Defective female reproductive function in 1,25(OH)2D-deficient mice results from indirect effect mediated by extracellular calcium and/or phosphorus

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1Laboratory of Reproductive Medicine, Research Center for Bone and Stem Cells and 2Department of Gerontology, First Affiliated Hospital, Nanjing Medical University, Nanjing, People’s Republic of China; and 3Departments of Medicine and Physiology, McGill University, Montreal, Quebec, Canada

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Sun W, Xie H, Ji J, Zhou X, Goltzman D, Miao D. Defective female reproductive function in 1,25(OH)2D-deficient mice results from indirect effect mediated by extracellular calcium and/or phosphorus. Am J Physiol Endocrinol Metab 299: E928–E935, 2010. First published August 31, 2010; doi:10.1152/ajpendo.00378.2010.—We used mice with targeted deletion of 25-hydroxyvitamin D 1α-hydroxylase [1α(OH)ase−/−] to investigate the effects of calcium and phosphorus on defects in the reproductive system of 1,25-dihydroxyvitamin D [1,25(OH)2D]-deficient female mice. The 1α(OH)ase−/− mice and their wild-type littermates were fed either a normal diet or a rescue diet (high calcium, phosphate, and lactose) starting from weaning until 3 mo of age. We then determined serum calcium and phosphorus levels, assessed gonadotropin and gonadal hormone production, and evaluated folliculogenesis, corpus luteum formation, ovarian angiogenesis, uterus development, and fertility. Results showed that hypocalcemic and hypophosphatemic female 1α(OH)ase−/− mice developed infertility accompanied by decreased estrogen and progestogen levels, elevated follicle-stimulating hormone and luteinizing hormone levels, defects in follicular development and corpus luteum formation, uterine hypoplasia, and decreased ovarian expression of angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-1 and -2, and Tie-2. When serum calcium and phosphorus were normalized by the rescue diet, the defective reproductive phenotype in the female 1α(OH)ase−/− mice, including the dysfunction in the hypothalamic-pituitary-ovarian axis, and ovarian angiogenesis were reversed. These results indicate that the infertility seen in 1,25(OH)2D-deficient mice is not a direct effect of active vitamin D deficiency on the reproductive system but is an indirect effect mediated by extracellular calcium and phosphorus.

vitamin D; infertility; folliculogenesis; corpus luteum formation; ovarian angiogenesis

VITAMIN D IS A PROHORMONE that can be metabolically converted from 25-hydroxyvitamin D by the enzyme 1α-hydroxylase [1α(OH)ase] to the active form of the vitamin, 1,25-dihydroxyvitamin D [1,25(OH)2D] (2, 4). It is well known that the vitamin D endocrine system plays an essential role in calcium homeostasis and bone metabolism. However, research in the past two decades has also revealed other biological actions of vitamin D, including cell differentiation, inhibition of cell growth, immunomodulation, and control of hormonal systems (5). Most of the biological activities of 1,25(OH)2D require the vitamin D receptor (VDR), a high-affinity nuclear receptor that alters the transcription rate of target genes (10). Nongenomic effects of 1,25(OH)2D, however, have also been reported (24, 26).

