was determined by radioimmunoassay (RIA; Gamma-B 1,25-dihydroxyvitamin D assay; Immunodiagnostic Systems, Fountain Hills, AZ, USA). This assay measures both D_3 and D_2 forms of 1,25-dihydroxyvitamin D. It has a sensitivity of 8 pM, and intra- and interassay CVs of 8% and 14%, respectively. Mouse serum intact PTH(1-84) was measured by enzyme immunoassay (EIA; Intact Mouse PTH; Alpco Diagnostics, Salem, NH, USA). The assay has a sensitivity of 4 pg/ml and intra- and interassay CVs of 3.5% and 8.5%, respectively.

RT and quantitative PCR

Kidneys were powdered under liquid nitrogen (WT, $n = 12$; ArKO, $n = 9$; ArKO + E, $n = 6$; independent samples). The powder was homogenized in TriReagent (Sigma-Aldrich), according to the manufacturer's instructions, to obtain protein, DNA, and RNA in three different phases. Total RNA was treated with DNase to prevent contamination of genomic DNA and finally resuspended in diethylpyrocarbonate-treated milliQ water. Thereafter, 2 μg of total RNA was subjected to reverse transcription using Moloney murine leukemia virus RT (Gibco BRL) as described previously.[12] Expression levels of *TRPV5, TRPV6, calbindin-D_{28k}, calbindin-D_{9k}*, plasma membrane calcium ATPase 1b (*PMCA-1b*), and *NCX1* mRNA were analyzed by quantitative real-time PCR, using the ABI Prism 7700 Sequence Detection System (PE Biosystems). With the use of standard curves, the amount of copy numbers of the target genes in each sample was calculated and expressed as a ratio to the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene. Primers and probes targeting the genes of interest were designed using Primer Express software (Applied Biosystems) and are listed in Table 1.[13,14]

Expression levels of ERα and ERβ

cDNA was synthesized from 2 μg total RNA isolated from kidney using multiscribe RT enzyme and random hexamer primers (Superscript II; Invitrogen) in a total vol-

ume of 20 μl. Negative controls included RNA without RT. Newly synthesized cDNA (50 ng) was used for real-time PCR reactions. To correct for sample-to-sample variation, mRNA amounts were normalized to cyclophilin as an endogenous reference. Primers for *ERα* and *ERβ* were designed using Primer Express software (Table 2). Primers that span an exon and Taqman probes were designed to avoid amplification of residual contaminating DNA. For each primer–probe set, serial dilutions of template were conducted to ensure equal efficiency of amplification over a wide range of template concentrations and to ensure that efficiency of amplification was equal to that of cyclophilin. Each real-time reaction included negative and positive controls: cDNA (pooled from four wildtype cycling animals) from mouse uterus and mouse bladder for ERα and ERβ, respectively. Real-time PCR reactions were conducted in triplicate using the TaqManTM PCR Reagent Kit. The ABI PRISMTM Sequence Detection System was used to monitor the increase of the reporter fluorescence (Perkin-Elmer, Applied Biosystems). Relative gene expression was calculated using the comparative Ct method as described in the Applied Biosystems User Bulletin 2. Expression levels of klotho and mouse PTH receptor 1 were determined by real-time PCR using Sybr Green and quantitated with the ddC_T method.[15] The primer sequences for *klotho* were based on accession NM 013823. The forward primer sequence was 5′-AATTATGTGAATGAGGCTCTGAA-AG-3′ and the reverse primer sequence was 5′-TACGCA-AAGTAGCCACAAAGG-3′. The primer sequences for mPTHR1 were based on NM 011199. The forward primer sequence was 5′-CAGGGACACTGTGGCAGATC-3′, and the reverse primer sequence was 5′-CAAAAAATC-CCTGGAAGGAGTT-3′. All investigators were blinded to genotypes of the animals during performance of this study.

Western blotting

Renal proteins were obtained from the organic phase of a TriReagent (Sigma) extract of the kidney powder. Protein was harvested from the organic phase by precipitation followed by solubilization in a buffered 1% SDS solution containing protease inhibitors. Protein concentration was determined using a detergent compatible assay (BioRad). The homogenates were subjected to SDS-PAGE on 12.5% polyacrylamide gels or Tris-Tricine gels 9.5% (for calbindin-D_{9k} only) followed by blotting onto PVDF membrane. The following antibodies were used in this study: mouse monoclonal anti-bovine calbindin-D_{28k} (Alpha Diagnostic International); rabbit polyclonal anti-calbindin-D_{9k} (Swant); and rat monoclonal anti-klotho KM2119.[16] Purified porcine intestinal calbindin-D9k was used as a standard for anti-calbindin-D_{9k} Western blot (Calbiochem). Recombinant rat calbindin-D_{28k} was used as a standard for anti-calbindin-D_{28k} Western blot (Swant). Recombinant klotho was used as a standard for the anti-klotho antibody. Membranes were probed with primary antibody at the following dilutions or concentration: anti-calbindin-D_{28k} monoclonal antibody (1:3000), anti-calbindin-D_{9k} (1:1000), and anti-klotho (3 μg/ml) overnight at 4°C. Subsequently, the membranes were probed with an appropriate horseradish per-

oxidase (HRP)-conjugated secondary antibody, and bands were detected by ECL system (Amersham).

Immunohistochemistry

Kidneys were bisected and embedded in paraffin by standard techniques. Seven-micrometer-thick sections were mounted on glass slides.

Anti-klotho immunohistochemistry: After deparaffinization and rehydration of kidney sections, endogenous peroxidase activity was neutralized by incubating the sections for 30 min with 0.3% H_2O_2 in methanol. The slides were blocked with normal rabbit serum for 20 min at room temperature. Subsequently, the slides were washed several times. The sections were incubated overnight with a rat monoclonal antibody against recombinant klotho protein.[16] The bound primary antibody was detected using an HRP-conjugated rabbit anti-rat IgG and DAB as substrate (Vector Laboratories). Sections were counterstained with 2% methyl green.

