

# Fluvastatin improves osteoporosis in fructose-fed insulin resistant model rats through blockade of the classical mevalonate pathway and antioxidant action

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**Abstract.** Feeding rats with a high-fructose diet induced insulin resistance, leading to hypertension or metabolic disorders. Although hypertension is known to accelerate osteoporosis, it is not obvious whether insulin resistance would accelerate osteoporosis. In this study, we evaluated whether osteoporosis might accelerate in fructose-fed rats (FFR), and examined the effect of fluvastatin through a blockade of the mevalonate pathway and an antioxidant action. Stimulation of recombinant receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL) expressed by osteoblasts/ stromal cells and macrophage-colony stimulating factor (M-CSF) significantly increased TRAP-positive multinuclear osteoclasts and pit formation, accompanied by an increase in reactive oxygen species as assessed by dichlorodihydrofluorescein (DCF) staining. Interestingly, it was completely abolished by treatment with fluvastatin, pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC), but not pravastatin. These actions of fluvastatin were partially abolished by co-treatment with geranylgeranylpyrophosphate (GGPP), but not farnesylpyrophosphate (FPP). In the estrogen-deficient model by ovariectomy, FFR exhibited a decrease in bone mineral density, activation of osteoclasts, and an increase in urinary deoxyypyridinoline. Importantly, the treatment of fluvastatin, but not pravastatin, attenuated FFR-induced osteoporosis. The present study demonstrates that fructose fed to rats induced insulin resistance and accelerated osteoporosis, while fluvastatin, but not pravastatin, significantly attenuated osteo-

clast differentiation and activation through a blockade of the classical mevalonate pathway and an antioxidant action, leading to prevention of osteoporosis.

## Introduction

Diabetic osteoporosis is increasingly recognized as a significant comorbidity of type I diabetes mellitus (DM), but not type II DM in the clinical view point (1,2). Thus, the analysis of type I DM may serve as a more straightforward system to understand the effect of suppressed insulin signaling, hyperglycemia, and metabolic abnormalities on regulation of bone formation. However, endocrine problems, such as, thyrotoxicosis or primary hyperparathyroidism, may cause osteoporosis in individuals with type I DM (3). Thus, it might be difficult to understand 'diabetic osteoporosis'.

There is also interest in whether pharmacotherapy used to lower blood lipids, 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), may increase bone mineral density. In 1999, experimental work produced the novel finding that statins have direct effects on bone formation through induction of bone morphogenetic protein-2 (BMP-2) in osteoblasts. In a cross-sectional population study, bone mineral density was approximately 7-8% higher in women taking statins than in controls (4), and a case control study showed that current use of statins is associated with a reduced risk of fracture (5,6). However, two large studies failed to demonstrate an association between statins and risk of fracture. In the LIPID study, there was no difference in fracture occurrence in the pravastatin group compared to the placebo group (7). In another study using General Practice database, no association was found between the use of statins and risk of fracture in 81,880 individuals (8). From these results, we speculate that individual statins have different effects on bone metabolism.

High blood pressure is associated with abnormalities of calcium metabolism, leading to an increase in calcium loss, secondary activation of the parathyroid gland, and increased movement of calcium from bone, thereby increasing the risk

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of osteoporosis (9,10). However, it is not understood whether insulin resistance or metabolic syndrome is associated with osteoporosis. A previous report suggests that increased dietary fructose in rodents recapitulates many aspects of metabolic syndrome with hypertension, insulin resistance and dyslipidemia (11). Thus, in this study, we evaluated whether osteoporosis would be accelerated in the fructose-fed rats (FFR), and examined the effect of statins on FFR-induced osteoporosis. In addition, we examined the effects of fluvastatin on osteoclasts, especially focusing on its antioxidant action, since fluvastatin has a scavenging effect on hydroxyl radicals due to its unique chemical structure (12). We demonstrated for the first time that fructose-feeding accelerated osteoporosis, while fluvastatin, but not pravastatin, attenuated osteoporosis and inhibited osteoclast differentiation and activation through both an antioxidant effect and inhibition of HMG-CoA reductase.

## Materials and methods

**Rat ovariectomy osteoporosis model.** Female adult Wistar rats (10 weeks old) were purchased from SLC Japan (Shizuoka, Japan). After the rats were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg), bilateral ovariectomy was performed. Rats were fed fructose-rich chow containing 60% fructose, 5% fat, and 20% protein (Oriental Yeast Co.) for 28 days. In some groups of rats, fluvastatin or pravastatin (5 mg/kg/day) was orally administered every day. Four weeks after the operation, systolic blood pressure was measured using the tail-cuff method (BP-98A, Softron), and they were deeply anesthetized and sacrificed to collect the femurs, tibias, and blood for biochemical analysis. Osteoclasts in trabecular bone under growth plate in proximal tibia were analyzed by light microscope and tartrate-resistant acid phosphatase (TRAP) stained area per field (300  $\mu\text{m}$  x 300  $\mu\text{m}$ ) was measured by Image J software, NIH.

**Dual energy X-ray absorptiometry (DEXA) and micro-computed tomography.** Bone density measurements were performed by dual-photon X-ray absorptiometry (DEXA) bone densitometry (GE-Lunar DPX-IQ, Madison, WI). High- and low-beam energies for all scans were 80 kV and 35 kV, respectively, at 0.5 mA as previously described (13). Bone mineral density (BMD) was obtained in  $\text{g}/\text{cm}^2$ .