To investigate the effects of 1,25(OH)2D and its molecular basis, we (18) and others (3) have previously generated a mouse model deficient in 1,25(OH)2D by targeted ablation of Cyp27b1, the gene encoding 1α(OH)ase. The mutant [1α(OH)ase−/−] mice developed hypocalcemia, hypophosphatemia, hyperparathyroidism, retarded growth, and the skeletal abnormalities characteristic of rickets when they were fed a diet of regular mouse chow after weaning. Other laboratories have reported mouse models with targeted ablation of the VDR gene (6, 15, 29). These VDR−/− mice developed manifestations similar to those of 1α(OH)ase−/− mice, although VDR−/− mice also display alopecia. To investigate whether deficiencies of 1,25(OH)2D and the VDR produce the same alterations in skeletal and calcium homeostasis and whether calcium could subserve the skeletal functions of 1,25(OH)2D and the VDR (17), we previously compared the phenotypes of 1α(OH)ase−/−, VDR−/−, and double mutants. These animals were fed either a normal diet containing 1% calcium and 0.67% phosphorus, on which the mice were hypocalcemic and hypophosphatemic, or a “rescue” diet containing 2% calcium, 1.25% phosphorus, and 20% lactose. Lactose in this high-calcium, high-phosphorus rescue diet facilitated mineral absorption and normalization of serum calcium and phosphorus (17). Normalization of serum calcium and phosphorus led to phenotypic changes in the parathyroid glands and skeleton that disclosed selective and overlapping modulation by these minerals and the vitamin D system of parathyroidal and skeletal function. In contrast, other studies using VDR−/− mice (14) and 1α(OH)ase−/− mice (30) have revealed that in the cardiovascular system the 1,25(OH)2D/VDR system, independent of ambient serum calcium and phosphorus levels, provides protection against the resulting hypertension by repressing the renin-angiotensin system.

Early studies reported that female vitamin D-deficient rats could give birth to litters that were slightly smaller than control litters from females maintained on a vitamin D-containing diet but concluded that vitamin D and its metabolites are not necessary for reproduction and fetal development in the rat (9). The VDR has, however, been detected in situ in the nuclei of both female reproductive tissues such as uterus, oviduct, ovary, mammary gland, and placenta and the pituitary gland and hypothalamus (25) and in vitro in a hamster ovarian cell line, suggesting a role for 1,25(OH)2D in female reproduction (27). When VDR−/− mice were used to investigate vitamin D function in female reproduction, the female VDR−/− mice were infertile and displayed uterine hypoplasia and impaired folliculogenesis, in addition to hypocalcemic rickets. With supplementation with estrogen, uterine weight was increased in the mice, suggesting that estrogen responsiveness was present.
but that the ovarian production of estrogen was impaired (29). Although one study in VDR-null mice reported that vitamin D is essential for full gonadal function in both sexes (12), another showed that the fertility of female VDR−/− mice fully recovered when serum calcium and phosphorus levels were kept in a normal range by dietary supplementation (11). Previous studies in our lab (18) also found that 1α(OH)ase−/− female mice were infertile and exhibited uterine hypoplasia and absence of corpora lutea. Therefore, although the regulatory function of 1,25(OH)2D is implicated in the female reproductive system, it remained to be further investigated whether the female infertility in 1,25(OH)2D-deficient mice is a direct effect of the 1,25(OH)2D deficiency or is secondary to hypocalcemia and hypophosphatemia. In the present study, we characterized functions of the female reproductive system in 1α(OH)ase−/− female mice. 1α(OH)ase−/− female mice and their wild-type female littermates were fed either a normal diet or a rescue diet from weaning and then mated with wild-type male mice on the same diet for 3 mo. The fertility efficiency of females was analyzed, gonadotropins and gonadal hormones were measured, and folliculogenesis, corpus luteum formation, ovarian angiogenesis, and uterus development were examined by histopathological and molecular techniques.

**MATERIALS AND METHODS**

*Animals and treatments.* Generation and characterization of 1α(OH)ase−/− mice were performed as previously described (18). 1α(OH)ase−/− mice were generated through breeding of heterozygous mice. The genotype of the mice was confirmed by PCR using mouse tail samples. Wild-type littersmates were used as control animals in all experiments. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Ten pairs of age- and sex-matched 1α(OH)ase−/− and wild-type littermates were randomly divided into two groups. After weaning they were fed with a normal diet containing 1% calcium and 0.67% phosphorus or a rescue diet (TD96348 Teklad, Madison, WI) containing 2% calcium, 1.25% phosphorus, and 20% lactose for 3 mo.

