Eldecalcitol replaces endogenous calcitriol but does not fully compensate for its action in vivo.

Calcirol (1α,25-dihydroxyvitamin D₃) is an essential hormone that works in cooperation with parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF-23) to regulate calcium and phosphorus homeostasis. Previous in vivo studies in rats have shown that eldecalcitol, a vitamin D analog, is more active than calcitriol in stimulating calcium and phosphorus absorption in the intestine and increasing serum calcium levels. However, simple replacement of eldecalcitol for calcitriol does not fully compensate for its action in vivo and in vitro.

Thus, we evaluated the biological activity of calcitriol and eldecalcitol in various models and methods. In VDR gene knockout mice, calcitriol and eldecalcitol did not affect either serum or urinary calcium levels. The biological activity in vivo of eldecalcitol and calcitriol in these models was comparable to that of calcitriol. Therefore, we tried to measure the true biological activity in vivo of each compound by comparing their biological activities with respect to their calcium concentrations in vivo.

In accordance with previous studies, eldecalcitol and calcitriol increased serum calcium and phosphorus levels when administrated acutely. In addition, eldecalcitol and calcitriol increased urinary calcium and phosphorus excretion. These results indicate that the actions of eldecalcitol and calcitriol are mediated by VDR. In normal rats, concentrations of both calcitriol and eldecalcitol remained stable even after acute administration. Therefore, the difference in the effectiveness of calcitriol and eldecalcitol might be due to the differences in the potency of the hormone, which is dependent on the VDR concentration and in vivo action. Therefore, we conclude that eldecalcitol is as effective as calcitriol in vivo.
1. Introduction

Calcitriol (1α,25-dihydroxyvitamin D₃, 1α,25(OH)₂D₃) exerts a wide variety of biological actions in many target organs. Calcitriol regulates calcium and phosphorus homeostasis, mineral metabolism, and bone metabolism. Through the action of calcitriol, in cooperation with parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF-23), the absorption of intestinal calcium and phosphorus, resorption of bone phosphorus and calcium, and reabsorption of renal calcium and phosphorus are increased, resulting in a rise in the serum calcium and phosphorus available for bone mineralization [1–3]. Vitamin D₃ is first metabolized to 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver, then to calcitriol (1α,25(OH)₂D₃) in the kidneys. In this pathway, renal 1α-hydroxylation (by 25-OHD-1α-hydroxylase, CYP27B1) is a rate-limiting step in the production of calcitriol. Degradation of 25(OH)D₃ and calcitriol is mediated by renal 24-hydroxylase (CYP24A1). The concentration of calcitriol in the blood is tightly regulated by a feedback loop controlling expression of renal CYP27B1 and CYP24A1. Expression of these enzymes is strictly regulated by PTH, FGF-23, and the vitamin D status of animals [3–7].

The biological actions of calcitriol are mediated through vitamin D receptor (VDR). VDR is a member of the nuclear hormone receptor family and is a ligand-dependent transcription factor [8–10]. The physiological importance of VDR in maintaining the integrity of mineral metabolism is indicated by the observation that patients with vitamin D deficiency and VDR gene knockout (VDRKO) mice both develop hypocalcemia and rickets or osteomalacia [11–13].

The intestinal and renal transepithelial transport of calcium in response to calcitriol is mediated by apical calcium ion channels of the transient receptor potential vanilloid subfamily 5 and 6 (TRPV5 and TRPV6), followed by cytosolic transport by calcium binding proteins (calbindin-D9k and calbindin-D28k) and extrusion across the basolateral membrane into the extracellular fluid by plasma membrane calcium ATPase (PMCA1b) and/or sodium-calcium exchanger (NCX1) [14].

Eldecalcitol (1α,25-dihydroxy-2β-(3-hydroxypropoxy) vitamin D₃), a new active vitamin D₃ analog, has recently been approved for the treatment of osteoporosis in Japan. A Phase III clinical trial in patients with osteoporosis showed that eldecalcitol increased bone mineral density (BMD) and reduced the incidence of vertebral fracture with an efficacy greater than that of alfalcaldiol [15]. It has also shown that eldecalcitol promotes urinary calcium excretion similarly to alfalcaldiol, but has a lower potency to suppress blood PTH [16]. Eldecalcitol increases BMD and reduces bone turnover markers in normal, ovariecctomized (OVX), and steroid-treated rats, and also in patients with osteoporosis [17–21]. Eldecalcitol is more active than calcitriol in stimulating calcium and phosphorus absorption in the intestine, as well as in increasing serum FGF-23 in normal rats [22]. However, administration of exogenous eldecalcitol or calcitriol affects the synthesis and/or degradation of endogenous calcitriol, and exogenous eldecalcitol or calcitriol competes with endogenous calcitriol for binding to VDR in target tissues. In the current study, we tried to evaluate the ‘true biological activity in vivo’ of each compound by comparing their biological activities with respect to their blood concentrations.

