Eldecalcitol prevents endothelial dysfunction in postmenopausal osteoporosis model rats

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Abstract
Postmenopausal women have high incidence of cardiovascular events as estrogen deficiency can cause endothelial dysfunction. Vitamin D is reported to be beneficial on endothelial function, but it remains controversial whether vitamin D is effective for endothelial dysfunction under the treatment for osteoporosis in postmenopausal women. The aim of this study was to evaluate the endothelial protective effect of eldecalcitol (ELD) in ovariectomized (OVX) rats. ELD (20 ng/kg) was orally administrated five times a week for 4 weeks from 1 day after surgery. After that, flow-mediated dilation (FMD) as an indicator of endothelial function was measured by high-resolution ultrasound in the femoral artery of living rats. ELD ameliorated the reduction of FMD in OVX rats. ELD inhibited the increase in NOX4, nitrotyrosine, and p65 and the decrease in dimer/monomer ratio of nitric oxide synthase in OVX rat femoral arteries. ELD also prevented the decrease in peroxisome proliferator-activated receptor gamma (PPARγ) in femoral arteries and cultured endothelial cells. Although PPARγ is known to inhibit osteoblastogenesis, ELD understandably increased bone mineral density of OVX rats without increase in PPARγ in bone marrow. These results suggest that ELD prevented the deterioration of endothelial function under condition of preventing bone loss in OVX rats. This endothelial protective effect of ELD might be exerted through improvement of endothelial nitric oxide synthase uncoupling, which is mediated by an antioxidative effect through normalization of vascular PPARγ/NF-κB signaling.

Key Words
- vitamin D
- flow-mediated dilation
- endothelial function
- estrogen deficiency

Introduction
In postmenopausal women, the risk of cardiovascular event and osteoporosis increases due to diminishing circulating levels of estrogen. Endothelial dysfunction is a powerful surrogate marker of cardiovascular events (Widlansky et al. 2003), and flow-mediated dilation (FMD), which is a useful indicator of endothelial function in clinical settings, is identified as an independent predictor of future cardiovascular events (Yeboah et al. 2007). FMD was identified as the arterial diameter changes after transient ischemia. Transient ischemia leads to reactive hyperemia with increase in flow velocity and vascular wall shear stress. Increased shear stress can induce nitric oxide (NO)
production from endothelium, leading to vasodilation (i.e. FMD). In estrogen-deficient postmenopausal women, brachial artery FMD was decreased as compared with premenopausal women (Moreau et al. 2012).

Many reports demonstrated that vitamin D, which is widely used as a supplement for osteoporosis, can exert a beneficial effect on endothelial function. Vitamin D deficiency is related to endothelial dysfunction in various diseases (Yiu et al. 2011, Chitalia et al. 2012) and is an independent predictor of cardiovascular disease and all-cause mortality (Dobnig et al. 2008). Vitamin D is effective in improving endothelial function as described above, whereas there are few trials using vitamin D in postmenopausal women. Although one randomized controlled trial among postmenopausal women with low serum vitamin D status reported no significant improvement of endothelial function by vitamin D₃ (Gepner et al. 2012), further studies are desired to elaborate the effectiveness of vitamin D on endothelial function in women after menopause (Liu et al. 2013).

Eldecalcitol (ELD), 2β-hydroxypropyloxy derivative of 1α,25-dihydroxyvitamin D₃, is currently approved as a drug for treatment of osteoporosis in Japan. Compared with 1α,25-dihydroxyvitamin D₃, ELD has a higher affinity for serum vitamin D-binding protein, binds more weakly to vitamin D receptor (VDR), and shows lower potency in suppression of serum PTH (Takahashi 2013). ELD is more efficacious than alfacalcidol (1α,25-dihydroxyvitamin D₃) in preventing vertebral and wrist fractures in osteoporotic patients (Matsumoto et al. 2011), however the effect of ELD on endothelial function has not been examined.

In the present study, we examined the effect of ELD on endothelial function by measurement of FMD under condition of preventing bone loss in ovariectomized (OVX) rats.