*Estrus cycle analysis and fertility assessment.* Estrus cycles were monitored by daily vaginal smears in 2-mo-old female mutant and wild-type mice over a period of 1 mo. A small drop of sterile saline was flushed into the vagina and then dried on a slide. Cells were fixed in methanol for 40 s, stained with 0.8% eosin for 40 s and 0.2% azur B for another 40 s, and rinsed to remove excess stain. Smears were taken daily between 9 and 10 AM and evaluated by two experienced observers blinded to the genotype of the mice. To assess the fertility of female mice, virgin females were housed continuously with wild-type males and numbers of litters and pups were counted after 3 mo.

*Measurements of serum calcium and phosphorus, gonadotropins, and gonadal hormones.* Serum calcium and phosphorus levels were analyzed by autoanalyzer (Beckman Synchron 67; Beckman Instruments), Serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured by enzyme-linked immunosorbent assays (Amersham Pharmacia Biotech, Aylesbury, UK). Serum estrogen and progesterone levels were measured by radioimmunoassay (Diagnostic Products, Los Angeles, CA).

*Quantification of ovarian follicles.* Quantification of ovarian follicles was performed as previously reported (20). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. For counting of follicle numbers, paraffin-embedded ovaries were serially sectioned at 5-μm thickness and stained with hematoxylin and eosin for observation. Ovarian follicles at different developmental stages were counted in all sections of an ovary judged by careful morphological analysis. The incidence of counting the same follicle twice or missing a follicle was low.

*Histology and electron microscopy.* Paraffin sections (5 μm) of uteri and ovaries from 1α(OH)ase−/− and wild-type mice were stained with hematoxylin and eosin. The ultrastructure of granulosa cells was examined by transmission electron microscopy.

*Immunohistochemistry.* Immunohistochemical staining was carried out for vascular endothelial growth factor (VEGF) with the avidin-biotin-peroxidase complex technique with affinity-purified rat anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, dried and rehydrated paraffin-embedded sections were incubated with methanol-hydrogen peroxide (1:10) to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). The slides were then incubated with the primary antibodies overnight at room temperature. After being rinsed with Tris-buffered saline for 15 min, tissues were incubated with secondary antibody for 45 min. Sections were then washed and incubated with the Vectastain Elite ABC reagent (Vector Laboratories, Burlington, ON, Canada) for 45 min. Staining was developed with 3.3’-diaminobenzidine (2.5 mg/ml), followed by counterstaining with Mayer’s hematoxylin.

*Superovulation.* Superovulation was induced in immature (22–23 days old) mice on a normal diet by intraperitoneal injections of 5 U of pregnant mare’s serum gonadotropin (Folligon, Intervet, Boxmeer, The Netherlands), followed by injection of 5 U of human chorionic gonadotropin (Chorulon, Intervet) 48 h later (8). Oviducts were collected from mice that underwent superovulation. Once recovered from the oviduct, the cumulus oocytes were observed under a binocular microscope (Leica Wild M650). The number of oocytes collected was counted.

*Western blot analysis.* Proteins were extracted from ovaries and quantified with a protein assay kit (Bio-Rad, Mississauga, ON, Canada). Thirty-microgram protein samples were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out as described elsewhere (28) with antibodies against VEGF, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), the angiopoietin receptor Tie-2, and β-tubulin (Santa Cruz). Bands were visualized with ECL chemiluminescence (Amersham) and analyzed by Scion Image Beta 4.02 (Scion, National Institutes of Health).

*RNA isolation and real-time RT-PCR.* RNA was isolated from mouse ovaries with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription reactions were performed with the SuperScript First-Strand Synthesis System (Invitrogen) as previously described (28). To determine the relative expression of genes of interest, real-time PCR was carried out using the appropriate primers and gene-specific controls.

