

# Caffeine decreases vitamin D receptor protein expression and 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated alkaline phosphatase activity in human osteoblast cells

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## Abstract

Of the various risk factors contributing to osteoporosis, dietary/lifestyle factors are important. In a clinical study we reported that women with caffeine intakes >300 mg/day had higher bone loss and women with vitamin D receptor (VDR) variant, were at a greater risk for this deleterious effect of caffeine. However, the mechanism of how caffeine affects bone metabolism is not clear. 1,25-Dihydroxy vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) plays a critical role in regulating bone metabolism. The receptor for 1,25(OH)<sub>2</sub>D<sub>3</sub>, VDR has been demonstrated in osteoblast cells and it belongs to the superfamily of nuclear hormone receptors. To understand the molecular mechanism of the role of caffeine in relation to bone, we tested the effect of caffeine on VDR expression and 1,25(OH)<sub>2</sub>D<sub>3</sub> mediated actions in bone. We therefore examined the effect of different doses of caffeine (0.2, 0.5, 1.0 and 10 mM) on 1,25(OH)<sub>2</sub>D<sub>3</sub> induced VDR protein expression in human osteoblast cells. We also tested the effect of different doses of caffeine on 1,25(OH)<sub>2</sub>D<sub>3</sub> induced alkaline phosphatase (ALP) activity, a widely used marker of osteoblastic activity. Caffeine dose dependently decreased the 1,25(OH)<sub>2</sub>D<sub>3</sub> induced VDR expression and at concentrations of 1 and 10 mM, VDR expression was decreased by about 50–70%, respectively. In addition, the 1,25(OH)<sub>2</sub>D<sub>3</sub> induced alkaline phosphatase activity was also reduced at similar doses thus affecting the osteoblastic function. The basal ALP activity was not affected with increasing doses of caffeine. Overall, our results suggest that caffeine affects 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated VDR protein expression and 1,25(OH)<sub>2</sub>D<sub>3</sub> mediated actions in human osteoblast cells.

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**Keywords:** 1,25(OH)<sub>2</sub>D<sub>3</sub>; Osteoblast cells; vitamin D

## 1. Introduction

Caffeine is a naturally occurring methyl-xanthine found in many beverages, food, medications and dietary supplements and is consumed regularly by most of the US population. Clinical studies have reported conflicting findings regarding caffeine as a risk factor for osteoporosis. Caffeine consumption has been reported to decrease bone mineral density (BMD) [1–3], increase the risk of hip fracture [4–7] and

negatively influence calcium retention [8]. However, some studies reported no association between caffeine intake and BMD and calcium metabolism [9–12]. In a recent study we observed that intake of caffeine in amounts >300 mg/day (>3 cups of coffee) accelerates bone loss at the spine in elderly women [13]. Furthermore, we observed that women with one particular variant of vitamin D receptor (VDR), were at a greater risk for this deleterious effect of caffeine on bone. Studies have shown that the polymorphisms in the 3' end of the VDR gene (encompassing the intronic site for BsmI, ApaI, a silent TaqI site in exon IX and a singlet (A) repeat in the portion of exon IX encoding the 3' UTR) correlate with BMD, bone turnover and bone loss [14,15].

In animal studies, caffeine was shown to alter the mechanical properties of the bone of young ovariectomized rats [16]

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and decrease the BMD [17]. Further, chronic administration of caffeine was noted to lead to a negative calcium balance due to impaired ability to increase the calcium absorption efficiency [18]. Using a chick osteoblast culture system [19], caffeine was reported to have a direct effect on osteoblast activity and bone matrix formation.

1,25(OH)<sub>2</sub>D<sub>3</sub> is one of the well known primary regulators of calcium homeostasis in the body and is recognized as a regulator of both osteoblast mediated bone formation and osteoclast mediated bone resorption. Osteoblasts express VDR [20,21] and 1,25(OH)<sub>2</sub>D<sub>3</sub> direct actions include modulation of cellular proliferation and stimulation of differentiation [22]. We hypothesized that caffeine decreases VDR expression and affects osteoblastic activity which in turn may contribute to decrease in BMD. In the present study we examined the effect of increasing doses of caffeine on VDR protein expression and alkaline phosphatase activity, a marker of osteoblast activity.

## 2. Materials and methods

### 2.1. Chemicals

Caffeine and the reagents for alkaline phosphatase assay were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). 1,25-Dihydroxy vitamin D<sub>3</sub> was a generous gift from Dr. M. Uskokovic (Roche Bioscience, Palo Alto, CA). The monoclonal antibody to VDR (MA1-710) was from Affinity Bioreagents Inc., Golden, CO. Horse radish peroxidase-conjugated secondary antibodies were purchased from BioRad laboratories (Richmond, CA) and the ECL chemiluminescence detection reagent was from Amersham Pharmacia (Piscataway, NJ).