Anti-PTHR1 immunohistochemistry: Deparaffinized and rehydrated sections of mouse kidney underwent antigen retrieval using Vector Laboratories antigen retrieval solution and the manufacturer's suggested procedure. After a 5-min wash in PBS, the slides were treated with 0.1% Triton X-100 in PBS for 20 min, washed, and incubated with 5 mM glycine for 10 min. The slides were further blocked by a 20-min incubation with 3% goat serum (Vector Laboratories). The sections were exposed to a 1:100 dilution of rabbit anti-PTHR1 antibody (Affinity Bioreagents) or to PBS (negative control) overnight at 4°C. Slides were washed for 5 min in PBS and treated with a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories) for 45 min. After a 5-min wash in PBS, slides were further treated with streptavidin-FITC (1:50; Molecular Probes) for 20 min. Slides were rinsed in PBS for 5 min, briefly rinsed in distilled water, shaken, blotted dry, and immediately cover-slipped with a xylene-based mounting medium. Sections were examined by confocal microscopy with a Zeiss LSM-510 confocal microscope at a wavelength of 488 nm and ×400 magnification. Human placenta, which does not express PTHR1, was used as a negative control and subjected to the same procedure.

PMCA enzymatic activity

PMCA activity was measured according to a previously published method.[17] Briefly, kidneys were harvested from WT, ArKO, and estradiol-treated ArKO mice after perfusion with cold PBS containing 1 mM EDTA + 1 mM PMSF. The kidneys were minced on ice in a hypotonic buffer containing protease inhibitors. After 15 min, the fragments were homogenized in an equal volume of 0.5 M sucrose + 0.3 M KCl + 1.5 mM EDTA. The homogenate was spun at 5000g to remove unbroken cells and mitochondria. The supernatant was centrifuged at 100,000g for 1 h. The pellet was resuspended in a solution containing 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM DTT, and 20 μM $CaCl_2$. Protein concentration was determined using the Bradford method (Bio-Rad). Membrane vesicles (50 μg of membrane protein) were added to 140 μl of medium con-

TABLE 1. SEQUENCES OF PRIMERS AND TAQMAN PROBES FOR QUANTITATIVE REAL-TIME PCR*

Gene	Forward primer	Reverse primer	Probe
TRPV5	5'-CGTTGGTTCTTACGGGTTGAAC-3'	5'-GTTTGGAGAACCACAGAGCCTCTA-3'	5'-TGTTTCTCAGATAGCTGCTCTTGTACTTCCTCTTTGT-3'
TRPV6	5'-ATCCGCCGCTATGCACA-3'	5'-AGTTTTTCTCCTGAATCTTTTTCCAA-3'	5'-TTCCAGCAACAAGATGGCCTCTACTCTGA-3'
CaBP-D28K	5'-AACTGACAGAGATGGCCAGGTTA-3'	5'-TGAACTCTTTCCCACACATTTTGAT-3'	5'-ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA-3'
CaBP-D9K	5'-CCTGCAGAAATGAAGAGCATTTT-3'	5'-CTCCATCGCCATTCTTATCCA-3'	5'-CAAAAATATGCAGCCAAGGAAGGCGA-3'
PMCA1B	5'-CGCCATCTTCTGCACCATT-3'	5'-CAGCCATTGCTCTATTGAAAGTTC-3'	5'CAGCTGAAAGGCTTCCCGCCAAA-3'
NCX1	5'-TCCCTACAAAACTATTGAAGGCACA-3'	5'-TTTCTCATACTCCTCGTCATCGATT-3'	5''-ACCTTGACTGATATTGTTTGACTATTTCATCATTCT-3'
ERα	5'-TGCCTGGCTGGAGATTCTG-3'	5'-TGCCTGGCTGCTCGTCTGGCGCTCC-3'	5'-GAGCTTCCCGGGTGTTC-3'
ERβ	5'-AAGCTGGCTGACAAGGAACTG-3'	5'-TGGCCCAGCCAATCATGTGCA-3'	5'-CCACAAAGCCAGGGATTTTC-3'
HPRT	5'-TTATCAGACTGAAGAGCTACTGTAAT GATC-3'	5'-TTACCAGTGTCAATTATATCTTCAAC AATC-3'	5'-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3'
Cyclophilin	5'-GCTTTTCGCCGCTTGCT-3'	5'-CAACCCCACCGTGTTC-3'	5'-TCGTCATCGGCCGTGAT-3'

* PCR primers and fluorescent probes (5'FAM-3'TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Applied Biosystems or Biolegio (Malden, The Netherlands). TRPV5/TRPV6, epithelial Ca^{2+} channel 1 and 2; CaBP-D28K, calbindin-D_{28k}; CaBP-D9K, calbindin-D_{9k}; PMCA1B, plasma membrane calcium ATPase; NCX1, sodium-calcium exchanger; ERβ estrogen receptor alpha; ERα estrogen receptor. HPRT, hypoxanthine–guanine phosphoribosyl transferase, C

TABLE 2. URINARY CALCIUM/CREATININE RATIOS IN ArKO AND WT MICE

	Ca/Cr (mean ± SE)		
Group	Spontaneous (mg/mg)	Fasting (mg/mg)	GF corrected
WT	0.123 ± 0.020 (n = 10)	0.108 ± 0.022 (n = 5)	0.011 ± 0.002 (n = 5)
ArKO	0.181 ± 0.020 (n = 10)	0.277 ± 0.050 (n = 5)	0.034 ± 0.008 (n = 5)
ArKO + E	—	0.078 ± 0.011 (n = 5)	0.012 ± 0.003 (n = 5)
p	0.001*	0.001*, 0.7†	0.03*, 0.9†

Urine was collected as an afternoon spontaneous void or during an 18-h fast. ArKO + E represents 20 µg of estradiol/mouse three times per week for 3 wk. GF-corrected values were obtained by multiplying the fasting Ca/Cr ratios by serum Cr.
* p for ArKO vs. WT.
† p for ArKO + E vs. WT.

taining 100 µM $CaCl_2$ and ouabain to inhibit Na-K ATPase, thapsigargin to inhibit SERCA pumps, oligomycin and NaN_3 to inhibit any remaining contaminating mitochondria, and sufficient EGTA to obtain a free calcium concentration of 100 nM. $^{45}CaCl_2$ was added at an activity of 50 µCi/mol of total Ca. The reaction was started by the addition of 6 mM ATP and allowed to proceed for 10 min. Radioactive Ca^{2+} accumulation in the membrane preparations was determined by filtration through 0.45-µm filters presoaked in 1 mM $CaCl_2$ to reduce background. The membranes were washed twice with 1 mM $CaCl_2$ before counting in a β-counter.