### Table 1. Effects of 1,25(OH)2D deficiency and dietary minerals on female fertility

<table>
<thead>
<tr>
<th></th>
<th>1α(OH)ase−/− Mice</th>
<th>Wild-Type Mice</th>
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<tbody>
<tr>
<td>n</td>
<td>Fertility, %</td>
<td>Survival of pups, %</td>
</tr>
<tr>
<td>ND</td>
<td>9</td>
<td>11 ± 0.34*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>97.7 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>97.82 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>97 ± 1.45†</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>98 ± 1.26</td>
</tr>
</tbody>
</table>

Data are means ± SD. 1,25(OH)2D, 1,25-dihydroxyvitamin D; 1α(OH)ase, 1α-hydroxylase; ND, normal diet; RD, rescue diet. *Different from wild-type littermates, P < 0.001; †different from genotype-matched mice on ND, P < 0.001.
RESULTS

Effects of 1,25(OH)2D deficiency and dietary minerals on female fertility. When maintained on a normal diet, 1α(OH)ase−/− female mice displayed a fertility rate of 11%, whereas their wild-type littermates were 97% fertile. Moreover, pups delivered by 1α(OH)ase−/− female mice died soon after birth. In contrast, 99.7% of pups delivered by the wild-type mice survived (Table 1). 1α(OH)ase−/− female mice and wild-type female mice were almost equally fertile, however, when fed the rescue diet (97% and 99% fertile, respectively), and almost all pups delivered by both 1α(OH)ase−/− female mice and wild-type female mice survived (98.5% and 99.8%, respectively) (Table 1).

Effect of 1,25(OH)2D deficiency and dietary minerals on follicular development, corpus luteum formation, and uterus development. We next examined the morphology of ovaries and uterus. The numbers of ovarian follicles at different developmental stages and corpora lutea were counted in 1-, 3-, and 15-wk-old wild-type and 1α(OH)ase−/− female mice. No apparent morphological differences were observed in ovaries and uterus between 1α(OH)ase−/− and wild-type pups at 1 wk of age (Fig. 1, A and B). Ovaries of both genotypes exhibited comparable numbers of mostly primordial follicles containing small oocytes surrounded by flattened pregranulosa cells and some primary follicles containing enlarged oocytes (Fig. 1, A and B, Table 2). At 3 wk of age, mostly primordial follicles and some primary and secondary follicles were seen in ovaries from both genotypes (Fig. 1C). Although the numbers of primordial follicles and primary follicles were not significantly different, the numbers of secondary follicles were reduced significantly in 1α(OH)ase−/− mice compared with their wild-type littermates (Table 2). No corpora lutea were observed in ovaries from either genotype at this age. The endometrium was hypoplastic, and uterine glands were diminished in the 3-wk-old 1α(OH)ase−/− mice compared with their wild-type littermates (Fig. 1D). At 15 wk of age, female 1α(OH)ase−/− mice showed obvious abnormalities in follicle development and corpus luteum formation (Fig. 1E) and uterine hypoplasia (Fig. 1F). The number of primordial follicles was lower in ovaries from 15-wk-old mice relative to 3-wk-old mice and was not altered significantly in the mutant mice. However, the numbers of primary follicles, secondary follicles, and mature follicles and the number of corpora lutea were decreased significantly in ovaries from 15-wk-old 1α(OH)ase−/− mice compared with their wild-type littermates (Table 2). On the rescue diet, compared with genotype-matched female mice on the normal diet, the morphology of ovaries (Fig. 1G) and uterus (Fig. 1F) and the numbers of ovarian follicles at different developmental

![Fig. 1](http://ajpendo.physiology.org/)

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in an Applied Biosystems Cycler with a SYBR Green PCR reagent kit. The PCR primers used were the same as previously reported (23). GAPDH was used as the internal control for each reaction. All primers were tested for their specificity by conventional RT-PCR before being used for real-time RT-PCR quantitative analysis. A melting curve was used to identify a temperature at which only the amplicon, and not primer dimers, accounted for the SYBR Green-bound fluorescence. Results were analyzed with SDS 7500 software, and the relative amount of mRNA was normalized to GAPDH mRNA.

