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ORIGINAL RESEARCH

# The Effect of Caffeine on Calcitriol-Inducible Vitamin D Receptor-Controlled Gene Expression in Intestinal and Osteoblastic Cells

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**Abstract**
Some epidemiological studies suggested caffeine consumption as the cause for bone mineral density loss. Certain genes involved in this process are regulated by vitamin D receptor (VDR). Therefore, we investigated if caffeine can affect inducible expression of VDR-regulated genes, some of them being involved in bone mineralization process. By employing reporter gene assay, polymerase chain reaction, and western blotting, we monitored the VDR activity and expression in cell cultures of intestinal (LS180), osteosarcoma (HOS), and normal human osteoblasts in vitro. While caffeine stimulated calcitriol-inducible VDR-dependent nanoluciferase activity in stable reporter cell line IZ-VDRE (derived from LS180), it rather modulated mRNA levels of target genes, like CYP24A1, BGLAP, SPP1, and TNSF11 in LS180 and HOS cells. However, caffeine significantly decreased calcitriol-inducible CYP24A1, TNSF11, and SPP1 transcripts in osteoblasts. This decrease had non-linear U-shaped profile. Our in vitro data demonstrate biphasic action of caffeine on the expression of certain calcitriol-inducible VDR-regulated genes in normal human osteoblasts.

**Keywords** Osteoblasts · HOS · IZ-VDRE · BGLAP · CYP24A1 · SPP1

## Introduction

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring alkaloid known for its stimulative effects. It can be found in coffee, tea, energetic drinks, or chocolate. An average daily income of caffeine is estimated at 186 mg per day [1] with 98% being ingested by drinks. The pharmacological effect of caffeine lies typically between 1 and 10 µM and the blood concentration is dependent on available amount and the form of caffeine. In the case of 180 ml of instant coffee with 96.34 mg caffeine, the maximal plasmatic concentration reaches 2.47 µg/ml (approx. 12.72 µM) after 26 min [2]. For higher doses, the data are controversial and are usually connected with toxic outputs from case reports. For example, drinking of a liter of black coffee containing approximately 565 mg of caffeine resulted in the plasma concentration of 4 µg/ml (approx. 20.6 µM) after 10 h [3]. Initial concentration was estimated to 16 µg/ml (approx. 83 µM) with rhabdomyolysis being the main toxic output. Higher doses (in the range of grams) of caffeine are toxic and are administered either accidentally or as suicide attempts. Concentrations in millimolar range were described *post mortem* [4].

The main cellular targets of caffeine are G-coupled adenosine receptors, which are antagonized by caffeine presence and this can lead to adenylate cyclase inhibition. Adenosine receptors can be found in the brain, but also in cardiovascular, pulmonary, gastrointestinal system [5] as well as in the cells of bone marrow, osteoblasts, and osteoclasts [6]. The most significant effect of caffeine is brain activity stimulation, which is characterized by shortening the reaction time, mitigating the fatigue, and increasing the vigilance.

Caffeine consumption has been associated with undesirable reproductive effects, cardiovascular disease, or osteoporosis. Many epidemiological studies demonstrated mutually contradictory effects, which can be described by U- or J-shaped curves, particularly for cardiovascular disease [7]. However, similar effect was described for bone health status as well, where certain studies show the

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relationship between osteoporosis occurrence and caffeine consumption [8, 9], but the others do not [10, 11].

Vitamin D receptor (VDR) is a ligand-activated transcription factor that mediates the action of 1α,25-dihydroxyvitamin D (calcitriol), the active form of vitamin D. VDR participates in mineral homeostasis and therefore, it is abundantly expressed in tissues participating in metabolism of calcium—like intestine, kidneys, and bones. Upon ligand binding, VDR binds to responsive elements in the upstream of target genes, many of them being involved in bone metabolism. Among these genes is BGLAP, the product of which is osteocalcin (OCN) [12, 13], a vitamin K-dependent carboxylated peptide that binds calcium ions and it forms the majority of non-collagen proteins in organic component of the bones [14]. Other important vitamin D responsive genes include SPP1 (protein product osteopontin; OPN) and TNSF11 (protein RANKL; receptor activator of nuclear factor kappaB ligand). First one participates in bone mineralization process, second one has been originally identified in T-lymphocytes [15], later described to be functional in bone metabolism as well [16].

Since VDR is involved in calcium homeostasis and it regulates the expression of genes involved in bone health, it is striking that the effect of caffeine on VDR signaling has been studied insufficiently. One study on elderly postmenopausal women demonstrated that women with high caffeine intake of more than 300 mg of caffeine daily (approx. 3 cups of coffee) have higher bone mass loss at the spine when carrying homozygous constitution of VDR gene (*tt*; defined by the presence of restriction site for *TaqI*) [17]. This research team, in an effort to find a molecular understanding of this observation, exposed the human osteoblast-like cell lines (U2-OS, MG-63) to different doses of caffeine in combination with calcitriol [18]. While 100 nM calcitriol resulted in the induction of alkaline phosphatase activity (ALP) and stabilization of VDR protein, the combination with caffeine resulted in the dose-dependent decrease in ALP activity as well as VDR protein amount. However, these effects were observed with caffeine in millimolar, i.e., toxic and physiologically unreachable concentrations (from 0.2 up to 10 mM).