**Materials and methods**

**Reagents**

ELD was synthesized in Chugai Pharma Manufacturing Co., Ltd (Tokyo, Japan). ELD was dissolved in medium-chain triglyceride (MCT; Nisshin Oillio, Tokyo, Japan) and diluted to the given concentration.

**Animals**

Female Sprague–Dawley rats (Charles River Japan, Yokohama, Japan; 12 weeks old, 200–270 g) were used. All rats were housed in polycarbonate cages with bedding and were fed ordinary laboratory chow and allowed free access to water under a constant 12 h light:12 h darkness cycle. Rats were randomized into three groups: sham-operated rats receiving vehicle (sham), bilaterally OVX rats receiving vehicle (OVX) and OVX rats receiving ELD (OVX + ELD). ELD (20 ng/kg) or vehicle (MCT) was orally administrated via gavage five times a week for 4 weeks from 1 day after surgery, and then rats were measured FMD (Fig. 1A). This dosage is comparable to clinical dose (0.75 μg, daily; Sanford & McCormack 2011), and prior reports indicated that ELD (15 and 30 ng/kg, daily) increased bone mineral density (BMD) in OVX rats (Sakai et al. 2012). All animal procedures were conducted in accordance with Chugai Pharmaceutical’s Ethical Guidelines for Animal Care, and all experimental protocols were approved by the Animal Care Committee of the institution and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

**Experimental surgical procedure**

Rats were anesthetized with isoflurane. The lower part of the back was shaved, and a single 0.5–1 cm incision was made in the skin to expose the back muscles. A small incision was made in the muscles overlying the ovaries on both sides, and the ovaries were isolated, tied with sterile suture, and removed. A sham operation was performed by exposing the ovaries without isolation. An analgesic agent such as flunixin meglumine (2.5 mg/kg; DS Pharma Animal Health, Osaka, Japan) was subcutaneously administered on the day and 1 day after surgery.

**Measurement of body and plasma parameters**

Systolic blood pressure and heart rate were measured by tail-cuff method (BP-98A Softron, Tokyo, Japan) before FMD measurement. Blood samples were collected from abdominal aorta under anesthesia after measurement of FMD, and plasma was stored in a −80 °C freezer. Plasma concentrations of calcium and inorganic phosphorus were measured using an autoanalyzer (Hitachi 7170S, Hitachi).

**Measurement of FMD**

FMD was measured randomly in rats as described previously (Serizawa et al. 2011, 2012). A graphic representing figure for measurement of FMD is shown in Fig. 1B. Rats were anaesthetized with thiobutabarbital (120 mg/kg, i.p.; Wako Pure Chemical Industries, Osaka, Japan) with...
constant monitoring of rectal temperature and monitoring of adequate anesthetic depth by pinching the toe. The animals were kept stable with a heated sheet and warming lamps directed at each rat. Femoral arterial diameter and Doppler flow were measured using a high-resolution ultrasound system (Vevo 770, VisualSonics, Toronto, ON, Canada). The femoral artery was visualized with a 40-MHz transducer. Ischemia and reperfusion of the hindlimb were achieved with a snare occluder positioned around the common iliac artery, through a trans-abdominal access. After an equilibration period, baseline recordings were taken and the common iliac artery was then occluded with the snare occluder. After 5 min of ischemia, the hindlimb was reperfused by releasing the occluder. The changes in flow velocity and the diameter of the femoral artery were monitored at 0, 0.5, 1, 2, and 3 min after reperfusion. In this study, FMD was decided as the peak changes of femoral artery diameter measured at 0.5 min after reperfusion (Corretti et al. 2002).