### 2.2. Cell culture

Osteoblast-like cell lines, U2-OS and MG-63 were obtained from the American Type Culture Collection and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and 100 µg/ml of streptomycin and 100 ml<sup>-1</sup> units of penicillin.

### 2.3. Western blot analysis

Forty-eight hours before the treatment, the U2-OS cells were plated at 5 × 10<sup>5</sup> density. Twelve hours before treatment, the medium was changed to DMEM with 10% dextran-coated charcoal-stripped FBS and antibiotics. The cells were treated with ethanol or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) + increasing doses of caffeine (0.2, 0.5, 1.0 and 10 mM).

After 24 h of treatment, the cells were harvested in PBS by scraping and cells were lysed in buffer containing 400 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% non-

idet P-40 (NP40), 10 mg/ml phenylmethylsulfonyl fluoride (PMSF) (10 µl/ml), aprotinin (30 µl/ml) and 100 mM sodium orthovanadate (10 µl/ml) by vortexing, followed by incubation on ice for 15–30 min. The lysates were centrifuged and about 20 µg of total protein from each sample were loaded and resolved on 10% SDS/PAGE and then transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell Inc, Keene, NH). The nitrocellulose membrane was blocked in blocking buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20 and 5% dried non-fat milk) overnight at 4 °C. The membrane was then incubated with VDR monoclonal antibody in incubation buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20 and 1% dried non-fat milk) for 1 h. After extensive washing, the membrane was incubated with appropriate horse radish peroxidase-conjugated secondary antibody for 1 h. Detection of specifically bound proteins was carried out using enhanced chemiluminescence detection system according to the instructions of the manufacturer. Membranes were exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

To quantify the expression levels of VDR protein, the Western blots were scanned by NIH Image 1.62 program; data was plotted as densitometric units.

### 2.4. Measurement of alkaline phosphatase activity

Twenty-four hours before treatment, MG-63 cells were plated in 6 well plates at 3 × 10<sup>5</sup> density in DMEM with 10% dextran-coated charcoal-stripped serum and antibiotics. The cells were treated with either ethanol or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) + increasing doses of caffeine (0.2, 0.5, 1.0 and 10 mM). After 72 h, the cells were washed twice with PBS. The enzyme reaction was started by addition of 500 µl of substrate/buffer mix [equal volumes of *p*-nitrophenol phosphate substrate (N 1891, Sigma Chemicals, St. Louis, MO) and alkaline buffer solution (A9226, Sigma Chemicals, St. Louis, MO)]. After 30 min of incubation at 37 °C, the reaction was stopped by adding equal volume of 0.05 M NaOH. The lysate from the wells were collected into individual eppendorf tubes and vortexed. The ALP activity was determined calorimetrically at 405 nm using *p*-nitrophenol standards (N7660, Sigma Chemicals, St. Louis, MO) 0–50 nmol. The protein concentration in the lysates was determined using the Bradford assay. The ALP activity is expressed as nmol *p*-nitrophenol (PNP) released/mg protein.

## 3. Results

Based on our clinical observation of increased bone loss in women with VDR genotype, tt compared to VDR genotype TT when their caffeine consumption was greater than 300 mg/day, experiments were carried out to determine if in vitro caffeine effects the VDR protein expression in human osteoblast cells. Osteoblast cells were treated with phys-

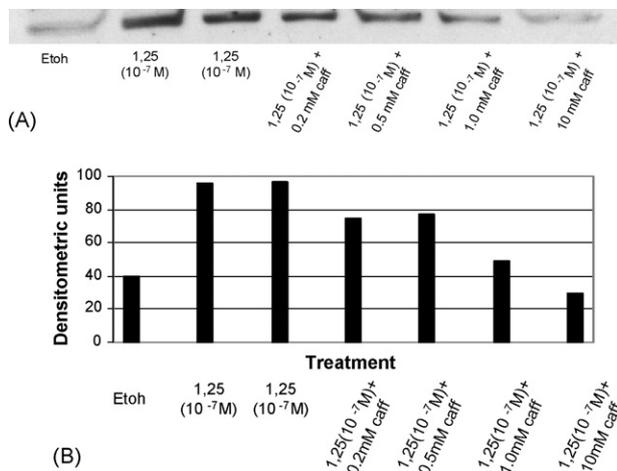


Fig. 1. Effect of increasing doses of caffeine on  $1,25(\text{OH})_2\text{D}_3$  induced VDR expression in osteoblast cells. Osteoblast cells were treated with ethanol or  $1,25(\text{OH})_2\text{D}_3$  or +caffeine (0.2, 0.5, 1.0 and 10 mM). After 24 h treatment, cells were lysed, centrifuged and 20  $\mu\text{g}$  protein from each treatment was resolved on 10% SDS–PAGE and transferred to nitrocellulose membrane. The immunoblots were probed with VDR monoclonal antibody (A) and quantitated by scanning by NIH image 1.62 program; data was plotted as densitometric units (B). Results are representative of three independent experiments.

