Eldecalcitol prevents endothelial dysfunction in postmenopausal osteoporosis model rats

Kenichi Serizawa¹, Kenji Yogo¹, Yoshihito Tashiro², Satoshi Takeda¹, Ryohei Kawasaki², Ken Aizawa² and Koichi Endo³

¹Product Research Department, Chugai Pharmaceutical Co., Ltd, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan

²Product Research Department, Chugai Pharmaceutical Co., Ltd, 200 Kajiwara, Kamakura, Kanagawa 247-8530, Japan

³Medical Science Department, Chugai Pharmaceutical Co., Ltd, 2-1-1 Nihonbashi-Muromachi, Chuo-ku, Tokyo 103-8324, Japan

Correspondence
should be addressed
to K Endo

Email
endokui@chugai-pharm.co.jp

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 (PPAR γ) in femoral arteries and cultured endothelial cells. Although PPAR γ is known to inhibit osteoblastogenesis, ELD understandably increased bone mineral density of OVX rats without increase in PPAR γ 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 PPAR γ /NF- κ B signaling.

Key Words

- ▶ vitamin D
- ▶ flow-mediated dilation
- ▶ endothelial function
- ▶ estrogen deficiency

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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), 2 β -hydroxypropyloxy derivative of 1 α ,25-dihydroxyvitamin D₃, is currently approved as a drug for treatment of osteoporosis in Japan. Compared with 1 α ,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 (1 α -hydroxyvitamin 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 administered 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 μ g, 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

Figure 1

Diagram of the experimental design. Study designs for treatment of ELD (A) and measurement of FMD (B) were shown as a graphic representing figure. ELD (20 ng/kg) or vehicle (MCT) was orally administered via gavage five times a week for 4 weeks from 1 day after surgery, and then rats were

measured FMD (A). After baseline recordings, the common iliac artery was occluded for 5 min. After that, the hindlimb was reperused and the changes in flow velocity and the diameter of the femoral artery were monitored for 3 min (B).

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 reperused 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, since clinical guideline for FMD assessment defined the peak changes in brachial arterial diameter as an endothelial function, which is occurred about 0.5–1 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 (PPAR γ) 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; Santa Cruz Biotechnology), or anti- β -actin antibodies (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/cm^2) 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) fetal bovine serum (FBS) or endothelial cell basal medium without phenol red indicator (Lonza) 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.* 1986). 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 N_2 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 *Ppar γ* (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 $\pm$ S.E.M. The *n* values refer to the number of individual animals on which

experiments were performed. The statistical significance 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

Table 1 Physiological parameters in sham-operated, OVX, or OVX rats treated with ELD for 4 weeks.

	Sham	OVX	OVX+ELD
<i>n</i>	7	10	8
Body weight (g)	294.0 $\pm$ 5.5	340.2 $\pm$ 4.7*	350.4 $\pm$ 5.8*
Systolic blood pressure (mmHg)	129.3 $\pm$ 4.2	118.5 $\pm$ 2.2	121.4 $\pm$ 4.4
Heart rate (b.p.m.)	398.3 $\pm$ 8.3	367.4 $\pm$ 8.5	405.2 $\pm$ 24.8
Plasma calcium (mg/dl)	8.8 $\pm$ 0.1	8.5 $\pm$ 0.1	9.1 $\pm$ 0.2 [†]
Plasma phosphorus (mg/dl)	6.5 $\pm$ 0.3	6.4 $\pm$ 0.2	6.7 $\pm$ 0.2

* $P < 0.05$ vs sham and [†] $P < 0.05$ vs OVX by Tukey's multiple comparison test.

Figure 2

Post-ischemic reactive hyperemia and flow-mediated dilation in femoral arteries of OVX rats. Ultrasound measurements were performed at femoral arteries. Representative ultrasound images show Doppler flow velocity (A) and femoral artery (D). Scale bar = 0.5 mm. Flow velocity was peaked at just after reperfusion (B), and increase rate of flow velocity was evaluated at

0 min (C). Vasodilation was peaked at 0.5 min after reperfusion (E), and peak vasodilation was evaluated as FMD (F). ELD prevented the reduction of FMD in OVX rats. * $P < 0.05$ vs sham and # $P < 0.05$ vs OVX by Tukey's multiple comparison test ($n = 7-10$).

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, $15.0 \pm 1.1\%$; 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- κ B, we also detected p65 expression which is NF- κ B 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 PPAR γ in femoral arteries of OVX rats or HCAECs

PPAR γ is a key molecule in regulating NOX4 expression through NF- κ B. In femoral arteries of OVX rats, ELD restored the decrease in PPAR γ expression (Fig. 4A).

Figure 3

Oxidative stress in femoral arteries of OVX rats. The expression of NOX4 was increased in OVX rat femoral arteries, and ELD prevented it (A). Nitrotyrosine accumulation was also increased in OVX rat femoral arteries,

and ELD prevented it (B). ELD also inhibited the increase in p65 expression in femoral arteries of OVX rats (C). * $P < 0.05$ vs sham and # $P < 0.05$ vs OVX by Tukey's multiple comparison test ($n = 7-10$).

In HCAECs, *Pparγ* 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 PPARγ (Sanz *et al.* 2012). *Pparγ* mRNA, however, was not changed in HCAECs by 9-*cis* RA, which is a ligand of RXRα.