Western blot analysis

Rats were euthanized by exsanguination under anesthesia with thiobutabarbital, and femoral arteries and bone marrow were harvested and frozen in liquid N2 immediately after isolation and stored in a –80 °C freezer. Equal amounts of protein extracts were separated on SDS–PAGE and immobilized on PVDF membranes. Immunoblotting was performed with anti-peroxisome proliferator-activated receptor gamma (PPARγ) antibodies (Abcam, Cambridge, UK), anti-endothelial NO synthase (anti-eNOS) antibodies (Santa Cruz Biotechnology), anti-p65 antibodies (Santa Cruz Biotechnology), anti-NOX4 antibodies (Epitomics, Burlingame, CA, USA), anti-nitrotyrosine antibodies (Santa Cruz Biotechnology), anti-adipocyte fatty acid-binding protein (A-FABP; Sigma–Aldrich). To investigate eNOS uncoupling state, low-temperature SDS–PAGE was performed (Grijalva et al. 2008). Briefly, protein extracts were mixed with sample buffer (without β-mercaptoethanol), and non-boiled samples were separated on SDS–PAGE on ice.
Measurement of BMD

After measurement of FMD, rats were euthanized by exsanguination and lumbar vertebrae (L2–L4), and femurs were excised and fixed in 70% (v/v) ethanol. The average BMD (mg/cm²) of the lumbar vertebrae (L2–L4) and right femur were measured by dual-energy X-ray absorptiometry (DCS-600EX, Aloka, Tokyo, Japan). During data analysis, the femur was divided into ten equal segments (one to ten) along its major axis. The mean values of the BMD for the three most proximal scanned areas (one to three), those for the next four scanned areas (four to seven), and those for the three most distal areas (eight to ten) were calculated as the densities of the proximal, middle, and distal parts of the femur respectively.

Cell culture and treatment

 Normal human coronary artery endothelial cells (HCAECs) were purchased from Lonza (Walkersville, MD, USA). HCAECs were cultured in endothelial cell basal medium-2 supplemented with 2% (v/v) charcoal stripped FBS or endothelial cell basal medium without phenol red indicator (Lonza) supplemented with 2% (v/v) fetal bovine serum (FBS) or HCAECs were cultured in endothelial cell basal medium-2 supplemented with 2% (v/v) charcoal stripped FBS (Biological Industries, Beit Haemek, Israel). Medium lacking phenol red was used to remove estrogenic activity because phenol red possesses mild estrogenic activity (Berthois et al. 1978). Charcoal stripped FBS was employed to remove steroid hormones, including estrogen (Horwitz & McGuire 1978). HCAECs were exposed to ELD (1 × 10⁻⁸ M), 9-cis retinoic acid (9-cis RA, 1 × 10⁻⁶ M; Enzo Life Sciences, Farmingdale, NY, USA) or vehicle (0.1% (v/v) ethanol). After 7 days, HCAECs were harvested and immediately frozen in liquid N2 and stored in a −80°C freezer until measurement of mRNA by real-time PCR analysis.

Real-time PCR analysis

Total RNA of HCAECs was isolated using an RNeasy Mini Kit (Qiagen). TaqMan real-time PCR was performed using TaqMan Gene Expression Assays for Pparγ (Hs01115513_m1; Applied Biosystems) in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Gene expression was normalized to β-actin (4352935E, Applied Biosystems).

Statistical analyses

All data are expressed as mean ± S.E.M. The n values refer to the number of individual animals on which experiments of differences between each group was determined using Tukey’s multiple comparison test, Dunnett’s multiple comparison test, and unpaired t-test. Test of no correlation was also performed. P < 0.05 was considered significant. Statistical analyses were performed using SAS Version 8.2 Software or JMP Version 10.0.0 Software (SAS Institute, Cary, NC, USA).

Results

Physiological parameters

At the measurement of FMD, the body weight in OVX rats was significantly higher than that in sham-operated rats. There was no significant influence of treatment with ELD on body weight. Systolic blood pressure and heart rate were not changed among the groups. Ovariectomy did not alter plasma calcium and phosphorus levels. Treatment with ELD increased plasma calcium level within normal limit but not phosphorus levels in OVX rats (Table 1).

FMD in femoral arteries of OVX rats

Reperfusion after 5 min ischemia instantaneously increased flow velocity (i.e. reactive hyperemia) compared with baseline femoral artery flow. This reactive hyperemia was peaked at just after reperfusion and rapidly decayed to baseline values at around 3 min. Reactive hyperemia did not differ among the groups (Fig. 2A, B and C). The increase in flow velocity was associated with a delayed increase in femoral arterial vasodilation that peaked at 0.5 min after reperfusion (Fig. 2D and E). This delayed vasodilation was observed as FMD. FMD was significantly decreased in OVX rats compared with sham-operated
rats despite the same level of reactive hyperemia, i.e., same stimulation to endothelium. ELD prevented the reduction of FMD in OVX rats to almost same level as in sham-operated rats (sham, 14.3 ± 2.5; OVX, 8.2 ± 1.5; and OVX + ELD, 15.0 ± 1.1%; Fig. 2F).

