Characterization of vitamin D-deficient klotho−/− mice: do increased levels of serum 1,25(OH)2D3 cause disturbed calcium and phosphate homeostasis in klotho−/− mice?

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Abstract

Background. Klotho−/− mice display disturbed Ca2+ and vitamin D homeostasis. Renal cytochrome p450 27b1 (Cyp27b1), the enzyme that catalyzes the hydrolysis to 1,25-dihydroxyvitamin D3, is increased in klotho−/− mice, and a 1,25(OH)2D3-deficient diet partially normalized Ca2+ homeostasis in these klotho−/− mice. The aim of the present study was to further delineate the interplay between 1,25(OH)2D3 and klotho and their relative contribution to the Ca2+ homeostasis of klotho−/− mice.

Methods. Double-klotho−/−/Cyp27b1−/− mice were generated and mice aged 8–12 weeks were housed in metabolic cages to collect 24-h urine. Blood samples were taken and the animals were sacrificed, and the kidney and duodenum tissues were sampled for RNA extraction. The bone was fixed in 10% v/v formalin and analysed by microcomputed tomography (μCT) scans.

Results. Klotho−/−/Cyp27b1−/− mice, like Cyp27b1−/− mice, displayed significantly decreased serum total calcium concentrations compared with wild-type mice (1.44 ± 0.03 and 2.25 ± 0.02 mM) along with normal urinary total calcium excretion. Hyperphosphatemia of klotho−/− mice normalized Ca2+ homeostasis in these klotho−/− mice. The mRNA levels of duodenal transient receptor potential vanilloid subtype 5 (TRPV5), TRPV6 and calcium-binding protein-D9K (calbindin-D9K), and renal calbindin-D28K and NCX1 were significantly reduced in the double knockouts compared with wild-type or klotho−/− mice. Elevated TRPV5 protein levels in klotho−/− mice normalized to wild type in klotho−/−/Cyp27b1−/− mice, but were decreased in Cyp27b1−/− mice. μCT scans showed that klotho−/−/Cyp27b1−/− mice, as Cyp27b1−/− mice, display significant bone hypomineralization and severely decreased bone mass. Klotho−/− mice show a reduced bone mass and increased trabecular numbers.

Conclusions. Klotho−/−/Cyp27b1−/− mice resemble Cyp27b1−/− mice. Since 1,25(OH)2D3 is absent in these mice, our results imply that Ca2+ homeostasis in klotho−/− mice is affected by their excessive 1,25(OH)2D3 levels.