Statistical analysis

Data are expressed as mean ± SE. Comparison of mean values for statistical significance was accomplished by one-way ANOVA. Posthoc analysis was performed by the two-tailed Dunnett's test, where the ArKO and estrogen-treated ArKO groups were compared with the WT group. Differences were considered significant at the $p < 0.05$ level.

RESULTS

Hypercalciuria and normophosphaturia in ArKO mice

Urinary calcium, expressed relative to creatinine, was significantly higher in ArKO mice compared with WT mice when examined in spot urine collections (Table 2). To better define this hypercalciuria, urine was collected under fasting conditions. In addition, a third group representing estradiol-treated ArKO mice was included. One-way ANOVA disclosed a significant interaction between the three groups ($p < 0.001$). Posthoc analysis disclosed that ArKO females again showed significant hypercalciuria compared with WT mice. Estrogen-treatment reduced urinary calcium excretion to a value not significantly different from WT. Similar results were also observed when urinary calcium was expressed relative to 100 ml glomerular filtration (Table 1). Urinary phosphate levels, expressed relative to creatinine, were not different between the groups (WT,
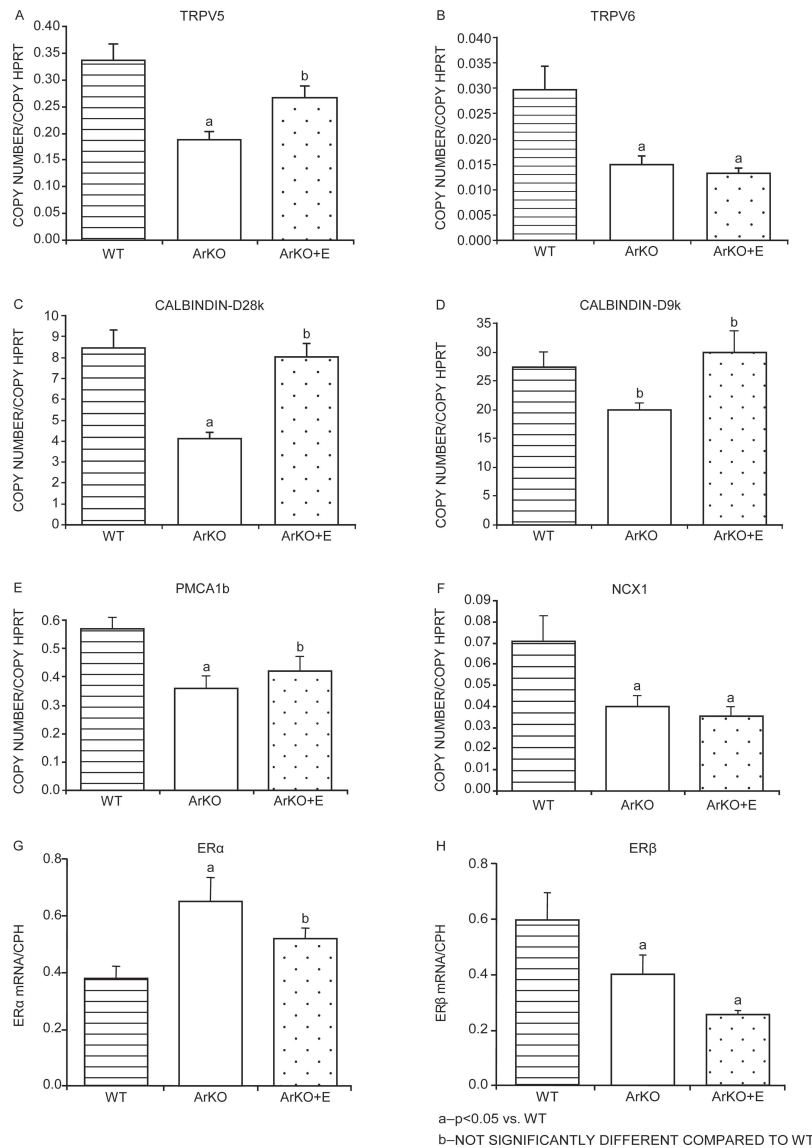
FIG. 1. Effect of aromatase deficiency and estradiol supplementation on mRNA levels of genes encoding proteins that regulate calcium transport in the kidney. RNA was prepared from whole kidneys and DNase treated to prevent contamination of genomic DNA. cDNA was prepared, and the copy number of the target gene was determined. The data are expressed as the ratio (mean ± SE) of copy number of target gene to the copy number of HPRT or cyclophilin (CPH). WT, wildtype ($n = 12$); ArKO, aromatase-deficient mice ($n = 9$); ArKO + E, ArKO mice treated with estradiol ($n = 6$), 20 μg/mouse three times per week for 3 wk. [a]ArKO vs. WT and [b]ArKO + E vs. WT.

4.65 ± 0.73; ArKO, 4.62 ± 0.36; ArKO + E, 4.89 ± 0.66; $n \geq$ 9 per group; ANOVA, $p = 0.9$). Total serum calcium was also not significantly different between the experimental groups (WT, 8.41 ± 0.18 mg/dl; ArKO, 8.45 ± 0.07 mg/dl; ArKO + E, 8.68 ± 0.27 mg/dl; $n \geq 9$ per group; ANOVA, $p = 0.6$).

PTH levels were unchanged between groups (WT, 75 ± 11; ArKO, 67 ± 10; ArKO + E, 61 ± 20 pg/ml; $n \geq 10$ per group; ANOVA, $p = 0.753$) as were 1,25OH$_2$D$_3$ levels (WT, 196 ± 30 pM; ArKO, 186 ± 35 pM; ArKO + E, 123 ± 14 pM; $n \geq 10$ per group; ANOVA, $p = 0.09$).