2. Materials and methods

2.1. Chemicals

Calcitriol was purchased from Wako Pure Chemical Industries (Osaka, Japan). Eldecalcitol was synthesized by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. Animals

2.2.1. Experiment 1

VDRKO mice were kindly provided by Dr. S. Kato [11]. VDRKO mice were fed ad libitum with a rescue diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (CLEA Japan, Tokyo, Japan) [23]; wild-type (WT) mice were fed normal rodent chow (CE-2; CLEA Japan). All animals were given free access to tap water and were maintained under specific pathogen free conditions with a 12-h light and dark cycle at 20–26 °C and humidity of 35–75%. The 9-week-old VDRKO and WT mice were divided into 3 groups based on body weight. Mice were administered either a vehicle control (medium chain triglyceride; The Nissin Oilio Group, Tokyo, Japan) (MCT), eldecalcitol (0.2 μg/kg), or calcitriol (2 μg/kg) by once-a-day oral gavage for 14 days (n = 5). Blood, kidney, and intestine samples were collected 6 h after the last dosing.

2.2.2. Experiment 2

Six-week-old male Sprague-Dawley rats were purchased from CLEA Japan. Animals were fed with normal rodent chow and tap water and acclimated to the above conditions for 1 week. Rats were divided into 11 groups based on body weight. Various doses of eldecalcitol (0.025, 0.05, 0.1, 0.25, and 0.5 μg/kg), calcitriol (0.25, 0.5, 1, 2.5, and 5 μg/kg), or MCT vehicle were administered by once-a-day oral gavage for 14 days (n = 6). On the 13th day, rats were transferred to and kept in metabolic cages for 24 h to collect urine samples. Blood, bone, kidney, and intestine samples were collected at 6 h after the last dosing on the 14th day.

Both animal studies were carried out in accordance with Chugai Pharmaceutical’s ethical guidelines of animal care, and the experimental protocols were approved by the animal care committee of the institution.

2.3. Biochemical analysis

Levels of calcium, phosphorus, and creatinine in serum and urine were determined by using an automatic analyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan). PTH in plasma was measured by rat intact PTH ELISA kit (Immutopics International, San Clemente, CA, USA). FGF-23 in serum was measured by FGF-23...
ELISA kit (Kainos Laboratories, Tokyo, Japan). Calcitriol in serum was measured by 1,25(OH)₂D RIA kit (TFB, Tokyo, Japan). Measurement of eldecalcitol in plasma was performed at the BoZo Research Center (Tokyo, Japan).

2.4. Quantitative RT-PCR

The right femur, intestine, and kidneys of mice, and the right femur, intestine, and kidneys of rats were excised and immediately frozen in liquid nitrogen. A small portion of each of the frozen tissues was soaked in TRIzol (Invitrogen, Carlsbad, CA, USA) and crushed in a homogenizer (Phycotron NS-310E; Microtec, Chiba, Japan). Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 200 ng of total RNA by reverse transcription PCR using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The reaction was performed at 37 °C for 1 h. Expression of mRNA in the tissues was detected using TaqMan Gene Expression Assays (Applied Biosystems). Target cDNA was amplified by 40 cycles (1 cycle: 95 °C for 15 s, 60 °C for 1 min) of PCR in an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The TaqMan probes used in this study were TRPV5, TRPV6, calbindin-D28k, and calbindin-D9k.
of mice and TRPV5, TRPV6, calbindin-D28k, calbindin-D9k, receptor activator of NF-κB ligand (RANKL), FGF-23, CYP27B1, CYP24A1, and VDR of rats. 18S rRNA was used as a control. Data are represented as expression relative to that in rats treated with vehicle control.

2.5. Statistical analysis

Data are presented as mean ± standard error (SE). Statistical analysis was performed using the SAS System for Windows (SAS Institute, Cary, NC, USA). Statistical significance was determined by Tukey's test in Section 2.2.1, and by Dunnett's test in Section 2.2.2.

3. Results

3.1. Effects of eldecalcitol on calcium metabolism in VDRKO mice

A dose of 2 μg/kg calcitriol or 0.2 μg/kg eldecalcitol administered daily by oral gavage for 14 days significantly increased serum calcium and urinary calcium excretion compared with vehicle administration in WT mice. However, neither eldecalcitol nor
calcitriol affected serum or urinary calcium in the VDRKO mice (Fig. 1A and B). Calcitriol and eldecalcitol significantly increased the expression of renal TRPV5 and calbindin-D28k mRNA and the expression of intestinal TRPV6 and calbindin-D9k mRNA in the WT mice. On the other hand, the expression of these genes in the VDRKO mice was not altered by the treatment (Fig. 1C–F). These results indicate that the calcemic actions of calcitriol and eldecalcitol are mediated by VDR.