Keywords: Ca2+ (re)absorption; hypocalcaemia; klotho; TRPV5; vitamin D

Introduction

Kuro-o et al. [1] generated klotho−/− mice, deficient for α-klotho (referred to as klotho), which display a phenotype comparable with human ageing. These mice are characterized by a mild hypercalcaemia, hyperphosphataemia, increased levels of serum 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) and bone abnormalities, e.g. increased metaphyseal trabecular bone mass and soft tissue calcifications. The expression of the renal and intestinal calcium (Ca2+) transport proteins, transient receptor potential vanilloid subtype 5 (TRPV5), TRPV6 and calcium-binding protein-D39 (calbindin-D39), is up-regulated in these mice [1–6]. Additionally, Alexander et al. [4] demonstrated that klotho−/− mice suffer from a primary renal Ca2+ leak. Klotho was shown to specifically stimulate the epithelial Ca2+ channels TRPV5 and TRPV6 in vitro [7], and channel activity was enhanced by the hydrolysis of the sugar residues at the extracellular domain of the channels by the β-glucuronidase and sialidase activity of klotho [8, 9]. Furthermore, klotho is essential for the recruitment of Na+ K+-ATPase to the plasma membrane upon reduced extracellular free Ca2+ ([Ca2+]o). Na+ K+-ATPase activity creates an electrochemical gradient that leads to parathyroid hormone release and transepithelial Ca2+ transport [10], thus demonstrating the regulatory role of klotho in Ca2+ homeostasis. Furthermore, it was reported that 1,25(OH)2D3 induces not only 24-hydroxylase and vitamin D receptor, but also klotho gene expression in wild-type mice [2], suggesting that klotho...
participates in a feedback circuit of 1,25(OH)2D3 [2, 11]. The question remains whether the disturbed Ca2+ homeostasis in klotho−/− mice is a consequence of the deletion of klotho or due to increased levels of 1,25(OH)2D3 or interplay of both. To address this, we generated a 1,25(OH)2D3-deficient klotho−/− mice and the klotho−/−/Cyp27b1−/− mice. Recently, Ohnishi et al. [12] studied the phenotype of klotho−/−/Cyp27b1−/− mice. In their study, klotho−/−/Cyp27b1−/− mice were characterized, demonstrating that the deletion of 1,25(OH)2D3 reversed the phenotype of klotho−/− mice. Klotho−/−/Cyp27b1−/− mice are larger in size, and body weight was increased compared with klotho−/− mice. Hypercalcemia and hyperphosphatemia were normalized, and soft-tissue calcifications disappeared [12]. Therefore, we hypothesized that the expression pattern of renal and intestinal Ca2+ transport proteins might be normalized to wild-type levels, and the bone phenotype could be restored as well in the klotho−/−/Cyp27b1−/− mice, implying a major role for 1,25(OH)2D3 in the phenotype of klotho−/− mice. 1,25(OH)2D3-deficient Cyp27b1−/− mice were generated by Dardenne et al. [13] as a model for vitamin D deficiency rickets type 1. The 1,25(OH)2D3 deficiency in these mice results in hypocalcemia, hypophosphatemia, hyperparathyroidism and severe bone abnormalities including diminished cortical bone mass, cortical porosity and hypomineralization of bone [13–17]. Further characterization of these Cyp27b1−/− mice revealed the down-regulation of the renal and intestinal Ca2+ transport proteins TRPV5, TRPV6, calbindin-D9K, calbindin-D28K and Na+/Ca2+ exchanger (NCX1) [13–15, 17, 18]. The expression levels of the Ca2+ transport proteins in the kidney and duodenum of the Cyp27b1−/− mouse are opposite to the klotho−/− mouse.

In the present study, we investigated the hypothesis that 1,25(OH)2D3 is the key player responsible for the phenotype of klotho−/− mice. Therefore, Ca2+ homeostasis was studied in klotho−/−/Cyp27b1−/− mice related to single klotho−/− and Cyp27b1−/− mice. Expression levels of intestinal and renal Ca2+ transport proteins were analyzed and the bone phenotype was assessed in detail in these mouse models.

Materials and methods

Animal studies

Klotho−/− mice (purchased from Mutant Mouse Regional Resource Centers, ID:011732-UCD) and Cyp27b1−/− mice (provided by René St-Arnaud, Shriners Hospital for Children, Montreal, Quebec, Canada) were bred to generate klotho−/−/Cyp27b1−/− mice in the second (F2) generation offspring. Genotypes were determined by polymerase chain reaction (PCR) analysis, using specific primers as described previously [4, 13]. Littermates were housed in a temperature- and light-controlled room with the standard pellet chow (SSNIFF spezialdiäten GmbH) and deionized drinking water available ad libitum. Wild-type (n = 8), klotho−/− (n = 7), Cyp27b1−/− (n = 8) and klotho−/−/Cyp27b1−/− (n = 5) mice aged 8–12 weeks were housed in metabolic cages and 24-h urine was collected, except for the klotho−/− mice, since they are too small and fragile. Blood samples were taken and the animals were sacrificed, and the kidney and duodenum tissues were sampled. The bone was fixed in 10% v/v formalin for 24 h at 4°C and stored in 70% v/v ethanol at 4°C. All experiments were performed in compliance with the animal ethics board of the Radboud University Nijmegen.