**Oxidative stress in femoral arteries of OVX rats**

Oxidative stress could play a crucial role in endothelial dysfunction. In OVX rats, the expression of NOX4, which is NADPH oxidase component, was significantly increased in femoral arteries, and this increase was consistent with augmented oxidative stress as reflected by the nitrotyrosine accumulation in femoral arteries. ELD prevented the increase in the expression of NOX4 and nitrotyrosine accumulation in OVX rat femoral arteries (Fig. 3A and B). Since NOX4 expression can be regulated by NF-κB, we also detected p65 expression which is NF-κB subunit. The expression of p65 was significantly increased in femoral arteries of OVX rats compared with sham-operated rats. ELD significantly decreased p65 expression (Fig. 3C).

**Expression of PPARγ in femoral arteries of OVX rats or HCAECs**

PPARγ is a key molecule in regulating NOX4 expression through NF-κB. In femoral arteries of OVX rats, ELD restored the decrease in PPARγ expression (Fig. 4A).
In HCAECs, \(\text{Ppar} \gamma\) mRNA was significantly decreased by culture in phenol red free medium, and ELD prevented it (Fig. 4B). VDR is known to heterodimerize with retinoid X receptor (RXR), and RXR ligand can activate PPAR\(\gamma\) (Sanz et al. 2012). \(\text{Ppar} \gamma\) mRNA, however, was not changed in HCAECs by 9-cis RA, which is a ligand of RXR\(\alpha\).

**eNOS uncoupling in femoral arteries of OVX rats**

Oxidative stress leads to eNOS uncoupling state. In this study, eNOS dimer/monomer ratio was significantly decreased in OVX rat femoral arteries. This result means the increase in eNOS uncoupling. ELD significantly restored the decrease in eNOS dimer/monomer ratio in OVX rat femoral arteries (Fig. 5).

**BMD in the lumbar spine and the femur**

To confirm the effect of ELD on bone loss, BMD of L2–L4 lumbar vertebrae and the femur were measured. In rats treated with ELD for 4 weeks, BMD of lumbar vertebrae and distal segment of femur were significantly increased compared with OVX rats (Fig. 6A and B).

**Expression of PPAR\(\gamma\) in bone marrow of OVX rats**

Activation of arterial PPAR\(\gamma\) exerts vasoprotective effects, whereas activation of PPAR\(\gamma\) in bone marrow is known to be a risk factor of bone loss and fracture via increased myeloid adipogenesis at the expense of osteoblast formation. Contrary to the effect of ELD in femoral arteries, ELD showed a tendency to prevent the increase in PPAR\(\gamma\) expression in bone marrow (Fig. 7A). The expression of PPAR\(\gamma\) in bone marrow showed positive correlation with the expression of A-FABP that is expressed in adipocytes (Fig. 7B).
Discussion