3.2. Effects of eldecalcitol on calcium and phosphorus metabolism in normal rats

Eldecalcitol (0.025, 0.05, 0.1, 0.25, and 0.5 μg/kg) or calcitriol (0.25, 0.5, 1, 2.5, and 5 μg/kg) administered daily by oral gavage for 14 days dose-dependently increased the blood concentration of each compound. The blood concentration of each compound correlated well with the administered dosage (eldecalcitol: y (pmol/L) = 29,834x (μg/kg) + 646.3, R² = 0.996; calcitriol: y (pmol/L) = 681.81x (μg/kg) + 402.1, R² = 0.971) (Fig. 2A and B). This result indicates that in order to reach the same concentration in the blood, the amount of eldecalcitol required is approximately 1/40 that of calcitriol. In the eldecalcitol-treated rats, serum concentration of calcitriol dose-dependently decreased and fell below the limit of detection at 0.1 μg/kg (Fig. 2C). Treatment with eldecalcitol and calcitriol significantly reduced renal CYP27B1 gene expression and dose-dependently increased renal CYP24A1 gene expression (Fig. 2D and E). These results suggest that the administration of eldecalcitol and calcitriol reduces endogenous production of calcitriol and stimulates degradation of calcitriol in the kidneys.

Blood concentrations of eldecalcitol and calcitriol correlated with urinary phosphorus excretion. Serum phosphorus slightly decreased along with the increase in eldecalcitol concentration in blood, whereas calcitriol concentration did not alter serum phosphorus (Fig. 3A and B). Serum calcium was significantly elevated at higher blood concentrations of calcitriol (≥7520 pmol/L) and calcitriol (≥1170 pmol/L) (Fig. 3C). Urinary calcium excretion correlated with blood concentrations of calcitriol and eldecalcitol (Fig. 3D). Serum FGF-23 increased at 15,800 pmol/L of eldecalcitol

Fig. 3. Effects of eldecalcitol or calcitriol on calcium and phosphorus metabolism in normal rats. Six-week-old male rats were administered either eldecalcitol or calcitriol for 14 days by oral gavage. Blood and urine samples were recovered for further analyses. Serum phosphorus (A), urinary phosphorus excretion (B), serum calcium (C), urinary calcium excretion (D), serum FGF-23 (E), and plasma PTH (F) are shown. Values represent means ± SE (n = 6 for each treatment group). (a) p < 0.05 (compared with the normal control group using Dunnett's multiple test).
and at ≥2480 pmol/L of calcitriol in blood (Fig. 3E). High concentrations of eldecalcitol in the blood (≥7520 pmol/L) suppressed plasma PTH concentration, whereas plasma PTH concentration was reduced from low blood calcitriol concentrations (≥590 pmol/L) (Fig. 3F).

3.3. Effects of eldecalcitol on target gene expression in normal rats

In the intestine, TRPV6 gene expression was induced from relatively low blood concentrations of eldecalcitol (≥1220 pmol/L) (Fig. 4A). Similarly, induction of TRPV6 gene expression was observed from low concentrations of calcitriol (590 pmol/L and ≥2480 pmol/L). Calbindin-D9k gene expression was unchanged by the administration of calcitriol or eldecalcitol (Fig. 4B). In the kidneys, TRPV5 mRNA expression was significantly elevated at the highest concentration of eldecalcitol (15,800 pmol/L) and at high concentrations of calcitriol (≥1170 pmol/L) (Fig. 4C). Calbindin-D28k mRNA was increased at the higher blood concentrations of eldecalcitol (≥7520 pmol/L) and calcitriol (≥1170 pmol/L) (Fig. 4D).

In bone, blood concentrations of calcitriol correlated with RANKL and FGF-23 gene expression; however, only the highest concentration of eldecalcitol (15,800 pmol/L) induced RANKL and FGF-23 gene expression (Fig. 4E).

Blood concentration of calcitriol correlated with VDR gene expression in the kidneys and bone (Fig. 5B and C), but calcitriol did not affect VDR gene expression in the intestine (Fig. 5A). Induction of VDR gene expression in the intestine and kidneys was associated with increasing concentration of eldecalcitol in the blood (Fig. 5A and B). In bone, significant induction of VDR gene expression was observed only at the highest concentration of eldecalcitol (Fig. 5C).