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 PPARγ in bone marrow of OVX rats

Activation of arterial PPARγ exerts vasoprotective effects, whereas activation of PPARγ 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 PPARγ expression in bone marrow (Fig. 7A). The expression of PPARγ in bone marrow showed positive correlation with the expression of A-FABP that is expressed in adipocytes (Fig. 7B).

Figure 4

Expression of PPARγ in femoral arteries of OVX rats and HCAECs. The expression of PPARγ was decreased in OVX rat femoral arteries, and ELD prevented it (A). PPARγ mRNA was also reduced in HCAECs cultured in phenol red free medium (PM) + charcoal stripped FBS (CS-FBS) compared with HCAECs cultured in normal medium (NM) + FBS (B). ELD inhibited the decrease in mRNA of PPARγ whereas 9-*cis* RA did not. * $P < 0.05$ vs sham and # $P < 0.05$ vs OVX by Tukey's multiple comparison test ($n = 7-10$). † $P < 0.05$ vs NM + FBS + vehicle by unpaired *t*-test, ‡ $P < 0.05$ vs PM + CS-FBS + vehicle by Dunnett's multiple comparison test ($n = 4$).

Figure 5

eNOS uncoupling in femoral arteries of OVX rats. Dimer and monomer eNOS were detected by low-temperature SDS-PAGE. In femoral arteries of OVX rats, eNOS uncoupling, indicated as reduction of dimer/monomer ratio, was decreased, and ELD prevented it. * $P < 0.05$ vs sham and # $P < 0.05$ vs OVX by Tukey's multiple comparison test ($n = 7-10$).

Discussion

Clinical dose of ELD prevents reduction of FMD in femoral arteries of OVX rats. ELD inhibited deterioration of oxidative stress with higher PPAR γ expression and lower NF- κ B 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 PPAR γ 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 PPAR γ /NF- κ B 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 addition, this is the first report indicated that *in vivo* endothelial function was evaluated by measurement of FMD under the study design, in which ELD could prevent bone loss in OVX rats.

Involvement of oxidative stress in endothelial dysfunction

A common mechanism underlying endothelial dysfunction is increased oxidative stress derived from vascular NADPH oxidase in vascular tissue. Recent reports demonstrated that increased oxidative stress via NADPH oxidase

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 tetrahydrobiopterin (BH $_4$) to dihydrobiopterin. Under conditions of BH $_4$ 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

Figure 6

BMD in lumbar vertebrae and femur of OVX rats. BMD was measured in L2-L4 lumbar vertebrae (A) and femur (B). ELD increased BMD in lumbar vertebrae and femur of OVX rats. * $P < 0.05$ vs sham and # $P < 0.05$ vs OVX by Tukey's multiple comparison test ($n = 7-8$).

Figure 7

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

1 α ,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 PPAR γ in endothelial dysfunction of OVX rats

PPAR γ is expressed in vascular endothelial cells and has been recognized to have vasculoprotective effects. Pioglitazone, which is a PPAR γ agonist, improved endothelial function in coronary arterial disease patients (Rizza *et al.* 2011). Beyer *et al.* (2008) demonstrated that endothelium-specific interference with PPAR γ caused endothelial dysfunction. In OVX mice, PPAR γ expression was significantly reduced in aortic tissue, and PPAR γ agonist prevented endothelial dysfunction in isolated aortic ring preparations (Tiyerili *et al.* 2012). Recent studies indicated that PPAR γ regulated NOX4 expression through NF- κ B (Lu *et al.* 2010). In this study, we demonstrated that PPAR γ expression was decreased in OVX rat femoral arteries accompanied by the increase in p65 and NOX4 expression. ELD restored PPAR γ expression and prevented the increase in p65 and NOX4 expression in femoral arteries of OVX rats. The increase in *Ppar γ* 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 PPAR γ /NF- κ B/NOX4 pathway in OVX rats.

PPAR γ 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 PPAR γ increased osteoblastogenesis and bone mass (Duque *et al.* 2013). In this study, the expression of PPAR γ was significantly increased in bone marrow of OVX rats associated with bone loss. ELD increased BMD and showed tendency to reduce the expression of PPAR γ in bone marrow of OVX rats. The expression of PPAR γ in bone marrow was positively associated with A-FABP expression. Our results are consistent with earlier report that 1 α ,25-dihydroxyvitamin D₃ inhibited bone marrow adipogenesis by decreasing PPAR γ expression in senescence accelerated mice (Duque *et al.* 2004). Therefore, the present study suggests that estrogen deficiency showed regiospecific influence in the PPAR γ expression, and ELD exerts bone mass increasing effect and endothelial protective effect by organ-specific regulation of PPAR γ .

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

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 PPAR γ /NF- κ B signaling pathway.

Declaration of interest

All authors are employees of Chugai Pharmaceutical Co., Ltd.

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Author contribution statement

Study design: K S, K Y, and K E; study conduct: K S, K Y, Y T, R K, and K A; data collection and analysis: K S and S T; data interpretation: K S, K Y, Y T, S T, R K, K A, and K E; drafting manuscript: K S; revising manuscript content: K S, K Y, and K E; approving final version of manuscript: K E; and K E takes responsibility for the integrity of the data analysis.

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