**Materials and cell culture.** Pravastatin was obtained from Wako (Osaka, Japan) and fluvastatin was obtained from Novartis. Other materials were obtained from Sigma Chemical Co., (St. Louis, MO).

Bone marrow cells were obtained from 3-day-old neonatal white rabbits as previously described (14). Briefly, rabbit bone marrow cells were flushed out from the femurs and tibiae, collected into tubes, and washed twice with PBS. The mononuclear-rich cell fraction was separated from marrow cells and cultured ( $1 \times 10^5$  cells/well of 24-well plate) in  $\alpha$ -MEM medium containing 10% fetal bovine serum.

Osteoclast differentiation was also examined using rat osteoclast culture system obtained from Primary Cell Company (Primary Cell Co., Ltd., Sapporo). Rat osteoclast precursor cells

seeded in a 24-well plate were incubated with macrophage-colony stimulating factor (M-CSF: 50 ng/ml) and receptor activator of nuclear factor-kappaB (NF- $\kappa$ B) ligand (RANKL: 50 ng/ml) expressed by osteoblasts/stromal cells containing medium with or without several stimulants for 5 days to examine the differentiation of osteoclasts. Cell viability of osteoclasts was measured using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay after 48 h of treatment with several stimulants. Approximately 100  $\mu\text{l}$  CellTiter 96 One Solution Reagent (Promega, Madison, WI) in 500  $\mu\text{l}$  Dulbecco's modified Eagle's medium (DMEM) was added to each well, and absorbance was measured at 490 nm.

**ROS (reactive oxygen species) production by dichlorodihydrofluorescein (DCF) staining.** To measure intracellular ROS production, rat osteoclasts were treated with M-CSF (50 ng/ml) and RANKL (50 ng/ml) and/or several stimulants for 24 h, and loaded with 10  $\mu\text{mol/l}$  CM-DCFH-DA for 15 min at room temperature in the dark. During loading, the acetate groups on CM-DCFH-DA are moved by intracellular esterase, trapping the probe inside the cells. Although several dihydrofluorescein derivatives were used to measure intracellular ROS generation (15,16), CM-DCFH-DA was chosen because it exhibits better retention in cells than other derivatives (17). The production of ROS was measured by fluorescence under fluorescence microscopy.

**Tartrate-resistant acid phosphatase (TRAP) staining.** After treatment with vitamin D3 ( $1 \times 10^{-8}$  M) and several stimulants for 5 days, mononuclear cells were fixed with 4.0% paraformaldehyde in PBS for 10 min at room temperature before being stained for TRAP. Rat osteoclasts were treated similarly after treatment with M-CSF (50 ng/ml) and RANKL (50 ng/ml) and several stimulants. Enzyme histochemical staining for TRAP was performed as previously reported (18).

**Pit formation.** Osteoclast activation was evaluated by the inhibitory effect on RANKL-induced pit-forming activity, as previously reported (19). We used osteologic calcium hydroxyapatite-coated slides (BD Biocoat; BD Biosciences, Bedford, MA). Rat osteoclasts ( $1 \times 10^5$  cells/well) were incubated with RANKL (50 ng/ml) and M-CSF (50 ng/ml) with several stimulants for 7 days. On day 8, cells were washed vigorously and the area of resorption of calcified matrix on each disc was measured.

**Statistical analysis.** All values are expressed as mean  $\pm$  SEM. Analysis of variance with subsequent Bonferroni's/Dunnett's test was employed to determine the significance of differences in multiple comparisons. Values of  $P < 0.05$  were considered statistically significant.

## Results

**Effects of statins on rat osteoclasts.** To examine the effects of fluvastatin and pravastatin, we employed rat osteoclast culture. Treatment with M-CSF (50 ng/ml) and RANKL (50 ng/ml) induced osteoclast differentiation as assessed by TRAP staining, whereas co-treatment with fluvastatin (100 nM), but