Clinical dose of ELD prevents reduction of FMD in femoral arteries of OVX rats. ELD inhibited deterioration of oxidative stress with higher PPARγ expression and lower NF-κB expression than those in femoral arteries of vehicle-treated OVX rats. ELD also ameliorated eNOS uncoupling state in OVX rat femoral arteries. ELD naturally increased BMD of lumbar vertebrae and femur with an increase in plasma calcium level. The mean value of plasma calcium was still within the normal range. In bone marrow of OVX rats, ELD did not increase PPARγ expression, unlike in femoral arteries. Therefore, it is suggested that ELD may prevent endothelial dysfunction through the reduction of oxidative stress via normalization of vascular PPARγ/NF-κB pathway in OVX rats. These beneficial effects of ELD correspond to previous report that calcitriol restored renovascular function in OVX rats (Dong et al. 2013). In contrast to the other NOX homologs, NOX4 is constitutively active (Ambasta et al. 2004), and the increase in NOX4 expression contributes to the activity (Serrander et al. 2007). Correspondingly, oxidative stress assessed by nitrotyrosine accumulation, as a marker of peroxynitrite, was increased in OVX rat femoral arteries. This result was associated with deterioration of eNOS uncoupling assessed by eNOS dimer/monomer ratio. ELD significantly improved those changes in OVX rat femoral arteries. Plasma NOx, which is NO metabolite, and plasma malondialdehyde as a marker of oxidative stress were not changed among the groups (data not shown). Therefore, it is suggested that ELD prevented endothelial dysfunction through the correction of NO availability by inhibition of oxidative stress in femoral arteries of OVX rats. These results are corresponding to the report that induced endothelial dysfunction in OVX rat (Camporez et al. 2011). Increased reactive oxygen species (ROS) production via NADPH oxidase can limit NO availability. Superoxide rapidly reacts with NO, resulting in formation of the peroxynitrite anion and loss of the amount of available NO (Gryglewski et al. 1986). Furthermore, increased peroxynitrite can oxidize avidly tetrahydrobipterin (BH₄) to dihydrobipterin. Under conditions of BH₄ deficiency, eNOS is in an uncoupled state, resulting in production of superoxide rather than NO (Landmesser et al. 2004). This reduction of NO availability can cause endothelial dysfunction. In clinical settings, enhanced NO availability by estrogen replacement therapy improved endothelial function in postmenopausal women (Tagawa et al. 1997). In the present study, we demonstrated that FMD was decreased in OVX rat femoral arteries, along with the increase in NOX4 expression. NOX4 is a component of NADPH oxidase and is highly expressed in vascular wall cells including smooth muscle and endothelial cells. In contrast to the other NOX homologs, NOX4 is constitutively active (Ambasta et al. 2004), and the increase in NOX4 expression contributes to the activity (Serrander et al. 2007). Correspondingly, oxidative stress assessed by nitrotyrosine accumulation, as a marker of peroxynitrite, was increased in OVX rat femoral arteries. This result was associated with deterioration of eNOS uncoupling assessed by eNOS dimer/monomer ratio. ELD significantly improved those changes in OVX rat femoral arteries. Plasma NOx, which is NO metabolite, and plasma malondialdehyde as a marker of oxidative stress were not changed among the groups (data not shown). Therefore, it is suggested that ELD prevented endothelial dysfunction through the correction of NO availability by inhibition of oxidative stress in femoral arteries of OVX rats. These results are corresponding to the report that
PPARγ has also been known to be involved in bone homeostasis. Mice treated with rosiglitazone exhibited bone loss accompanied by an increase in marrow adipocyte formation and a decrease in the ratio of osteoblasts to osteoclasts (Ali et al. 2005). Pharmacological inhibition of PPARγ increased osteoblastogenesis and bone mass (Duque et al. 2013). In this study, the expression of PPARγ was significantly increased in bone marrow of OVX rats associated with bone loss. ELD increased BMD and showed tendency to reduce the expression of PPARγ in bone marrow of OVX rats. The expression of PPARγ in bone marrow was positively associated with A-FABP expression. Our results are consistent with earlier report that 1α,25-dihydroxyvitamin D3 inhibited bone marrow adipogenesis by decreasing PPARγ expression in senescence accelerated mice (Duque et al. 2004). Therefore, the present study suggests that estrogen deficiency showed regiospecific influence in the PPARγ expression, and ELD exerts bone mass increasing effect and endothelial protective effect by organ-specific regulation of PPARγ.

Limitations of this study

OVX model is the most commonly used and extensively studied animal model of postmenopausal osteoporosis. FDA guidelines state that OVX rat model is appropriate for evaluation of agents to prevent bone loss in postmenopausal women (Thompson et al. 1995). However, OVX model does not completely replicate postmenopausal women in terms of the pattern of estrogen deficiency. Because it is known that surgical ovariectomy in premenopausal women also caused endothelial dysfunction (Takahashi et al. 2007), it is possible that endothelial dysfunction in OVX rats is more close to that in women who underwent surgical ovariectomy. Additional examination may be needed to clarify whether ELD can prevent the deterioration of endothelial function by a gradual estrogen depletion.