Analytical procedures

Serum and urine total calcium and magnesium concentrations were determined using a colourimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, the Netherlands). Serum Na+ and K+ concentrations were measured using a flame spectrophotometer (FCA 6343, Eppendorf, Hamburg, Germany). Serum phosphate (Pi) concentrations were measured by the phosphomolybdate method with an Aeroset analyser (Abbott Diagnostics, Abbott Park, IL).

Quantitative real-time PCR analysis

Total RNA was isolated from the kidney and colon using TriZol Total RNA Isolation Reagent (Gibco BRL, Breda, the Netherlands) as described previously [19]. cDNA was subsequently used to determine mRNA expression levels of TRPV5, TRPV6, calbindin-D9K, calbindin-D28K, NCX1 and plasma membrane Ca2+-ATPase 1b (PMCA1b). The mRNA expression of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an endogenous control. The mRNA levels were quantified by TaqMan qPCR on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The sequences of primers and fluorescent probes (Biologeo, Malden, the Netherlands) are listed in van Abel et al. [19], Nijenhuis et al. [20] and Amling et al. [26].

Immunohistochemistry

Staining of the kidney sections for TRPV5 was performed on cryosections of peridate-lysine-paraformaldehyde-fixed kidney samples as previously described [21] with amplification using the tyramide signal amplification fluorescent system (Perkin Elmer, Groningen, the Netherlands). Images were semi-quantified with the Image J 1.43 software for Mac OS X (Media Cybernetics, Silver Spring, MD).

Western blot

Total kidney lysates were prepared as described [22]. Immunoblotting for calbindin-D28K and β-actin (20 μg of kidney protein lysate) was performed as described previously [23]. After exposure of the blots in the ChemiDoc XRS system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), western blots were quantified with Quantity One® software (Bio-Rad). Band intensities were expressed as the ratio of β-actin expression.

Bone structural analysis

After fixation in 10% v/v formalin, femurs from wild-type, klotho−/−, Cyp27b1−/− and klotho−/−/Cyp27b1−/− mice (n = 4) were studied in detail by scanning them in an in vivo microcomputed tomography (μCT) scanner (Skyscan 1076, Skyscan, Aartselaar, Belgium) at 40 kV and 250 μA using a 1-μm aluminum filter, a size 9 μm voxel with a frame averaging of 3. Dissimilar composition of the distal femurs from all four genotypes (Figure 4A–D) hampered consistent assessment of the metaphyseal region. Therefore, we decided to analyse the mid-diaphysis (±500 μm, indicated by dashed lines in Figure 4A) of the mice femurs in detail. Scans were processed and three-dimensional morphometric analyses of the bones were performed by software packages, including Nrecon, Dataviewer and CT-Analyser (all from http://www.skyscan.be/products/downloads.htm), to quantify the different bone parameters, which were expressed according to the bone histomorphometry nomenclature [24].