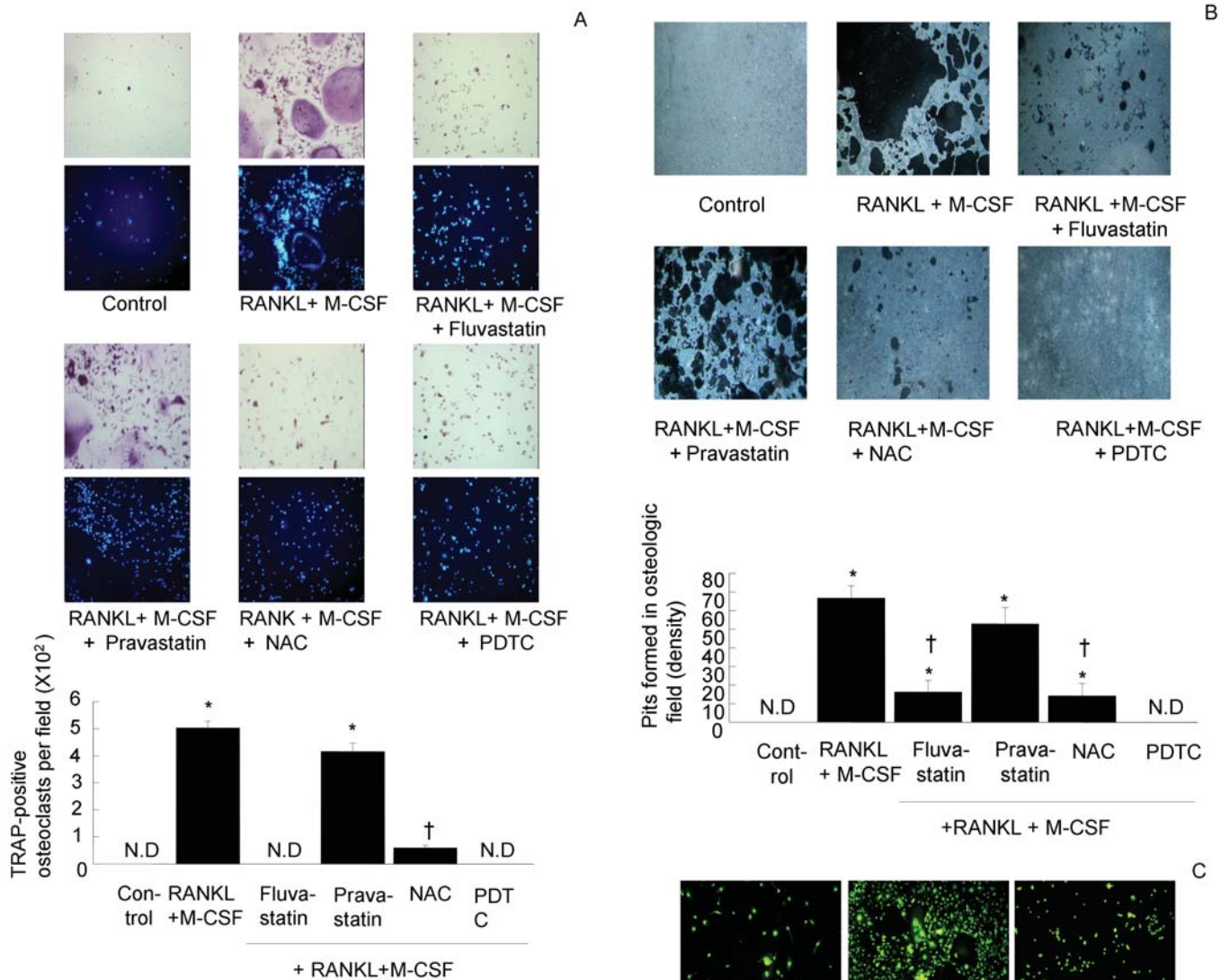
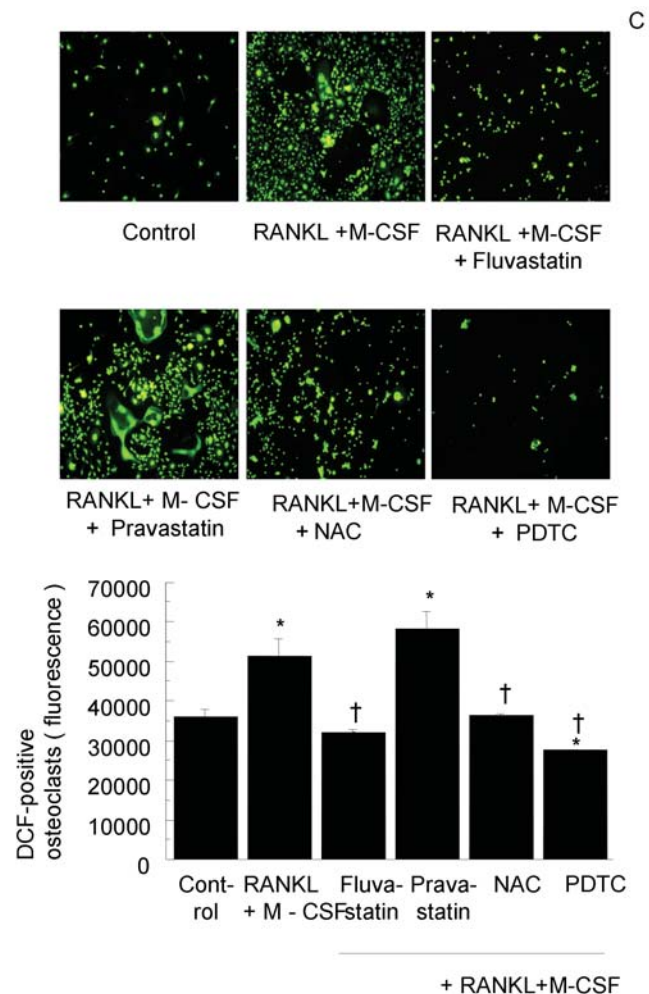


Figure 1. Effects of fluvastatin, pravastatin, PDTC and NAC on rat osteoclast differentiation and activation. (A) Representative photomicrographs and number of TRAP-positive staining cells. (B) Pit formation assay (C) DCF staining. Control, no treatment; RANKL+M-CSF, RANKL (50 ng/ml) and M-CSF (50 ng/ml). Under treatment with RANKL and M-CSF, fluvastatin (100 nM), pravastatin (100 nM), PDTC (10  $\mu$ M) and NAC (10 mM) were administered. \* $P < 0.01$  vs. control; † $P < 0.01$  vs. RANKL+M-CSF. N.D., not detected. n=4-8 per group.