Obesity is known to be a risk factor of cardiovascular disease (CVD) events. On the other hand, it is also reported that plasma adipocytokine levels such as leptin were positively associated with vascular endothelial function in overweight patients (Morioka et al. 2014). Therefore, adipose tissue may have some influence on endothelial function. Tarcin et al. (2009) demonstrated that vitamin D replacement increased serum leptin levels accompanied by improving FMD in asymptomatic subjects. In the present study, ELD did not change the increased body weight in OVX rats. However, it is unclear whether ELD

1α,25-dihydroxyvitamin D3 protects vascular function through the inhibition of oxidative stress such as NOX4 and nitrotyrosine in hypertensive patients and animals (Dong et al. 2012). We recently reported that the other vitamin D analog, 22-oxacalcitriol, also prevents progression of endothelial dysfunction through antioxidative effects in type 2 diabetic rats (Hirata et al. 2013).

Involvement of PPARγ in endothelial dysfunction of OVX rats

PPARγ is expressed in vascular endothelial cells and has been recognized to have vasculoprotective effects. Pioglitazone, which is a PPARγ agonist, improved endothelial function in coronary arterial disease patients (Rizza et al. 2011). Beyrer et al. (2008) demonstrated that endothelium-specific interference with PPARγ caused endothelial dysfunction. In OVX mice, PPARγ expression was significantly reduced in aortic tissue, and PPARγ agonist prevented endothelial dysfunction in isolated aortic ring preparations (Tiyerili et al. 2012). Recent studies indicated that PPARγ regulated NOX4 expression through NF-κB (Lu et al. 2010). In this study, we demonstrated that PPARγ expression was decreased in OVX rat femoral arteries accompanied by the increase in p65 and NOX4 expression. ELD restored PPARγ expression and prevented the increase in p65 and NOX4 expression in femoral arteries of OVX rats. The increase in Pparγ mRNA by ELD was also observed in HCAECs cultured in phenol red free medium. Therefore, it is suggested that ELD prevented endothelial dysfunction through the improvement of endothelial PPARγ/NF-κB/NOX4 pathway in OVX rats.

Figure 7
Expression of PPARγ in bone marrow of OVX rats. The expression of PPARγ was increased in bone marrow of OVX rat, and ELD showed a tendency to reduce it. The expression of PPARγ in bone marrow showed positive correlation with the expression of A-FABP. *P<0.05 vs sham by Tukey’s multiple comparison test (n=6–8).

1α,25-dihydroxyvitamin D3 protects vascular function through the inhibition of oxidative stress such as NOX4 and nitrotyrosine in hypertensive patients and animals (Dong et al. 2012). We recently reported that the other vitamin D analog, 22-oxacalcitriol, also prevents progression of endothelial dysfunction through antioxidative effects in type 2 diabetic rats (Hirata et al. 2013).
affected the function of adipose tissue such as the capacity for adipocytokine production. Although endothelial VDR is important for endothelial function (Ni et al. 2014), it is not yet known which mechanism is more important in endothelial protection in OVX rats, endothelial VDR activation, or adipocytokine production.

There are many reports demonstrated that vitamin D3 is beneficial for endothelial function. On the other hand, it is reported that vitamin D3 has less to do with vascular function. Siasos et al. (2014) indicated that vitamin D2 concentrations were positively associated with endothelial function in coronary artery disease patients, although vitamin D status was not associated with arterial wall properties. Because vitamin D2 also exerts its effect via VDR, VDR activation is certainly important for endothelial function. However, it may be controversial that vitamin D3 is absolutely essential for endothelial function in clinical settings.

Conclusion

The present study demonstrated that ELD prevented the deterioration of endothelial function in OVX rats through improvement of eNOS uncoupling by an antioxidative effect. This antioxidative effect may be mediated by normalization of vascular PPARγ/NF-κB signaling pathway.

Declaration of interest

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Author contribution statement


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