Statistical analysis

Data are expressed as the mean ± SEM. Statistical comparisons were analysed by one-way analysis of variance and Tukey’s multiple comparison post hoc test. P-values of <0.05 were considered statistically significant.
Table 1. Characteristics of the klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Klotho&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Cyp27b1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Klotho&lt;sup&gt;−/−&lt;/sup&gt;/Cyp27b1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Serum</td>
<td></td>
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<td>Total calcium (mM)</td>
<td>2.25 ± 0.02</td>
<td>2.07 ± 0.05</td>
<td>1.44 ± 0.03&lt;sup&gt;*,#&lt;/sup&gt;</td>
<td>1.40 ± 0.03&lt;sup&gt;*,#&lt;/sup&gt;</td>
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<tr>
<td>Total magnesium (mM)</td>
<td>1.57 ± 0.03</td>
<td>1.73 ± 0.05</td>
<td>1.62 ± 0.06</td>
<td>1.57 ± 0.14</td>
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<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt; (mM)</td>
<td>139 ± 1</td>
<td>137 ± 1</td>
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<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt; (mM)</td>
<td>8.6 ± 0.2</td>
<td>7.6 ± 0.3</td>
<td>8.4 ± 0.3</td>
<td>7.9 ± 0.6</td>
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<tr>
<td>Pi (mM)</td>
<td>2.5 ± 0.1</td>
<td>4.0 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.1 ± 0.2&lt;sup&gt;*,#&lt;/sup&gt;</td>
<td>2.4 ± 0.5</td>
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<tr>
<td>Urine</td>
<td></td>
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<tr>
<td>Diuresis (mL/24 h)</td>
<td>1.3 ± 0.2</td>
<td>ND</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>Total calcium (μmol/24 h)</td>
<td>4.4 ± 0.6</td>
<td>ND</td>
<td>3.2 ± 0.6</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>Total magnesium (μmol/24 h)</td>
<td>28.0 ± 3.4</td>
<td>ND</td>
<td>27.6 ± 6.4</td>
<td>13.6 ± 5.1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.4 ± 1.9</td>
<td>9.2 ± 0.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.3 ± 1.1&lt;sup&gt;*,#&lt;/sup&gt;</td>
<td>16.5 ± 1.0&lt;sup&gt;*,#&lt;/sup&gt;</td>
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Results are displayed as the mean ± SEM of wild-type (n = 8), klotho<sup>−/−</sup> (n = 7), Cyp27b1<sup>−/−</sup> (n = 8) and klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> (n = 5) mice. ND, not determined. *P < 0.05 indicates significant difference from wild-type mice. #P < 0.05 indicates significant difference from klotho<sup>−/−</sup> mice.

All analyses were performed using the SPSS Statistical Package software (Chicago, IL).

Results

Klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice displayed hypocalcemia

Klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> and Cyp27b1<sup>−/−</sup> mice showed a significant hypocalcemia, normocalciuria and magnesium and normal serum total magnesium, Na<sup>+</sup>, K<sup>+</sup>, and Pi concentrations compared with wild-type mice (Table 1). Serum total calcium, magnesium, Na<sup>+</sup> and K<sup>+</sup> levels of klotho<sup>−/−</sup> mice were not different from wild-type mice (Table 1), while serum Pi concentrations were significantly increased compared with wild types. The klotho<sup>−/−</sup> mice have a low body weight compared with the other mice strains (Table 1). They were considered too small to be placed in a metabolic cage for 24 h to prevent needless discomfort plus the risk of early dying of the klotho<sup>−/−</sup> mice. The body weight of klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> was increased to that of Cyp27b1<sup>−/−</sup> mice (Table 1). Although both were still lower compared with wild-type mice, the appearance of these mice was improved versus the klotho<sup>−/−</sup> mice. The kidney sections of klotho<sup>−/−</sup> mice show massive Ca<sup>2+</sup>-precipitates compared with wild-type mice (Figure 1). No staining could be observed in Cyp27b1<sup>−/−</sup> or klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice (Figure 1).

TRPV5 protein expression is normalized to wild-type levels in klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice

To investigate the molecular mechanism resulting in hypocalcaemia in klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice in more detail, the expression levels of Ca<sup>2+</sup> transport proteins in the kidney were examined. TRPV5 mRNA and protein expression were significantly up-regulated in klotho<sup>−/−</sup> mice compared with wild-type mice (Figure 2A–C). In Cyp27b1<sup>−/−</sup> and klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice, TRPV5 gene expression was similar to wild-type mice (Figure 2A), and significantly reduced compared with klotho<sup>−/−</sup> mice. On the protein level, TRPV5 expression was decreased in Cyp27b1<sup>−/−</sup> mice (Figure 2B and C), but normalized to wild-type levels in klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice (Figure 2B and C). Calbindin-2<sub>8K</sub> and NCX1 expression were reduced in the kidneys from klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> and Cyp27b1<sup>−/−</sup> mice compared with wild-type mice (Figure 2D–G), while in klotho<sup>−/−</sup> mice, the expression levels resembled that of wild types (Figure 2D–G).