not pravastatin (100 nM), significantly attenuated the number of TRAP-positive multinuclear cells induced by M-CSF and RANKL ( $P < 0.01$ , Fig. 1A). To elucidate the differential effects of statins on the number of TRAP-positive cells, we focused on the role of oxidative stress. Importantly, a potent antioxidant, pyrrolidine dithiocarbamate (PDTC: 10  $\mu$ M) as well as N-acetylcysteine (NAC: 10 mM), significantly decreased the number of TRAP-positive multinuclear cells induced by M-CSF and RANKL ( $P < 0.01$ , Fig. 1A). To examine further functional aspects, we employed the pit formation assay, whose results are well correlated with the function of TRAP-positive cells in rat osteoclasts under treatment with M-CSF and RANKL. Similarly, treatment with fluvastatin, PDTC or NAC significantly attenuated the area of pit formation on an osteologic field induced by M-CSF and RANKL, while pravastatin did not (Fig. 1B). To



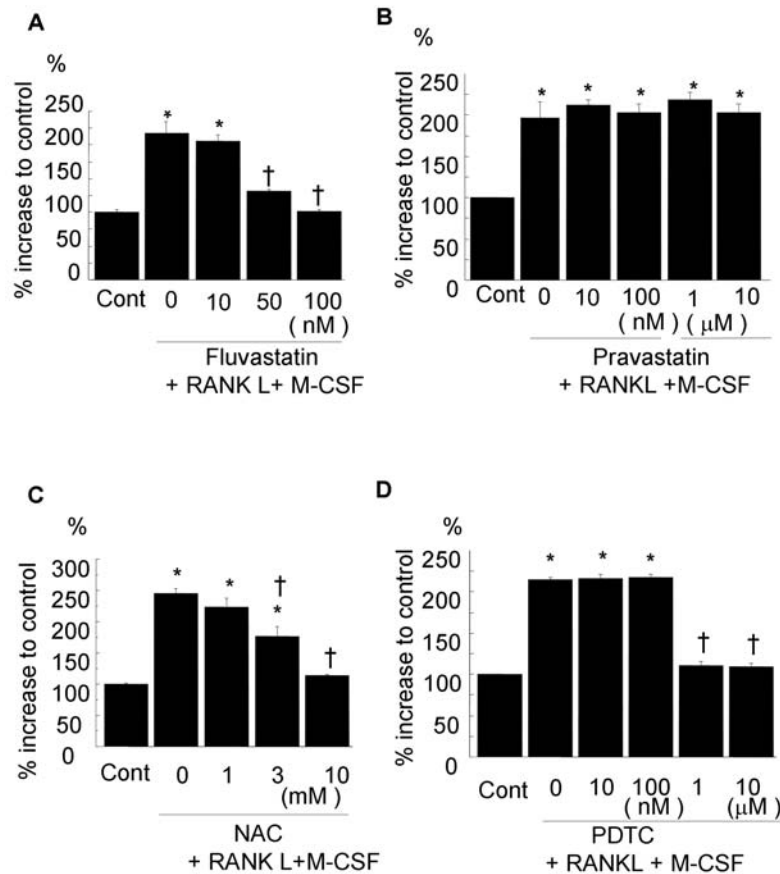


Figure 2. Effect of fluvastatin, pravastatin and PDTC on cell growth as assessed by MTS assay in a dose-dependent manner. Control, no treatment; RANKL+M-CSF, RANKL (50 ng/ml) and M-CSF (50 ng/ml). Under treatment with RANKL and M-CSF, fluvastatin, pravastatin, PDTC and NAC were administered in several doses. \* $P < 0.01$  vs. control; † $P < 0.01$  vs. RANKL+M-CSF.  $n = 4-8$  per group.

clarify whether the inhibitory effects of fluvastatin might be due to antioxidant effects, we measured ROS production as assessed by DCF staining. As shown in Fig. 1C, treatment with M-CSF and RANKL significantly induced ROS production, whereas co-treatment with fluvastatin, PDTC or NAC completely inhibited ROS production. In contrast, pravastatin did not decrease ROS production.

Since the number of TRAP-positive cells correlated with cell viability, assessed by MTS assay in rat osteoclasts, we examined cell viability under treatment with M-CSF and RANKL. As shown in Fig. 2, co-treatment with fluvastatin or NAC, but not pravastatin, significantly attenuated an increase in cell viability induced by treatment with M-CSF and RANKL in a dose-dependent manner. Treatment with PDTC completely blocked it even at a low concentration (1  $\mu$ M).

It is well known that statins reduce the synthesis of other isoprenoid intermediates in the mevalonate pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), and the prenylation of small GTP-binding protein (20,21), which block osteoclast activity and inhibit osteoblast apoptosis. Thus, we examined the effects of these isoprenoid intermediates in rat osteoclasts. The inhibitory effects of fluvastatin on osteoclast differentiation and activation assessed by the number of TRAP-positive cells and pit formation, were partially abolished by co-treatment with GGPP, but not FPP (Fig. 3A and B). Moreover, co-treatment with GGPP, but not

FPP, partially attenuated antioxidant effects of fluvastatin as assessed by DCF staining (Fig. 3C).