ArKO mice express lower levels of some proteins involved in transepithelial calcium reabsorption in the DCT that is normalized with estradiol therapy

To determine whether the hypercalciuria was associated with abnormal expression of proteins that mediate transcellular calcium reabsorption, we performed real-time PCR analysis of the expression of *TRPV5* and *6, calbindins D$_{28k}$* and *D$_{9k}$, PMCA1b, NCX1,* and the PTH receptor isoform *PTHR1.*

For the real-time PCR analysis of expression of each protein analyzed, one-way ANOVA disclosed a significant interaction between the three study groups ($p = 0.029$ or less), with the exception of PTHR1 (WT, 0.82 ± 0.08; ArKO, 1.08 ± 0.11; ArKO + E, 0.99 ± 0.11; ANOVA, $p = 0.140$). As expected, *TRPV5* was more highly expressed than *TRPV6* for both genotypes. ArKO mRNA levels were significantly decreased to 50–55% of WT levels for both proteins (Fig. 1). *Calbindin-D$_{28k}$* mRNA level was significantly decreased in ArKO mice to ~50% of the WT level. The mRNA level of *calbindin-D$_{9k}$* in ArKO mice was reduced to ~70% of WT, but the difference was not significant ($p = 0.054$, WT versus ArKO). In ArKO kidneys, the mRNA levels of the basolateral membrane proteins, *NCX1* and *PMCA1b,* were decreased. ArKO mRNA level of
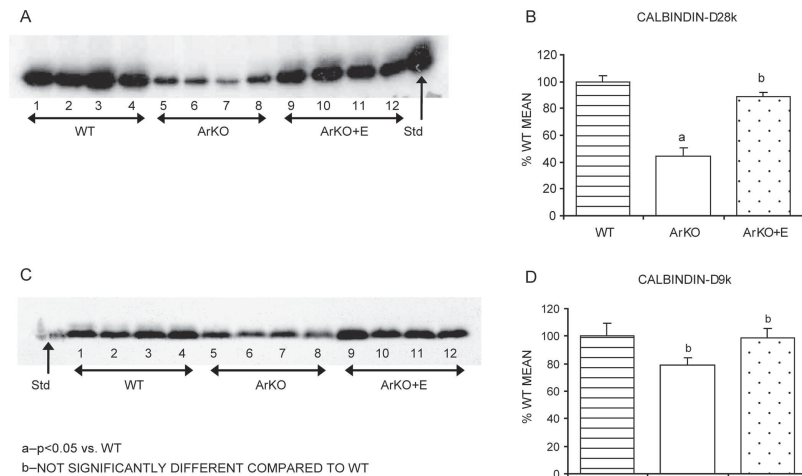
FIG. 2. Effect of aromatase deficiency and estradiol supplementation on protein expression levels of calbindin-D_{28k} and calbindin-D_{9k}. Detergent lysates were prepared from whole kidneys and equal amounts of protein subjected to SDS-PAGE followed by Western blotting. Membranes were probed with antibody specific for calbindin-D_{28k} (A) or calbindin-D_{9k} (C). Quantitative analysis (B and D) of protein expression was made by calculating the ratio of the band intensity for protein of interest to that for WT mean. WT, wildtype; ArKO, aromatase-deficient mice, ArKO + E, ArKO mice treated with estradiol, 20 μg/mouse three times per week for 3 wk. [a]ArKO vs. WT and [b]ArKO + E vs. WT.

a—p<0.05 vs. WT
b—NOT SIGNIFICANTLY DIFFERENT COMPARED TO WT

NCX1 was significantly reduced to ~55% of WT (Fig. 1). *PMCA1b* mRNA level was significantly lower in ArKO animals, being ~68% of WT (Fig. 1). After treatment with estradiol, there was no significant difference in the mRNA levels of *TRPV5, calbindin-D_{28k},* and *PMCA1b* between treated ArKO mice and WT mice (Fig. 1). Whereas *calbindin-D_{28k}* and *D_{9k}* mRNA levels were increased by estradiol therapy such that neither was significantly different from WT, both were significantly higher than untreated ArKO mice (ArKO versus ArKO + E, $p < 0.04$ by Tukey's posthoc analysis). *NCX1* and *TRPV6* mRNA levels in kidneys of estradiol treated ArKO mice remained significantly lower than those of the WT mice (Fig. 1).

Because estradiol acts through receptors ERα and ERβ, mRNA levels of both receptors were assessed. Normalized mRNA level for *ERα* was higher in ArKO kidney than WT, whereas normalized *ERβ* level was lower (Fig. 1). After estradiol treatment, there was no significant difference between *ERα* mRNA levels of WT and treated ArKO mice (Fig. 1). Expression of *ERβ* in ArKO kidney did not significantly change with estradiol therapy (Fig. 1).

Protein levels of the calbindins in whole kidney extracts were also assessed by Western blot analysis. PTHR1 receptor protein levels and distribution were evaluated by immunohistochemistry. At the protein level, calbindin-D_{28k} was significantly decreased in ArKO mice (Fig. 2). Estradiol therapy raised the levels to those of WT. The protein level of calbindin-D_{9k} in ArKO was not significantly different than that of WT ($p = 0.1$). Estradiol therapy raised the mean level of calbindin-D_{9k} in ArKO mice closer to the WT mean. In agreement with the mRNA data, there was no obvious difference in PTHR1 protein expression, by immunofluorescent histochemistry, between the three groups (Fig. 3). Moreover, we did not detect any difference in the cellular distribution of the protein.

Given the resolution of hypercalciuria after estradiol treatment of ArKO mice with a modest increase of PMCA1b in mRNA and no normalization of NCX1, we measured PMCA1 activity in all three experimental groups. The interaction between groups was significant (ANOVA, $p = 0.02$). Untreated ArKO mice had lower mean levels of PMCA activity than WT mice, but the difference was not significant ($p = 0.08$; Fig. 4). On the other hand, ArKO mice treated with estradiol had significantly higher PMCA activity levels compared with WT ($p = 0.04$) and untreated ArKO mice ($p = 0.01$).