Statistical analysis. Data from image analysis are presented as means ± SD. Statistical comparisons were made by two-way ANOVA, with P < 0.05 being considered significant.
stages and corpora lutea were all normal in 15-wk-old 1α(OH)ase−/− mice fed the rescue diet (Table 2).

Effect of 1,25(OH)2D deficiency and dietary minerals on function of hypothalamic-pituitary-ovarian axis. To determine the abnormalities of the hypothalamic-pituitary-ovarian axis (19) associated with infertility of 1α(OH)ase−/− female mice and how the reduction of infertility by the rescue diet related to this axis, serum calcium, phosphorus, estrogen, progestogen, FSH, and LH were measured. Our results showed that serum estrogen and progestogen levels were reduced, while FSH and LH levels were increased, in the hypocalcemic and hypophosphatemic 1α(OH)ase−/− female mice on the normal diet (Fig. 2). However, in 1α(OH)ase−/− female mice fed a rescue diet, which normalized serum calcium and phosphorus, both pituitary and ovarian hormones returned to normal (Fig. 2).

We also examined the estrus cycle by vaginal smears in 2-mo-old wild-type and 1α(OH)ase−/− mice fed either the normal diet or the rescue diet for 1 mo, and superovulation experiments were performed. Estrus cycles were irregular and longer in female 1α(OH)ase−/− mice fed the normal diet than in their wild-type littermates on the same diet. They exhibited a decreased time of proestrus, estrus, and metestrus periods and a prolonged diestrus, and these abnormalities were normalized in female 1α(OH)ase−/− mice fed the rescue diet (Table 3). Results from superovulation experiments showed that 24- to 25-day-old 1α(OH)ase−/− mice on the normal diet had impaired formation of the corpus luteum (Fig. 2G), an abnormal endometrium (Fig. 2H), reduced levels of estrogen and progesterone (Fig. 2, J and K), and a decreased number of ovulated oocytes (Fig. 2L). The ultrastructure of granulosa cells from both genotypes was examined after superovulation. Fewer lipid droplets, which correlate with estrogen biosynthesis, were seen in granulosa cells from 1α(OH)ase−/− mice on the normal diet than in granulosa cells from their wild-type littermates (Fig. 2H). Thus the irregular estrus cycles and insufficient response to superovulation confirmed impaired function of the hypothalamic-hypophysial-ovarian axis in 1α(OH)ase−/− mice on a normal diet.

Effect of 1,25(OH)2D deficiency and dietary minerals on ovarian angiogenesis. The cyclical growth of blood vessels associated with the development of the ovarian corpora lutea is essential for ovarian function and for maintaining fertility (1, 21, 22). We therefore examined the expression of angiogenic factors at metestrus periods. On the normal diet, in ovaries of 1α(OH)ase−/− mice compared with wild-type littermates, VEGF-immunopositive cells were reduced in corpora lutea (Fig. 3, A and B) and expression of VEGF, Ang-1, Ang-2, and Tie-2 at both protein and mRNA levels were significantly downregulated (Fig. 3, C–K). In contrast, when 1α(OH)ase−/− mice were fed the rescue diet, VEGF-immunopositive cells in the corpora lutea and the protein and mRNA expression of VEGF, Ang-1, and Tie-2 were all increased to levels seen in wild-type mice on the normal diet. However, on the rescue diet, these parameters were significantly enhanced in wild-type mice and were higher than in 1α(OH)ase−/− mice on the rescue diet (Fig. 3). The protein and mRNA expression levels for Ang-2 were not enhanced in wild-type mice fed the rescue diet, but they were significantly upregulated in 1α(OH)ase−/− mice on the rescue diet compared with those on the normal diet, although not to levels seen in wild-type mice on the normal diet (Fig. 3, C, F, and J). These results indicated that normal ambient calcium and phosphate were required for ovarian angiogenesis, but 1,25(OH)2D was also needed for optimal ovarian angiogenesis.