*Effect of fluvastatin on rabbit mononuclear cells.* We also examined the effects of fluvastatin in rabbit mononuclear cells which may include pre-osteoblasts and pre-osteoclasts. Treatment with vitamin D3 ( $1 \times 10^{-8}$  mol/l) significantly increased the number of TRAP-positive mononuclear cells, whereas treatment with fluvastatin, PDTC or NAC abolished them (Fig. 4A). In addition, the inhibitory effect of fluvastatin on osteoclast differentiation assessed by the number of TRAP-positive cells, was only partially abolished by co-treatment with GGPP, but not FPP (Fig. 4B). These results suggest that the inhibition of osteoclast differentiation and activation by treatment with fluvastatin might be due to both an antioxidant effect and the loss of GGPP.

*Effect of statins on osteoporosis of fructose-fed rats.* Finally, we examined the effects of fluvastatin and pravastatin in *in vivo* osteoporosis model. Initially, to clarify whether fructose-fed insulin resistance would accelerate osteoporosis, we employed rat ovariectomy model of estrogen deficiency as a model of osteoporosis with fructose-fed rat. We previously confirmed that at 28 days after bilateral ovariectomy, serum estradiol level was significantly decreased in the ovariectomy group, while there was no significant difference in body weight (21).

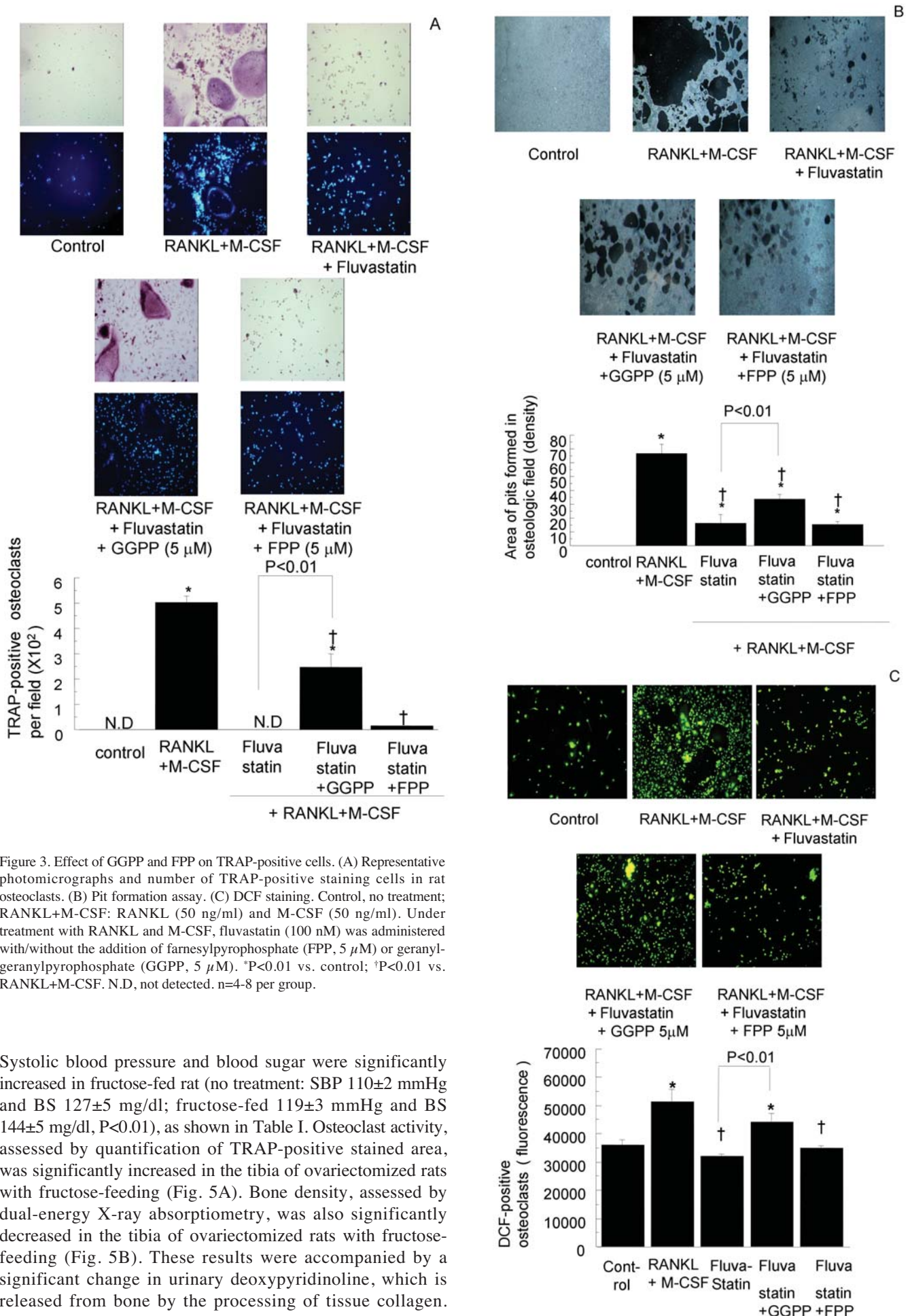


Figure 3. Effect of GGPP and FPP on TRAP-positive cells. (A) Representative photomicrographs and number of TRAP-positive staining cells in rat osteoclasts. (B) Pit formation assay. (C) DCF staining. Control, no treatment; RANKL+M-CSF: RANKL (50 ng/ml) and M-CSF (50 ng/ml). Under treatment with RANKL and M-CSF, fluvastatin (100 nM) was administered with/without the addition of farnesylpyrophosphate (FPP, 5  $\mu$ M) or geranylgeranylpyrophosphate (GGPP, 5  $\mu$ M). \* $P$ <0.01 vs. control; † $P$ <0.01 vs. RANKL+M-CSF. N.D., not detected.  $n$ =4-8 per group.