Increased level of klotho protein in ArKO mice

The klotho protein has recently been shown to be a regulator of renal calcium handling through its regulation of TRPV5. Therefore, we studied its expression in the experimental groups. Immunohistochemistry was performed to examine renal klotho expression. The staining was observed in the distal convoluted tubule. Positive staining was both more diffuse and consistently more intense in ArKO kidneys (Fig. 5A). This increased expression was confirmed by real-time PCR and Western blot analysis (Figs. 5B–5D). The klotho standard used does not have the transmembrane domain and thus is smaller than the endogenous protein, accounting for the smaller apparent molecular weight on the gels. Estradiol treatment of ArKO mice decreased klotho expression in the kidney at both the mRNA and protein levels (Figs. 5B–5D).

DISCUSSION

In the mammalian kidney, 90% of calcium reabsorption occurs in the proximal tubule and cortical thick ascending limb by paracellular diffusion. The remaining 10% takes place in the distal convoluted tubule through active transcellular transport and is believed to be responsive to calcium-regulating hormones such as PTH and vitamin D. The lack of significant change in vitamin D or PTH levels in the ArKO model suggests that estrogen deficiency is the cause of the hypercalciuria in the ArKO mice. The normal level of urinary phosphorous suggests that the proximal nephron function is intact in ArKO mice. The mechanism(s) by which estrogens may regulate renal calcium reabsorption in the DCT is not completely understood, but decreased expression of proteins involved in renal calcium reabsorption may lead to increased urinary calcium. Indeed, the ArKO mice showed hypercalciuria in both spontaneous voids or
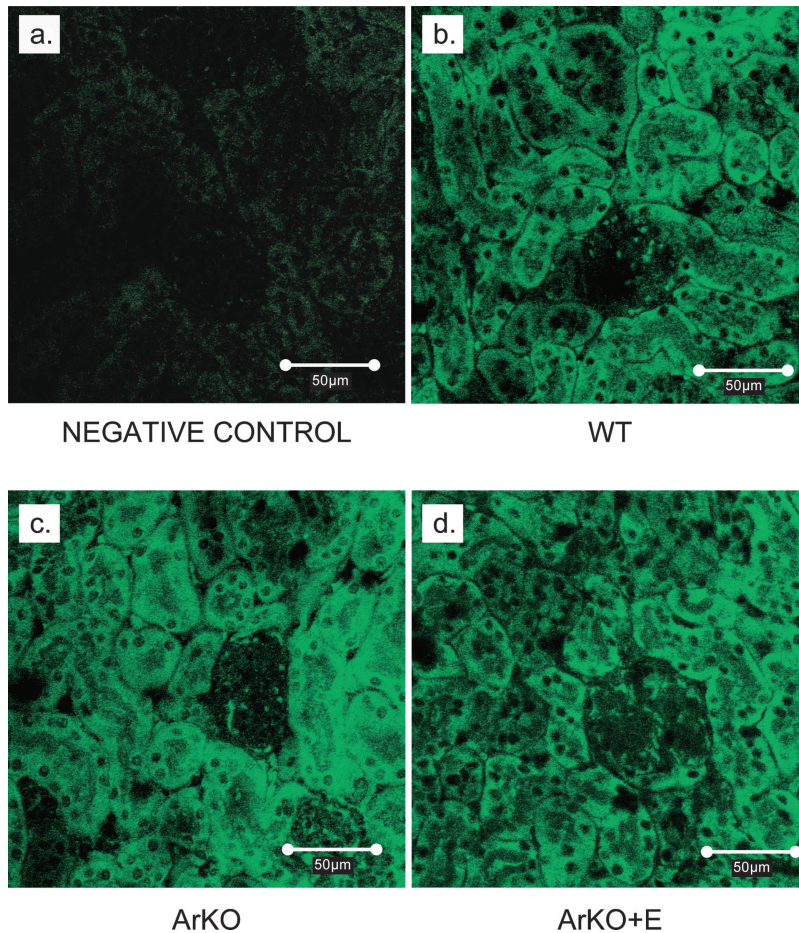
FIG. 3. Effect of aromatase deficiency and estradiol supplementation on PTHR1 receptor expression. Kidneys were fixed and bisected. Immunofluorescent histochemistry was performed using negative control (no primary antibody, panel A) or rabbit anti-PTHR1 primary (B–D) followed by detection with biotinylated goat anti-rabbit IgG and streptavidin-FITC. WT, wildtype; ArKO, aromatase-deficient mice; ArKO + E, ArKO mice treated with estradiol, 20 μg/mouse three times per week for 3 wk.

with fasting. Although we believe that the observed hypercalciuria in the ArKO female is caused principally by a renal calcium leak in the distal nephron, the current experimental design does not rule out the possibility that an increased renal filtered load of calcium could contribute to the observed hypercalciuria. On the other hand, increased intestinal calcium absorption is not expected to be contributing to an increased filtered load, because serum 1,25-dihydroxyvitamin D concentration was observed to be normal in these mice and fasting hypercalciuria was evident.

The decreased expression of TPRV5, calbindin-D_{28k}, PMCA1b, and NCX1, proteins involved in transcellular reabsorption pathway of calcium, during estrogen deficiency as shown here is consistent with a coordinated regulation. Our findings are reminiscent of the coordinated regulation of the transcellular calcium reabsorption pathway after parathyroidectomy in rats. Similar to these findings during estrogen deficiency, van Abel et al.[18] showed that parathyroidectomized rats have decreased mRNA and protein levels of TRPV5 and calbindin-D_{28k}, and decreased mRNA levels of *NCX1*. Although urine calcium was not reported in the study of van Abel et al., serum calcium levels were low, and presumably the animals were hypercalciuric. One difference between the parathyroidectomized rats and the ArKO mice is that *PMCA1b* mRNA levels were lower in ArKO mice but unchanged in the rats. The cause for this

difference between animal models is not known, but clearly, marked deficiency in either PTH or estrogens is sufficient to cause simultaneous decrease of some proteins involved in transcellular calcium transport.