1.25(OH)<sub>2</sub>D<sub>3</sub> deficiency defines the expression level of intestinal Ca<sup>2+</sup> transporters

Next, the expression of genes encoding proteins involved in intestinal Ca<sup>2+</sup> absorption were assessed. The mRNA expression levels of TRPV6, calbindin-D<sub>2K</sub> and PMCA1b in the duodenum from klotho<sup>−/−</sup> mice were similar to wild-type mice (Figure 3A–C). In Cyp27b1<sup>−/−</sup> mice, these genes were significantly reduced compared with both wild-type and klotho<sup>−/−</sup> mice (Figure 3A–C). Additional deletion of the klotho gene in these vitamin D-deficient mice resulted in mRNA levels similar as observed in Cyp27b1<sup>−/−</sup> mice; both TRPV6 and calbindin-D<sub>2K</sub> were significantly decreased compared with wild-type and klotho<sup>−/−</sup> mice (Figure 3A and B). In contrast, PMCA1b levels were not significantly affected in klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice (Figure 3C).

Klotho inactivation in Cyp27b1<sup>−/−</sup> mice does not restore bone abnormalities

Since TRPV5 protein expression was normalized to wild-type levels in klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice, we hypothesized that the severe bone phenotype of Cyp27b1<sup>−/−</sup> mice might also be restored. To this end, femoral X-ray images were analysed using μCT. Klotho<sup>−/−</sup> mice displayed reduced bone mass compared with wild-type mice, although the metaphysis appeared to contain more trabeculae (Figure 4A and B, open arrow). This was more severe in mice lacking Cyp27b1 or both klotho and Cyp27b1, which also suffered from epiphyseal widening (arrow heads) and hypomineralization of bones (Figure 4C and D). Femurs from both klotho<sup>−/−</sup> and Cyp27b1<sup>−/−</sup> mice as well as the klotho<sup>−/−</sup>/Cyp27b1<sup>−/−</sup> mice were
shorter compared with wild-type femurs (Figure 4I). Cortical thickness was reduced in klotho−/− mice compared with wild-type mice (Figure 4E and F), whereas cortical porosity became apparent (Figure 4F), both contributing to significantly diminished bone strength (moment of inertia, Figure 4K). These parameters were further reduced in Cyp27b1−/− and klotho−/−/Cyp27b1−/− mice [Figure 4G and H (arrows) and J and K]. In addition, klotho−/−, Cyp27b1−/− and klotho−/−/Cyp27b1−/− mice had strongly reduced cortical volume, which corresponded to reduced diaphyseal bone volume in these mice compared with wild-type animals (Table 2). There were no differences between the Cyp27b1−/− and the klotho−/−/Cyp27b1−/− mice for any of the bone parameters that were assessed.

Discussion

The present study demonstrated that increased serum 1,25(OH)2D3 levels can explain the phenotype of klotho−/− mice. Klotho−/− mice with a 1,25(OH)2D3 deficiency have a phenotype resembling Cyp27b1−/− mice. Gene expression levels of renal and intestinal Ca2+ transport proteins are similar in both mice, and the bone phenotype is indistinguishable. Furthermore, despite normalization of the renal TRPV5 protein expression in klotho−/−/Cyp27b1−/− mice compared with 1,25(OH)2D3-deficient mice, Ca2+ homeostasis is not restored in the double-knockout mice.