ological level of  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-7}$  M) and increasing doses of caffeine and VDR protein expression was determined by Western blotting. It was observed that caffeine dose dependently decreases the  $1,25(\text{OH})_2\text{D}_3$  induced VDR expression (Fig. 1A). Compared to VDR expression seen with  $1,25(\text{OH})_2\text{D}_3$  (lanes 2 and 3), caffeine decreased the VDR protein expression by 50 and 70% at the doses of 1 and 10 mM, respectively (lanes 6 and 7) (Fig. 1B).

Next we examined if caffeine has any effect on the osteoblast activity by examining the effect of increasing doses of caffeine on alkaline phosphatase activity, a well known marker of osteoblast differentiation. At 0.5 mM caffeine did not have an effect on alkaline phosphatase activity, however at 1 mM concentration caffeine decreased  $1,25(\text{OH})_2\text{D}_3$  stimulated ALP activity by about 50% (Fig. 2). Treatment of

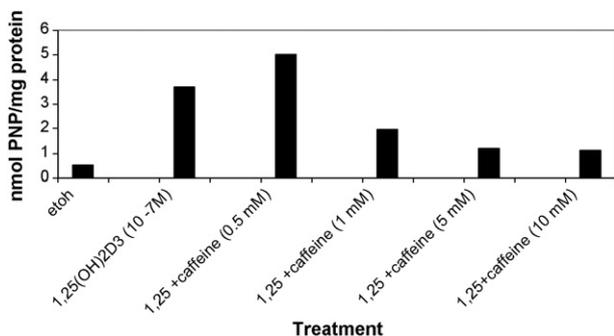


Fig. 2. Effect of increasing doses of caffeine on  $1,25(\text{OH})_2\text{D}_3$  stimulated alkaline phosphatase activity in osteoblast cells. Osteoblast cells were treated with ethanol or  $1,25(\text{OH})_2\text{D}_3$  or +caffeine (0.2, 0.5, 1.0 and 10 mM). After 72 h treatment, the alkaline phosphatase activity was determined calorimetrically at 405 nm. Results are representative of three independent experiments.

osteoblast cells with increasing doses of caffeine alone did not have any effect on the basal activity (data not given).

Overall these results suggest that caffeine decreases VDR protein expression and affects the osteoblast function.

#### 4. Discussion

The results presented in the paper demonstrate for the first time that caffeine dose dependently decreases VDR protein expression and alkaline phosphatase enzyme activity, a marker of osteoblast differentiation in osteoblast cells. The effect of caffeine on VDR expression was evident at the lowest dose of caffeine tested (0.2 mM), though about 50% and 70% reduction in VDR protein expression was observed at 1 and 10 mM concentrations of caffeine, respectively. However, a significant effect of caffeine on alkaline phosphatase activity was observed at 1 mM dose. Earlier studies on caffeine on osteoblast growth [19,23] have used caffeine doses in the range of 0.1–0.4 mM; while studies of caffeine on the release of intracellular calcium have used much higher concentrations of caffeine (4 and 10 mM) [24,25].

The role of caffeine as a risk factor for osteoporosis is controversial, though several studies have shown that caffeine consumption is associated with decreased BMD. In a recent study, we also demonstrated increased bone loss of –8% in women with VDR genotype tt compared to VDR genotype TT when their caffeine consumption was >300 mg/day [13]. There are very few studies in literature which explored the molecular basis for the effect of caffeine on bone. Tassinari et al. [19] reported that caffeine inhibits the formation of a competent extracellular matrix during the osteoblast differentiation sequence leading to delayed ossification. Caffeine has also been demonstrated to inhibit osteoblast cell proliferation [23]. In a recent study, Focking et al. [26] demonstrated that caffeine drastically amplified glucocorticoid receptor transcriptional activity in osteoblastic cells. They propose that caffeine may act via modulating glucocorticoid receptor activity, thus increasing the risk for osteoporosis.

In summary, our data provides evidence of a direct effect of caffeine on VDR protein expression and osteoblast activity, which could be one of the probable responsible molecular mechanisms for the role of caffeine in osteoporosis.

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