TRPV5 is thought of as a gatekeeper of epithelial calcium uptake[19,20] and was upregulated at the mRNA level by estradiol treatment of the ArKO mouse. This finding is similar to those of van Abel et al., who showed that estradiol treatment of ovariectomized rats increased renal *TRPV5* mRNA and protein expression.[14] In addition, 17β-estradiol was shown to increase renal mRNA levels of *calbindin-D_{28K}* and the basolateral membrane calcium pump *PMCA1b*. However, when similar studies were performed in male 25-hydroxyvitamin D_3-1α-hydroxylase-knockout mice, only TRPV5 expression remained responsive to estrogen. Combined, the two studies suggest a coordinated regulation pattern by estradiol that is independent of vitamin D. In our study, estradiol therapy of ArKO mice also restored *TRPV5, calbindin-D_{28k},* and *PMCA1b* mRNA levels. Because ArKO mice have normal serum 1,25-dihydroxyvitamin D concentrations, this study supports the conclusion that estrogen regulation of the gatekeeper TRPV5, calbindin-D_{28k}, and PMCA1b in the kidney occurs in a coordinated fashion and can occur independently of vitamin D.

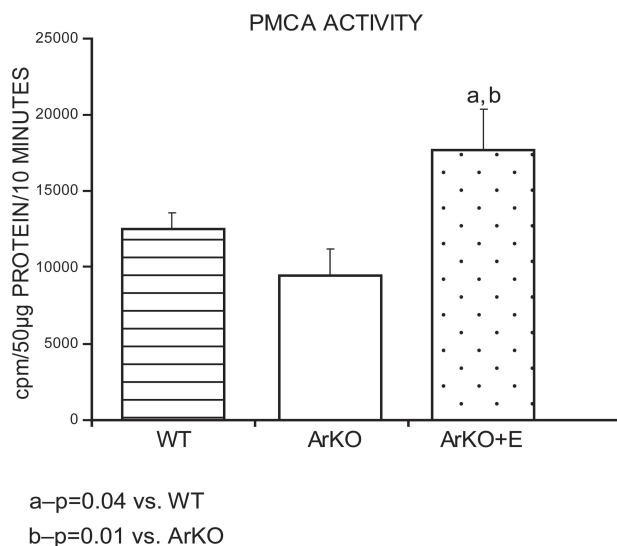The TRPV6 calcium channel is also expressed in the kid-

PMCA ACTIVITY

FIG. 4. Effect of aromatase deficiency and estradiol supplementation on PMCA activity. Membrane vesicles (50 μg of membrane protein) prepared from whole kidneys were added to a buffer containing CaCl$_2$ and inhibitors of Na-K ATPase, SERCA pumps, and mitochondrial calcium transport. ^{45}CaCl$_2$ was added at an activity of 50 μCi/mol of total Ca. The PMCA reaction was initiated by the addition of 6 mM ATP, allowed to proceed for 10 min, and terminated by filtration through 0.45-μm filters. WT, wild-type; ArKO, aromatase-deficient mice; ArKO + E, ArKO mice treated with estradiol, 20 μg/mouse three times per week for 3 wk. ^{a}ArKO + E vs. WT and ^{b}ArKO + E vs. ArKO.

ney but at much lower levels compared with TRPV5. Weber et al.[21] studied the response of renal TRPV6 in ovariectomized mice with and without estradiol treatment at doses similar to ours. Expression was higher in the ovariectomized mice. After estradiol administration, the expression level decreased to a point between that of the sham and ovariectomized groups. Unfortunately, the authors did not perform statistical analysis to determine the significance of the increase but concluded that expression of TRPV6 in the kidney is independent of estradiol levels. In contrast, we observed significantly decreased TRPV6 expression in ArKO mice that was unchanged with estradiol therapy. Taken together, the results suggest that ovarian factors other than estradiol maybe involved in regulation of renal TRPV6 and that TRPV6 is not important for normalizing transepithelial calcium transport after an estradiol challenge.

Although TRPV5 and TRPV6 are involved in calcium uptake at the apical surface, PMCA1b and NCX1 are responsible for transport of calcium into the blood at the basolateral surface. In the ArKO mice, estradiol therapy increased the mRNA levels of *PMCA1b* to a point not statistically different from WT levels, although mean levels were still lower. Moreover, this treatment increased PMCA1b activity significantly above that in WT and untreated ArKO mice. This observation is reminiscent of *in vitro* studies showing estradiol can enhance the activity of PMCA1b in a renal tubule cell line without changes in expression levels.[22] On the other hand, the same dose failed to increase NCX1 expression. These observations, together

with normalization of urine calcium level in treated ArKO mice, suggested that, whereas both NCX1 and PMCA1b are important in calcium extrusion under basal conditions, it is PMCA1b that is responsible for extruding calcium in response to an estrogen challenge. However, we can not exclude that NCX1 is an estrogen regulated protein because increased expression of NCX1 mRNA has been reported by van Abel et al.[14] after estradiol therapy at doses of 250 μg/d and higher, a dose that exceeded ours by 4-fold. Nevertheless, the observed increased enzymatic activity and modest changes in PMCA mRNA level after estradiol treatment seem sufficient for normal calcium efflux.

Recent reports of the glucuronidase klotho regulating TRPV5 activity[23] prompted us to compare the levels of klotho expression in WT and ArKO kidneys. The ArKO mice had increased levels of the klotho protein. Clues to the function of the klotho protein were originally discovered as a result of random insertional mutagenesis in mice resulting in many phenotypes seen in aging.[24] To our knowledge, this is the first report that estrogens regulate renal klotho expression. The increased expression of klotho in aromatase deficiency may be a compensatory physiological response to increased urinary calcium to compensate for decreased expression of the TRPV5 gatekeeper by prolonging its presence and activity on the surface membrane.[23] The fact that mRNA levels decreased after estradiol therapy suggests that estradiol regulates klotho expression at the transcriptional level. In addition to the results shown in this report, estradiol regulates klotho expression in the MDBK and MDCK kidney cell lines (A Hajibeigi and OK Öz, unpublished results, 2007). In contrast to our findings of suppression of klotho expression by estradiol, others have shown upregulation of klotho expression after a single injection of vitamin D.[25] Although klotho-deficient mice have been shown to have defective PTH-induced luminal calcium reabsorption,[26] to our knowledge, the regulation of klotho protein expression in the kidney by PTH in the intact mouse has not been reported. Thus, klotho regulation by calcitropic hormones needs further study but is likely to be different for each hormone.