Taken together, these results show that, in comparison to calcitriol, relatively higher concentrations of eldecalcitol in the blood were required to stimulate expression of vitamin D target genes in the kidneys (VDR, TRPV5, and calbindin-D28k) and bone (VDR, RANKL, and FGF-23).
3.4. Comparison of the 'true in vivo biological activity' of eldecalcitol and calcitriol

In order to compare the true biological activity of calcitriol and eldecalcitol in vivo, the blood concentration required to elicit a 50% response in each activity was calculated from the raw data above. The ratio of biological activity was obtained by dividing the 50% response concentration of calcitriol by that of eldecalcitol. Based on these calculations, eldecalcitol was approximately 1/4 to 1/7 as active as calcitriol in increasing serum calcium and FGF-23, in stimulating urinary calcium excretion, and in suppressing plasma PTH (Table 1). Eldecalcitol was approximately 1/3 to 1/8 as active as calcitriol in stimulating expression of target genes in the kidneys (VDR, TRPV5, and calbindin-D28k) and bone (VDR, FGF-23, and RANKL). The biological activities of eldecalcitol in increasing intestinal TRPV6 and VDR gene expression were comparable to those of calcitriol (Figs. 4A and 5A).

4. Discussion

The half-life of eldecalcitol in the blood is much longer than it is for calcitriol [23]. Although eldecalcitol strongly induces CYP24A1 in the intestine and kidneys [24], eldecalcitol itself is hardly degraded by CYP24A1 [25]. At the same time, eldecalcitol strongly suppresses CYP27B1 in the kidneys. Blood concentration of eldecalcitol during treatment in clinical trials was reported to be relatively high (200–250 pg/mL) in comparison to the normal range of calcitriol (30–60 pg/mL) in humans [15]. An approximately 50% reduction in blood calcitriol was observed during eldecalcitol treatment in the clinical trial.

| Table 1 | Relative biological activity of eldecalcitol compared to that of calcitriol. |
|---------|--------------------------------------------------------------------------------|
|         | Blood concentration required for a 50% response (pmol/L) | Relative activity |
|         | Eldecalcitol | Calcitriol |
| **Biochemical parameters** | | |
| Serum calcium | 8841 | 1288 | 0.15 |
| Urinary calcium excretion | 4856 | 1096 | 0.23 |
| Serum FGF-23 | 11,232 | 1779 | 0.16 |
| Plasma PTH | 3876 | 563.7 | 0.15 |
| **Target gene** | | |
| **Bone** | | |
| VDR | 10,791 | 1328 | 0.12 |
| FGF-23 | 11,506 | 1668 | 0.14 |
| RANKL | 10,711 | 1753 | 0.16 |
| **Kidney** | | |
| VDR | 6711 | 917 | 0.14 |
| TRPV5 | 11,033 | 1850 | 0.17 |
| Calbindin-D28k | 6768 | 1946 | 0.29 |
In the present study, we demonstrated by using VDRKO mice that the calcemic actions of calcitriol and eldecalcitrol were mediated solely by VDR. Administration of small amounts of eldecalcitrol in rats markedly reduced serum concentration of calcitriol, which fell to below the limit of detection at 0.1 μg/kg eldecalcitrol. Plasma concentration of eldecalcitrol increased dose-dependently and reached 3820 pmol/L by 0.1 μg/kg eldecalcitrol administration. These observations indicate that, after administration of eldecalcitrol, the eldecalcitrol rapidly replaces calcitriol in blood and exerts biological activities in target organs. It was observed in an earlier study that the binding activity of eldecalcitrol to VDR is approximately 1/8 of that of calcitriol in vitro [26] and that the distribution capacity of eldecalcitrol to target organs is much lower than that of calcitriol in rats.

In this study, based on the concentration of each compound in the blood, the relative biological activities of eldecalcitrol, such as its activity in increasing serum calcium, FGF-23, and urinary calcium excretion, and in suppressing plasma PTH in vivo were only 15–26% of that of calcitriol (Table 1). Eldecalcitrol stimulated the expression of target genes in the kidneys (VDR, TRPV5, and calbindin-D28k) and bone (VDR, FGF-23, and RANKL) much less than did calcitriol. Stimulation of target genes in the intestine by eldecalcitrol treatment was comparable to that of calcitriol. These results indicate that eldecalcitrol is primarily a weak agonist of VDR as compared with calcitriol in vivo.

Thus, we conclude that administration of eldecalcitrol rapidly suppresses endogenous calcitriol and replaces it. However, eldecalcitrol may not fully compensate for the action of calcitriol in the kidneys and bone.

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