DISCUSSION

In the present study, we employed a combination of genetic and dietary manipulations to assess the effects of 1,25(OH)2D deficiency versus calcium and phosphorus deficiency on murine female reproductive capacity. We found that the absence of 1,25(OH)2D resulted in uterine hypoplasia and female infertility that could be corrected by normalizing serum calcium and phosphorus alone. These results are consistent with previous studies that reported that calcium and phosphorus normalization in vitamin D-deficient rats also corrects infertility (13). Furthermore, reproductive phenotypes in 1α(OH)ase−/− female mice, which include infertility and uterine hypoplasia, are similar to those in VDR−/− female mice (29), and infertility in VDR−/− female mice has also been shown in some studies (11) to be normalized by a rescue diet or by a high-calcium diet alone, suggesting that ambient calcium per se might be the active moiety.

We also explored possible mechanisms of 1,25(OH)2D and mineral action on the female reproductive system that might underlie the infertility. We found that 1,25(OH)2D-deficient female mice that developed hypocalcemia and hypophosphatemia displayed multiple disorders. Thus follicle develop-
ment and corpus luteum formation were impaired, estrus cycles were irregular and longer, and responses to a superovulation challenge were insufficient. Estrogen and progestogen levels were reduced significantly, and although FSH and LH levels were elevated in infertile 1α(OH)ase−/− female mice the ovaries were not responsive to the elevated gonadotropins. The defects were therefore in large part related to altered hypothalamic-pituitary-ovarian axis function. These results are consistent with previous studies that reported that VDR−/− female mice have reduced expression of the Cyp19 gene encoding aromatase cytochrome P-450 and reduced activity of aromatase cytochrome P-450, therefore impeding their capacity...
for estrogen biosynthesis in reproductive tissues. Nevertheless, in our studies complete normalization of defects in ovarian folliculogenesis and corpus luteum development, of low estrogen and progestogen levels, and of elevated pituitary hormones were achieved by correction of the low ambient calcium and phosphorus in these mice, even in the continued absence of 1,25(OH)2D. In contrast, in the VDR−/− model hypergonadotropic hypogonadism was only partly corrected by increasing calcium levels to near normal with a rescue diet (12), suggesting that 1,25(OH)2D is essential for full gonadal function.

### Table 3. Effects of 1,25(OH)2D deficiency and dietary minerals on estrus cycle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>1α(OH)ase−/− (n = 7)</th>
<th>RD</th>
<th>1α(OH)ase−/− (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle length, days</td>
<td>4.7 ± 0.8</td>
<td>8.6 ± 0.3*</td>
<td>4.9 ± 0.7</td>
<td>4.9 ± 0.9‡</td>
</tr>
<tr>
<td>Cycles in 30 days</td>
<td>4.3 ± 0.4</td>
<td>2.1 ± 0.3*</td>
<td>4.1 ± 0.8</td>
<td>4.1 ± 0.6‡</td>
</tr>
<tr>
<td>Percent time</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diestrus</td>
<td>10 ± 2</td>
<td>63 ± 8†</td>
<td>9 ± 9</td>
<td>9 ± 7§</td>
</tr>
<tr>
<td>Proestrus</td>
<td>40 ± 5</td>
<td>15 ± 9†</td>
<td>40 ± 8</td>
<td>40 ± 9§</td>
</tr>
<tr>
<td>Estrus</td>
<td>17 ± 5</td>
<td>7 ± 2*</td>
<td>18 ± 9</td>
<td>18 ± 3‡</td>
</tr>
<tr>
<td>Metestrus</td>
<td>32 ± 7</td>
<td>15 ± 10*</td>
<td>32 ± 2</td>
<td>32 ± 7‡</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. Vaginal smears of virgin mice aged 2 mo were checked daily for 30 days. Different from wild-type littermates: *P < 0.01, †P < 0.001. Different from genotype-matched mice on ND: ‡P < 0.01, §P < 0.001.