Systolic blood pressure and blood sugar were significantly increased in fructose-fed rat (no treatment: SBP 110 $\pm$ 2 mmHg and BS 127 $\pm$ 5 mg/dl; fructose-fed 119 $\pm$ 3 mmHg and BS 144 $\pm$ 5 mg/dl,  $P$ <0.01), as shown in Table I. Osteoclast activity, assessed by quantification of TRAP-positive stained area, was significantly increased in the tibia of ovariectomized rats with fructose-feeding (Fig. 5A). Bone density, assessed by dual-energy X-ray absorptiometry, was also significantly decreased in the tibia of ovariectomized rats with fructose-feeding (Fig. 5B). These results were accompanied by a significant change in urinary deoxypyridinoline, which is released from bone by the processing of tissue collagen.

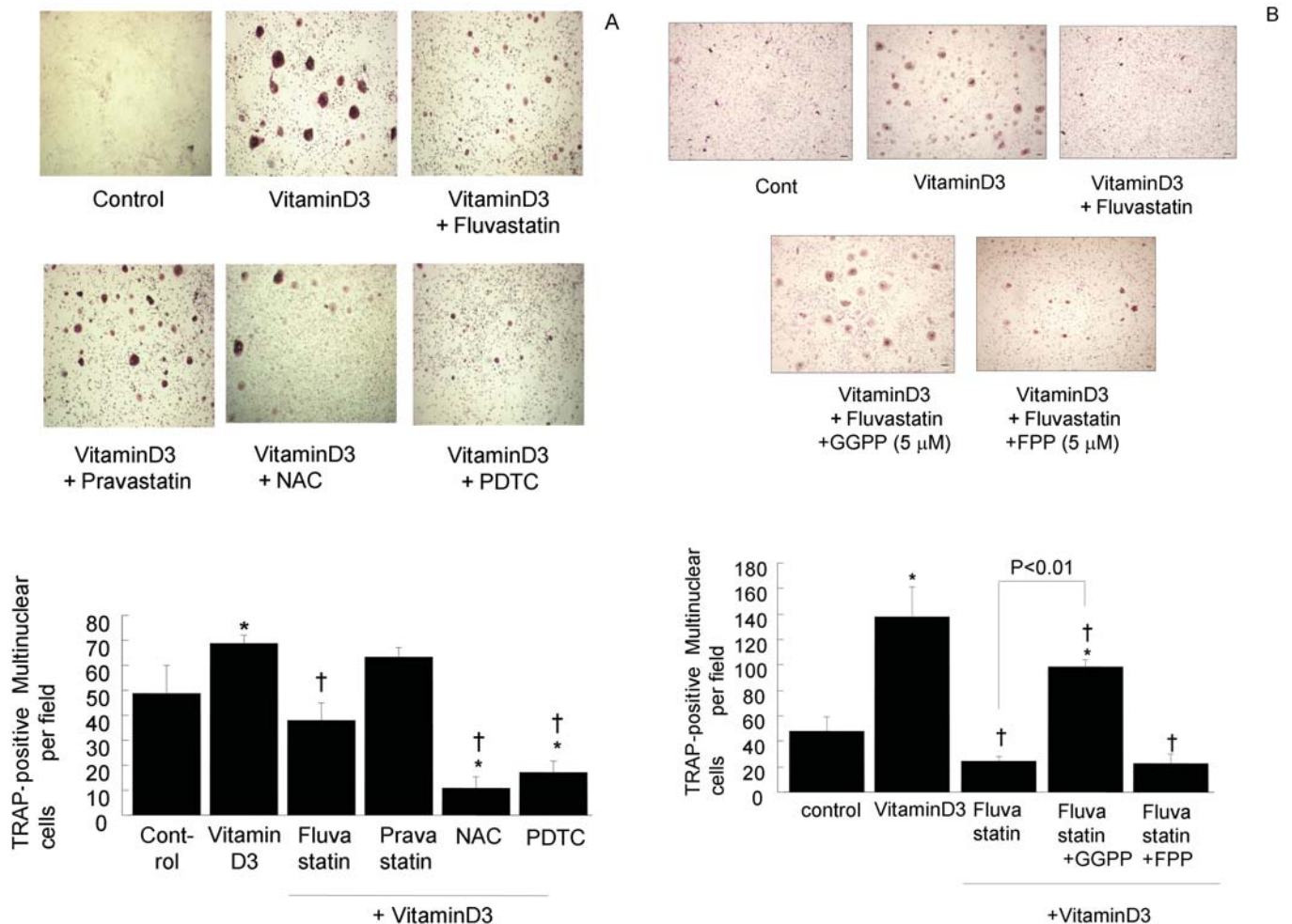


Figure 4. Effects of fluvastatin, pravastatin, PDTC and NAC on osteoclast differentiation in rabbit mononuclear cells. (A and B) Representative photomicrographs and numbers of TRAP-positive staining cells in rabbit mononuclear cells. Control, no treatment; RANKL+M-CSF: RANKL (50 ng/ml) and M-CSF (50 ng/ml). Under treatment with RANKL and M-CSF, fluvastatin (100 nM), pravastatin (100 nM), PDTC (10 μM) and NAC (10 mM) were administered. Under treatment with RANKL and M-CSF, fluvastatin (100 nM) was administered with/without farnesylpyrophosphate (FPP: 5 μM) or geranylgeranylpyrophosphate (GGPP: 5 μM). \*P<0.01 vs. Control; †P<0.01 vs. RANKL+M-CSF. N.D., not detected; n=4-8 per group.

Table I. Body weight, blood pressure and serum cholesterol levels in FFR-induced rat with or without the treatment of fluvastatin.

	Wild+ovx	FFR+ovx	FFR+ovx +Fluvastatin	FFR+ovx +Pravastatin
Body weight (g)	301±4	297±3	287±3	298±6
SBP (mmHg)	110±2	119±3 <sup>a</sup>	116±2	113±3
DBP (mmHg)	82±2	86±2	83±1	83±2
Glucose (mg/dl)	127±5	144±5 <sup>a</sup>	132±5	147±6 <sup>a</sup>
Insulin (ng/ml)	0.2±0.02	0.4±0.03 <sup>a</sup>	0.3±0.02	0.3±0.02

SBP, systolic blood pressure; DBP, diastolic blood pressure. <sup>a</sup>P<0.05 vs. Wild+ovx.

Fructose-feeding significantly induced the ovariectomy-induced increase in urinary deoxyypyridinoline (Fig. 5C). These results suggest that fructose-fed insulin resistance directly accelerated estrogen deficiency-induced osteoporosis

independent of blood pressure. Interestingly, the treatment of fluvastatin, but not pravastatin, significantly decreased osteoclast activity and improved bone mineral density, accompanied by the decrease in urinary deoxyypyridinoline. In addition, fruvastatin decreased blood sugar induced by fructose feeding (fructose-fed: 144±5 mg/dl; fructose-fed+fluvastatin 132±5 mg/dl, P<0.01).

## Discussion

Recent therapy for diabetes, including improved glucose monitoring, insulin delivery methods, and pharmacologic treatments, are increasing the lifespan of patients, but increasing the risk of complications from extended exposure to diabetic conditions. Well known diabetic complications include retinopathy, neuropathy, and nephropathy. However, recently attention also focused on diabetic bone pathology. In the clinical view point, diabetic osteoporosis is mainly recognized as a significant comorbidity of type I diabetes mellitus (DM), but not type II DM (1,2). In this study, we focused on bone pathology of insulin resistance, which could be involved in pre-hypertension and pre-diabetes. The