We report a significant hypocalcaemia in klotho−/−/Cyp27b1−/− mice similar to Cyp27b1−/− mice. In both mice, this is probably a consequence of reduced Ca2+ absorption in duodenum. Indeed, reduced mRNA levels of the intestinal Ca2+ transport proteins in klotho−/−/Cyp27b1−/− mice mimic that of Cyp27b1−/− mice [14, 17]. This suggests that impaired Ca2+ absorption, due to 1,25(OH)2D3 deficiency, is the underlying mechanism of the Ca2+ malabsorption. Moreover, in mice lacking klotho, which display increased levels of serum 1,25(OH)2D3, increased expression of TRPV6 and subsequently intestinal hyperabsorption occurs [4]. Apparently, 1,25(OH)2D3 is the major regulator of disturbed Ca2+ homeostasis in klotho−/− mice, suggesting that 1,25(OH)2D3 dominates the klotho-mediated effects. Indeed, additional ablation of klotho in 1,25(OH)2D3-deficient mice could not compensate for the reduced mRNA levels of TRPV6 and calbindin-D9K and hence the malabsorption of Ca2+ in mice lacking 1,25(OH)2D3.

Furthermore, klotho−/−/Cyp27b1−/− mice suffered from a renal Ca2+ leak, they are normocalciuric despite a hypocalcaemia. For klotho−/− mice, a renal Ca2+ leak has been described [4] recently. Therefore, the renal Ca2+ waste could be a consequence of the deletion of klotho in these double-knockout mice. However, the Cyp27b1−/− mice are also hypocalcaemic along with normocalciuria, a phenomenon that was observed in TRPV5−/−/Cyp27b1−/− mice as well [17]. Since TRPV5 function seems also affected in these mice strains, we cannot exclude that renal Ca2+ wasting in the klotho−/−/Cyp27b1−/− mice is only
Vitamin D defines the phenotype of klotho−− mice

Fig. 2. Deletion of 1,25(OH)2D3 in klotho−− mice normalizes TRPV5 protein expression to wild-type levels. Renal mRNA expression levels of TRPV5 (A), calbindin-D28K (D) and NCX1 (G) in wild-type (n = 8), klotho−− (n = 7), Cyp27b1−− (n = 8) and klotho−−/Cyp27b1−− (n = 5) mice. mRNA expression was assessed by qPCR analysis as the ratio to HPRT mRNA levels. Representative immunohistochemical images of TRPV5 staining of the kidney cortex sections of wild-type, klotho−−, Cyp27b1−− and klotho−−/Cyp27b1−− mice are shown. (B) Images were semiquantified by the computer analysis of the integrated optical density (IOD) and are expressed as the percentage of wild type (C). Representative immunoblot for calbindin-D28K expression in the kidney lysates of wild-type, klotho−−, Cyp27b1−− and klotho−−/Cyp27b1−− mice are shown. (E) Band intensities were quantified, normalized for β-actin and are expressed as the percentage of wild-type mice (F). Data are presented as the mean ± SEM. *P < 0.05 indicates significant difference from wild-type mice. #P < 0.05 indicates significant difference from klotho−− mice. $P < 0.05 indicates significant difference from Cyp27b1−− mice.
caused by the absence of klotho. Disturbances in TRPV5 function or even a combination of both TRPV5 and klotho might contribute to the renal Ca\(^{2+}\) leak. However, it is evident that it is not the result of 1,25(OH)\(_{2}\)D\(_3\) deficiency, because the various mice models displayed both hypo- and hypervitaminosis D.

In the absence of 1,25(OH)\(_{2}\)D\(_3\), the body weight of klotho\(^{-/-}\) mice was substantially increased, serum Pi levels were normalized and renal calcifications disappeared. The abrogation of enhanced levels of serum 1,25(OH)\(_{2}\)D\(_3\)-mediated disturbances in klotho\(^{-/-}\) mice significantly improved their appearance, and in the same line their body weight is increased to some extent. Nevertheless, the body weight of these klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice resembled that of Cyp27b1\(^{-/-}\) mice and was not fully recovered to wild-type levels, which confirms that these mice still suffer from physiological defects. Likewise, Ohnishi et al. [12] reported a partial restoration of body weight in klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice. Interestingly, klotho\(^{-/-}\) mice on a diet low in Ca\(^{2+}\), phosphorous and deficient for 1,25(OH)\(_{2}\)D\(_3\) showed body weights identical to wild-type mice on either this diet or regular food [4]. However, serum 1,25(OH)\(_{2}\)D\(_3\) levels normalized to wild-type levels in klotho\(^{-/-}\) mice on the 1,25(OH)\(_{2}\)D\(_3\)-deficient diet [4], and hence 1,25(OH)\(_{2}\)D\(_3\) is not completely absent as in the present study. This suggests that the lack of 1,25(OH)\(_{2}\)D\(_3\) is responsible for the reduced body weight of klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice compared with wild-type mice.

In klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice, TRPV5 protein was normalized to wild-type levels compared with klotho\(^{-/-}\) and Cyp27b1\(^{-/-}\) mice, which showed elevated and reduced TRPV5 expression, respectively. Since it was previously shown that TRPV5 is not retained at the plasma membrane in the absence of klotho [4, 8, 9], this restoration is probably functionally insignificant. This notion is further supported by the reduced expression of calbindin-D\(_{28K}\) and NCX1 in klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice. The expression of these renal Ca\(^{2+}\) transport proteins is known to be down-regulated as a consequence of impaired Ca\(^{2+}\) reabsorption via TRPV5 [25]. This is reflected in the overall observation that Ca\(^{2+}\) homeostasis is not restored in these klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) mice.

The bone phenotype of mice lacking both klotho and 1,25(OH)\(_{2}\)D\(_3\) is identical to mice in which only 1,25(OH)\(_{2}\)D\(_3\) is absent. Klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\), like Cyp27b1\(^{-/-}\), mice [13–15, 17] suffer from severe bone abnormalities including reduced cortical bone mass and porosity and decreased bone mineralization. Moreover, 1,25(OH)\(_{2}\)D\(_3\) deficiency leads to disrupted chondrocyte differentiation and subsequent removal by metaphyseal chondroclasts, resulting in epiphyseal widening and stunted bone growth [16, 17, 26]. Thus, the femurs of klotho\(^{-/-}\)/Cyp27b1\(^{-/-}\) and Cyp27b1\(^{-/-}\) mice are indistinguishable. Apparently, the presence of klotho is insignificant for the bone phenotype of 1,25(OH)\(_{2}\)D\(_3\)-deficient mice. On the contrary, the bone appearance in klotho\(^{-/-}\) mice, e.g. increased metaphyseal trabecular bone mass [5, 6], is altered by additional ablation of Cyp27b1 to the phenotype of mice lacking 1,25(OH)\(_{2}\)D\(_3\). This finding, together with other studies [27], implies that high 1,25(OH)\(_{2}\)D\(_3\) levels in klotho\(^{-/-}\) mice are instrumental in the
perturbation of the bone development. Thus, our studies indicate that the deficiency of 1,25(OH)2D3 rather than the absence of klotho determines the bone phenotype of the klotho−/−/Cyp27b1−/− mice.

The klotho−/−/Cyp27b1−/− mice phenotypically resembled the Cyp27b1−/− mice, which is partially in line with the observations of Ohnishi et al. [12]. Compared with our study and others [13–17], they did not report a hypocalcaemia in Cyp27b1−/− or klotho−/−/Cyp27b1−/− mice. This suggests that there might be some differences between the two studies. The genetic background of the knockout animals is identical in both studies, but the age

![Image of bone phenotype](https://ndt.oxfordjournals.org/)

**Fig. 4.** The severely disturbed bone phenotype of 1,25(OH)2D3-deficient mice is independent of klotho. Representative sagittal X-ray images of whole femurs (A–D) as well as mid-diaphyseal cross sections (E–H) from wild-type (A and E), klotho−/− (B and F), Cyp27b1−/− (C and G) and klotho−/−/Cyp27b1−/− mice (D and H, n = 4 for each genotype). Femur length (I), cortical thickness (J) and moment of inertia (K) were measured in these mice. The area between the dashed lines comprised the selected area of interest for μCT analyses (A). Many trabeculae (B, open arrow) were observed in the metaphysis of klotho−/− mice compared with wild-type mice. Epiphyseal widening in both mice models lacking Cyp27b1 is indicated by arrowheads (C and D). Arrows indicate affected cortices in all three knockout mouse models (F–H). Data are presented as the mean ± SEM. *P < 0.05 indicates significant difference from wild-type mice, #P < 0.05 indicates significant difference from klotho−/− mice.