### Fig. 3. Effects of 1,25(OH)2D3 deficiency and dietary calcium on ovarian angiogenesis.

A: representative micrographs of paraffin-embedded sections of ovaries from 15-wk-old WT and 1α(OH)ase−/− mice fed with either ND or RD stained immunohistochemically for vascular endothelial growth factor (VEGF). Scale bars, 25 µm. B: VEGF-immunopositive area as % of the field. C: representative Western blots of ovarian extracts for expression of VEGF, angiopoietin (Ang)-1, Ang-2, and Tie-2. β-Tubulin was used as loading control for Western blots. D–G: VEGF (D), Ang-1 (E), Ang-2 (F), and Tie-2 (G) protein levels relative to β-tubulin protein level were assessed by densitometric analysis and expressed relative to the levels of 15-wk-old WT mice on ND. Real-time RT-PCR was performed on ovarian extracts for expression of VEGF, Ang-1, Ang-2, and Tie-2 mRNA as described in MATERIALS AND METHODS. H–K: mRNA expression of VEGF (H), Ang-1 (I), Ang-2 (J), and Tie-2 (K) assessed by real-time RT-PCR analysis calculated as a ratio to the GAPDH mRNA level and expressed relative to the levels of 15-wk-old WT mice on ND. Each value is the mean ± SE of determinations in 8 mice of each group. Different from wild-type mice on the same diet: *P < 0.05, **P < 0.01, ***P < 0.001; different from genotype-matched mice on ND: #P < 0.05, ##P < 0.01, ###P < 0.001.
Whether the differences in results in our study versus the study in VDR\(^{-/-}\) mice reside in the level of serum calcium achieved or in the duration of the normocalcemia before analysis (8 wk in the VDR\(^{-/-}\) study vs. 15 wk in ours) or are due to other differences in the models remains to be determined.

The ovary is known as an adult tissue that continuously undergoes physiological angiogenesis. New blood vessels provide substrate for steroidogenesis, allowing for delivery of endocrine and paracrine factors to and from the follicle and corpora lutea and supplying the preovulatory oocyte with oxygen and other metabolic needs. Ovarian function is therefore critically dependent on angiogenesis for follicular development, ovulation, and corpus luteum function (7, 21). VEGF is the primary modulator of angiogenesis in the ovary, and inhibition of VEGF action disrupts folliculogenesis and formation and function of the corpus luteum. In addition to VEGF, however, the coordinated action of multiple factors, such as angiopoietins Ang-1 and Ang-2, which act via the tyrosine kinase receptor Tie-2 (16), can enhance the maturation and stabilization of newly formed blood vessels. Our results revealed that VEGF, Ang-1, Ang-2, and Tie-2 at both the protein and mRNA expression levels were all significantly downregulated in ovaries from 1\(\alpha\)(OH)ase\(^{-/-}\) mice on the normal diet. After repletion of serum calcium and phosphorus levels, however, the levels of these factors returned to the normal levels found in wild-type mice fed a normal diet. Therefore, evidence from the present study suggests that hypocalcemia and hypophosphatemia caused by 1,25\((\text{OH})_2\)D deficiency impair ovarian angiogenesis and contribute to the reduction of estrogen and progesterone production, as well as defective follicle development and corpus luteum formation.

In summary, we demonstrated that the absence of 1,25\((\text{OH})_2\)D results in female infertility with impaired ovarian follicular development, corpus luteum formation, and uterine hypoplasia due to a defective hypothalamic-hypophysial-ovarian axis and impaired ovarian angiogenesis. These abnormalities in the mouse female reproductive system were normalized by dietary supplementation of calcium and phosphate. These results support the concept that the regulation of 1,25\((\text{OH})_2\)D in the female mouse reproductive system is mediated through extracellular calcium and phosphate.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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