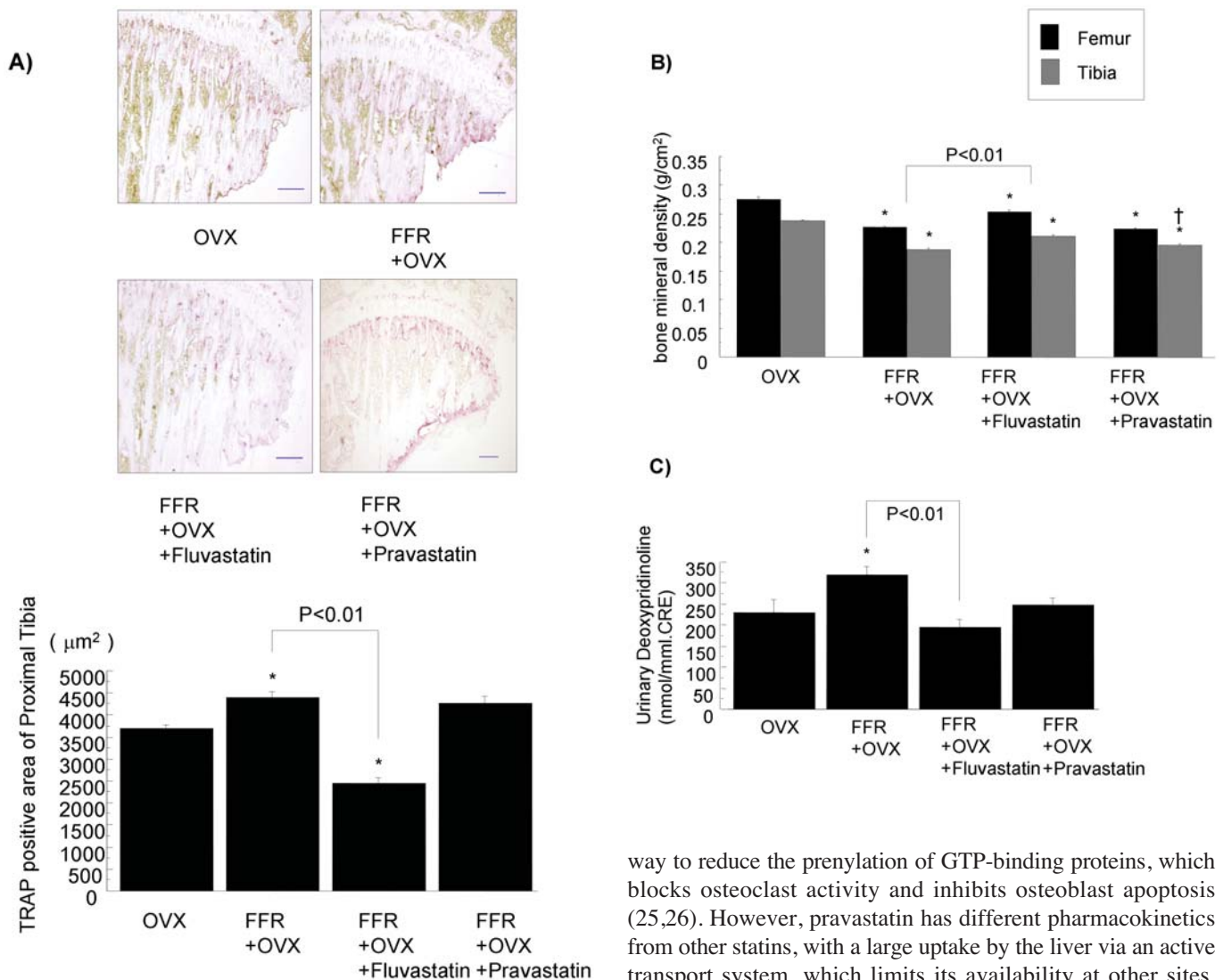


Figure 5. Effect of fluvastatin or pravastatin on Fructose-fed rats with ovariectomy. (A) TRAP staining of proximal tibia (Scale bar shows  $300\ \mu\text{m}$ ), (B) bone mineral density by DEXA, and (C) urinary deoxyypyridinoline after 28 days of each treatment. OVX, bilateral ovariectomy; FFR, fructose fed rat; Fluvastatin, treated with fluvastatin ( $5\ \text{mg}/\text{kg}/\text{day}$ ); Pravastatin, treated with pravastatin ( $5\ \text{mg}/\text{kg}/\text{day}$ ). Urinary deoxyypyridinoline was adjusted for urinary creatinine concentration. \* $P<0.05$  vs. OVX; † $P<0.05$  vs. FFR + OV.  $n=6-10$  per group.

present study demonstrated that fructose-fed rat, one of the insulin resistance models with ovariectomy showed accelerated osteoporosis. Interestingly, fluvastatin attenuated the development of osteoporosis.

Simvastatin, mavastatin, fluvastatin, and lovastatin stimulate bone formation (22), and simvastatin and pitavastatin increase human osteoblast differentiation (23,24). In addition to stimulating bone formation, statins may also inhibit resorption, in a similar way as described for some bisphosphonates (25,26). Because statins also reduce the synthesis of other isoprenoid intermediates, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are important lipid attachments for post-translational modification of the small GTP-binding protein Ras and Ras-like proteins (i.e. Rho and Rac). Nitrogen-containing bisphosphonates also act on the mevalonate path-

way to reduce the prenylation of GTP-binding proteins, which blocks osteoclast activity and inhibits osteoblast apoptosis (25,26). However, pravastatin has different pharmacokinetics from other statins, with a large uptake by the liver via an active transport system, which limits its availability at other sites. This may explain the lack of effects seen in the analysis of the LIPID study (7), in which all the patients were taking pravastatin. Indeed, the present study demonstrated that fluvastatin, but not pravastatin, significantly decreased ROS production, and the antioxidant effect of fluvastatin is necessary to inhibit osteoclast activation and differentiation, in addition to the classical well-known mechanism through a reduction in prenylation of GTP-binding proteins.