Estrogens act through two receptors, namely ERα and ERβ. Estrogen receptors have been found in the mammalian kidney.[27] A prior study in αERKO$^{AF-1-/-}$ mice reported mRNA levels of renal calcium transport proteins were also decreased.[28] The ERα splice variant expressed by these mice lacks the AF-1 domain but retains the ability to signal through classical genomic mechanisms. To our knowledge, there are no published studies that show changes in expression of calcium transport proteins in the kidneys of ERβ knockout mice. Therefore, it seems that ERα is the mediator of estrogen action at the kidney, and regulation of the calcium reabsorption pathway by estrogens may not involve classical genomic signaling. One advantage to the ArKO model is the ability to study effects on molecular expression after estradiol treatment, which allowed us to determine the estrogen responsiveness of urinary calcium excretion and protein expression abnormalities. The facts that both ArKO and αERKO$^{AF-1-/-}$ mice show decreased levels of the same calcium transport proteins and αERKO$^{AF-1-/-}$ female mice have elevated estra-

A

WT

ArKO

B

KLOTHO PROTEIN

a–p<0.05 vs. WT
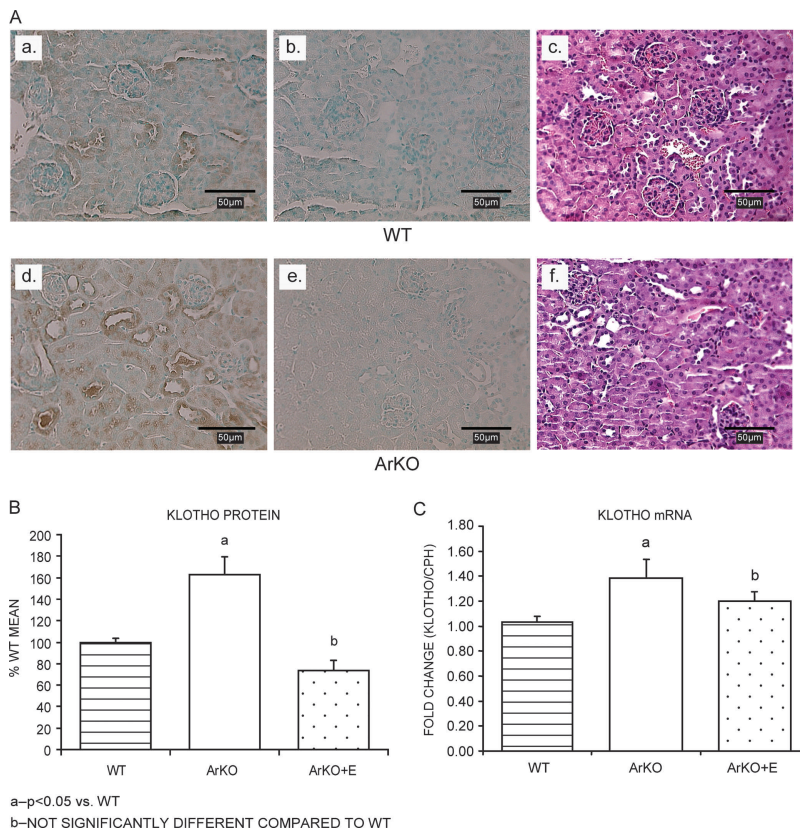b–NOT SIGNIFICANTLY DIFFERENT COMPARED TO WT

C

KLOTHO mRNA

FIG. 5. Effect of aromatase deficiency and estradiol supplementation on klotho expression. (A) Immunohistochemical analysis. Kidneys were fixed and bisected. Immunohistochemistry (a–f) was performed using a rat monoclonal anti-klotho (a and d); no primary antibody (b and e); and H&E stain of renal tissue (c and f). The magnification bar represents 50 μm. (B) Anti-klotho Western blot. Detergent lysates were prepared from whole kidneys and subjected to SDS-PAGE followed by Western blotting. The blot was probed with rat anti-klotho antibody followed by quantitative analysis of the Western blot. (C) Real-time PCR analysis of klotho expression. WT, wildtype; ArKO, aromatase-deficient mice; ArKO + E, ArKO mice treated with estradiol, 20 μg/mouse three times per week for 3 wk. [a]ArKO vs. WT and [b]ArKO + E vs. WT.

diol levels, whereas ArKO mice have undetectable estradiol levels, establish that ligand-dependent pathways acting through ERα are important regulators of renal calcium transport in vivo.

In summary, estrogen deficiency, produced by aromatase inactivation, is sufficient to cause renal calcium loss. The calcium loss is independent of changes in vitamin D and PTH. The decreased calcium reabsorption is associated with coordinated decreased expression of the genes for some renal calcium transport proteins including *TRPV5 and 6, calbindin-D$_{28k}$, NCX1,* and *PMCA1b;* however, normocalciuria after estradiol therapy is associated with a coordinated increase in expression only for TRPV5, calbindin-D$_{28k}$, and PMCA1b. This implies that, whereas TPRV6 and NCX1 maybe involved in basal calcium transport in the kidney, they are not responsible for estradiol-mediated increases in renal calcium reabsorption. Taken together with previous work by other groups, we suggest that estrogen regulates renal calcium reabsorption at the transcriptional level through ERα acting to regulate these genes in a coordinated ligand dependent fashion but the regulation may not be solely through classical estrogen response elements. In addition, estradiol may regulate the process through nontranscriptional based regulatory mechanisms such as klotho regulating activity of TRPV5 and estradiol regulation of PMCA enzymatic activity. The study findings may provide an explanation for the increased risk of nephrolithiasis and the observed increased incidence in females at the menopause. Patients with congenital aromatase deficiency or those being treated with aromatase inhibitors for

breast cancer may also be at increased risk for hypercalciuria and nephrolithiasis. The findings also suggest attention to urinary calcium levels in patients being treated with aromatase inhibitors for delaying growth plate closure. Finally, deficiency of calcitropic hormones seems to have common and unique effects on the expression of renal calcium transport proteins.[29] Understanding the molecular basis for differences in expression of calcium transport proteins after parathyroidectomy, aromatase inactivation, or in vitamin D insufficiency may be helpful in understanding the complex physiological regulation of renal calcium reabsorption in the distal convoluted tubule leading to improved health care for patients at risk for nephrolithiasis as well as bone loss.