**Table 2.** μCT analysis of femoral cortices from mice lacking either or both klotho and Cyp27b1

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Klotho−/−</th>
<th>Cyp27b1−/−</th>
<th>Klotho−/−/Cyp27b1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur length (mm)</td>
<td>16.1 ± 0.3</td>
<td>12.1 ± 0.2*</td>
<td>13.3 ± 0.3*</td>
<td>13.5 ± 0.1*#</td>
</tr>
<tr>
<td>Ct.Th (μm)</td>
<td>206 ± 12</td>
<td>121 ± 6*</td>
<td>60 ± 2*</td>
<td>59 ± 4*</td>
</tr>
<tr>
<td>Ct.V (mm³)</td>
<td>0.52 ± 0.05</td>
<td>0.27 ± 0.02*</td>
<td>0.10 ± 0.01*</td>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td>Ec.V (mm³)</td>
<td>0.40 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>0.60 ± 0.03*</td>
<td>0.59 ± 0.01*</td>
</tr>
<tr>
<td>Dp.V (mm³)</td>
<td>0.93 ± 0.08</td>
<td>0.67 ± 0.03*</td>
<td>0.71 ± 0.03*</td>
<td>0.69 ± 0.01*</td>
</tr>
<tr>
<td>MOI (mm⁴)</td>
<td>0.42 ± 0.08</td>
<td>0.24 ± 0.02*</td>
<td>0.11 ± 0.02*</td>
<td>0.10 ± 0.02*</td>
</tr>
</tbody>
</table>

Femoral diaphyses from wild-type, klotho−/−, Cyp27b1−/− and klotho−/−/Cyp27b1−/− (n = 4) mice were analyzed in detail by μCT. Parameters measured included cortical thickness (Ct.Th), moment of inertia (MOI), cortical volume (Ct.V), endocortical volume (Ec.V) and diaphyseal volume (Dp.V). Data are presented as the average ± SEM.

*P < 0.05 indicates significant difference from wild-type mice.

#P < 0.05 indicates significant difference from klotho−/− mice.
of the mice at the time of the experiment might explain the differences. We investigated mice aged 8–12 weeks, while Ohnishi et al. [12] analysed 3- and 6-week-old mice. Serum Ca\(^{2+}\) levels slightly increased for wild-type and klotho\(^{-/-}\) mice in this time span of 3 weeks [12]. Hence, additional ageing for 4–6 weeks could further raise serum Ca\(^{2+}\) concentrations in these mice and subsequently result in a hypocalcaemia in Cyp2b1\(^{-/-}\) and klotho\(^{-/-}\)/Cyp2b1\(^{-/-}\) animals as described in our study. In addition, different techniques were used to sample blood from the mice, and the dietary origin and contents might vary.

In conclusion, this study provides detailed insight into the role of klotho and 1,25(OH)\(_2\)D\(_3\) in the Ca\(^{2+}\) disturbances of klotho\(^{-/-}\) mice. We describe a phenotype for klotho\(^{-/-}\)/Cyp2b1\(^{-/-}\) mice almost indistinguishable from Cyp2b1\(^{-/-}\) mice. Hence, our study suggests that 1,25(OH)\(_2\)D\(_3\) is the major contributor to the phenotype of klotho\(^{-/-}\) mice. Furthermore, our data imply that klotho has a minor role in Ca\(^{2+}\) homeostasis in 1,25(OH)\(_2\)D\(_3\)-deficient animals.

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Conflict of interest statement. None declared.

References


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