Overall, the present study demonstrated potent inhibitory effects of fluvastatin on osteoclast activation and differentiation due to both an antioxidant effect and the reduction in prenylation of GTP-binding proteins. These favorable aspects of fluvastatin suggest the beneficial effects on osteoporosis and the reduction in bone fractures in human patients. Although the present study did not include a clinical evaluation, a previous study clearly demonstrated the superiority of fluvastatin compared to pravastatin for prevention of osteoporosis in patients (27). Furthermore, according to a pharmacokinetic study, the mean highest plasma concentration of fluvastatin after oral administration at a dose of  $40\ \text{mg}/\text{day}$  for 6 days was almost  $1\ \mu\text{M}$  (28), while the inhibitory effect of fluvastatin on osteoclast activation occurs at  $100\ \text{nM}$  ( $1/10$  the plasma concentration at a therapeutic dosage) as shown in this study. Of importance, the effective concentration of fluvastatin in this study seems to be much lower than the concentration in human plasma. In contrast, although simvastatin also

has inhibitory effects on osteoclast activation and differentiation, its effective dose (100 nM, data not shown) is much lower than the concentration in human plasma (the mean highest plasma concentration of simvastatin after oral administrations at a dose of 20 mg/day for a period of 6 days was almost 17.8 nM). Further clinical studies comparing bone turnover, density, and risk of fracture among various statins in prospective trials are necessary. The present study demonstrates that fluvastatin significantly attenuated osteoclast differentiation and activation through blockade of the classical mevalonate pathway and an antioxidant action, leading to prevention of osteoporosis.

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