REFERENCES

1. Churchill DN, Maloney CM, Bear J, Bryant DG, Fodor G, Gault MH 1980 Urolithiasis—a study of drinking water hardness and genetic factors. J Chronic Dis **33:**727–731.
2. Williams RE 1963 Long-term survey of 538 patients with upper urinary tract stone. Br J Urol **35:**416–437.
3. Marshall V, White RH, De Saintonge MC, Tresidder GC, Blandy JP 1975 The natural history of renal and ureteric calculi. Br J Urol **47:**117–124.
4. Johnson CM, Wilson DM, O'Fallon WM, Malek RS, Kurland LT 1979 Renal stone epidemiology: A 25-year study in Rochester, Minnesota. Kidney Int **16:**624–631.
5. Robertson WG, Longhorn SE, Whitfield HN, Unwin RJ, Mansell MA, Neild GH 1999 The changing pattern of the age of onset of urinary stone disease in the UK. In: Borghi L, Meschi T, Briganti A, Schianchi T, and Novarinini A (eds.) Proceeding of the 8th European Symposium on Urolithiasis. Editoriale Bios, Cosena, Italy, pp. 165–168.
6. Heller HJ, Sakhaee K, Moe OW, Pak CY 2002 Etiological role of estrogen status in renal stone formation. J Urol **168:**1923–1927.
7. Nordin BE, Polley KJ 1987 Metabolic consequences of the menopause. A cross-sectional, longitudinal, and intervention study on 557 normal postmenopausal women. Calcif Tissue Int **41**(Suppl 1)**:**S1–59.
8. Nordin BE, Need AG, Morris HA, Horowitz M, Robertson WG 1991 Evidence for a renal calcium leak in postmenopausal women. J Clin Endocrinol Metab **72:**401–407.
9. McKane WR, Khosla S, Burritt MF, Kao PC, Wilson DM, Ory SJ, Riggs BL 1995 Mechanism of renal calcium conservation with estrogen replacement therapy in women in early postmenopause—a clinical research center study. J Clin Endocrinol Metab **80:**3458–3464.
10. Hoenderop JG, Nilius B, Bindels RJ 2002 Molecular mechanism of active Ca2+ reabsorption in the distal nephron. Annu Rev Physiol **64:**529–549.
11. Fisher CR, Graves KH, Parlow AF, Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci USA **95:**6965–6970.
12. Hoenderop JG, Hartog A, Stuiver M, Doucet A, Willems PH, Bindels RJ 2000 Localization of the epithelial Ca(2+) channel in rabbit kidney and intestine. J Am Soc Nephrol **11:**1171–1178.
13. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G 2001 Duodenal calcium absorption in vitamin D receptor-knockout mice: Functional and molecular aspects. Proc Natl Acad Sci USA **98:**13324–13329.
14. van Abel M, Hoenderop JG, Dardenne O, St Arnaud R, Van Os CH, Van Leeuwen HJ, Bindels RJ 2002 1,25-dihydroxy-vitamin D(3)-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. J Am Soc Nephrol **13:**2102–2109.
15. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF 2006 High-throughput real-time quantitative reverse transcription PCR. In: Moore DD, Seidman JG, Smith JA, Struhl K (eds.) Current Protocols in Molecular Biology, Supplement 74, Volume 2. John Wiley & Sons, Cambridge, MA, USA, pp. 15.18.11–15.18.28.
16. Kato Y, Arakawa E, Kinoshita S, Shirai A, Furuya A, Yamano K, Nakamura K, Iida A, Anazawa H, Koh N, Iwano A, Imura A, Fujimori T, Kuro-o M, Hanai N, Takeshige K, Nabeshima Y 2000 Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. Biochem Biophys Res Commun **267:**597–602.
17. Liu BF, Xu X, Fridman R, Muallem S, Kuo TH 1996 Consequences of functional expression of the plasma membrane Ca2+ pump isoform 1a. J Biol Chem **271:**5536–5544.
18. van Abel M, Hoenderop JG, van der Kemp AW, Friedlaender MM, van Leeuwen JP, Bindels RJ 2005 Coordinated control of renal Ca(2+) transport proteins by parathyroid hormone. Kidney Int **68:**1708–1721.
19. Hoenderop JG, Nilius B, Bindels RJ 2002 ECaC: The gatekeeper of transepithelial Ca2+ transport. Biochim Biophys Acta **1600:**6–11.
20. Hoenderop JG, Nilius B, Bindels RJ 2005 Calcium absorption across epithelia. Physiol Rev **85:**373–422.
21. Weber K, Erben RG, Rump A, Adamski J 2001 Gene structure and regulation of the murine epithelial calcium channels ECaC1 and 2. Biochem Biophys Res Commun **289:**1287–1294.
22. Dick IM, Liu J, Glendenning P, Prince RL 2003 Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. Mol Cell Endocrinol **212:**11–18.
23. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG 2005 The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. Science **310:**490–493.
24. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI 1997 Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature **390:**45–51.
25. Tsujikawa H, Kurotaki Y, Fujimori T, Fukuda K, Nabeshima Y 2003 Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. Mol Endocrinol **17:**2393–2403.
26. Tsuruoka S, Nishiki K, Ioka T, Ando H, Saito Y, Kurabayashi M, Nagai R, Fujimura A 2006 Defect in parathyroid-hormone-induced luminal calcium absorption in connecting tubules of Klotho mice. Nephrol Dial Transplant **21:**2762–2767.
27. Dubey RK, Jackson EK 2001 Estrogen-induced cardiorenal protection: Potential cellular, biochemical, and molecular mechanisms. Am J Physiol Renal Physiol **280:**F365–F388.
28. Van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, Dijcks FA, Ederveen AG, Carmeliet P, Verhaeghe J, Bouillon R, Carmeliet G 2003 Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. J Bone Miner Res **18:**1725–1736.
29. Lambers TT, Bindles RJM, Hoenderop JGJ 2006 Coordinated control of renal Ca2+ handling. Kidney Int **69:**650–654.

Address reprint requests to:
Orhan K Öz, MD, PhD
Department of Radiology
UT Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, TX 75390-9153, USA
E-mail: Orhan.Oz@UTSouthwestern.edu