Not many studies tried to link caffeine effects with VDR-regulated genes involved in bone matrix formation. Therefore, we investigated the effects of caffeine on VDR transcription activity and the inducible expression of VDR target genes in human intestinal adenocarcinoma cells (LS180), human osteosarcoma cells (HOS), and primary culture of human osteoblasts. Our hypothesis that caffeine impacts VDR activity was based on the fact that adenosine receptors inhibition by caffeine results in the change of cAMP concentration, which results in the change of protein kinase A (PKA) activity that is known to affect VDR [19].

## Materials and Methods

### Compounds and Reagents

Dimethylsulfoxide (DMSO), 1α,25-dihydroxycholecalciferol (calcitriol; D1530), DMEM medium (D6546), Charcoal-stripped Fetal Bovine Serum (CS-FBS; F6765), and caffeine (C0750) were purchased from Merck/Sigma-Aldrich (Prague, Czech Republic). Oligonucleotide primers used in RT-PCR reactions were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). LightCycler 480 Probes Master was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

### Cell Cultures

Human Caucasian adenocarcinoma (LS180; ECACC 87021202) and human Caucasian osteosarcoma (HOS; ECACC 87070202) were purchased from Public Health England and with IZ-VDRE cells (derived from LS180) cultured in DMEM medium supplemented with 10% of regular or charcoal-stripped (for treatment purposes) fetal bovine serum, 2 mM L-glutamine, and 1% non-essential amino acids. Cells were maintained at 37 °C and 5% $CO_2$ in a humidified incubator.

Normal human osteoblasts (NHOst; CC-2538) were cultivated in Osteoblast Growth Media Bulletkit (CC-3207) containing osteoblast basal medium and supplements and growth factors (FBS, ascorbic acid, gentamicin/amphotericin-B), both purchased from Lonza (Eastport, Czech Republic). Cells were maintained at 37 °C and 5% $CO_2$ in a humidified incubator.

### Cell Viability Assay (MTT)

Cell line IZ-VDRE was treated with increasing concentrations of caffeine (0.1–100 mM) and/or vehicle (DMSO; 0.1% v/v) for 24 h. Thereafter, the medium was replaced by PBS with MTT (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in final concentration of 0.3 mg/ml. The solution was discarded after 30–40 min of incubation and replaced with DMSO for dissolution of the formazan crystals. Absorbance was measured at 570 nm with Infinite M200 (TECAN, Austria). Tested concentrations, which decreased the viability under 80% of negative control, were excluded from further experimentation. Four independent experiments (i.e., consecutive passages) were performed.

### Reporter Gene Assay

For the assessment of VDR transcriptional activity, we employed recently established gene reporter cell line

IZ-VDRE (kindly provided by Dr. Z. Dvorak) [20]. Following the plating in medium with CS-FBS, cells were stabilized overnight and then treated with caffeine (0.0001–5 mM) in the absence or presence of calcitriol (75 nM) and/or vehicle (DMSO; 0.1% v/v) for 24 h in medium with CS-FBS. After the treatments, cells were lysed and luciferase activity was measured with Infinite M200 (TECAN, Austria). Up to 8 independent experiments (i.e., consecutive passages) were performed.

### Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated using *TRI Reagent*® (Molecular Research Center, USA). cDNA was synthesized from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (M0253S, New England BioLabs) at 42 °C for 60 min in the presence of random hexamers (S1230, New England BioLabs). qRT-PCR was carried out on Light Cycler 480 II apparatus (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP24A1, BGLAP, SPP, TNSF11, and GAPDH mRNAs were determined using primers and probes from Universal Probes Library (UPL; Roche Diagnostic Corporation, Prague, Czech Republic) (Table 1). The following program was used for monitoring the expression of both genes: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s; annealing with elongation at 60 °C for 30 s). The measurements were performed in triplicates. Gene expression was normalized per *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene. Data were processed with the delta–delta method. Results are expressed as fold induction over DMSO-treated cells. At least 3 independent experiments (i.e., consecutive passages) were performed for transcript quantification.

### SDS-PAGE and Western Blotting

The isolation of total protein extracts and SDS-PAGE procedure were described elsewhere [21].

The following proteins were detected either by classic western blot technique (HOS cells) or by fully quantitative Sally Sue Simple western system [22] (human osteoblasts—due to the limited amount of cells and limited number of division cycles): osteopontin (mouse monoclonal antibody, Santa Cruz, sc-73631), osteocalcin (mouse monoclonal antibody, Santa Cruz, sc-74495), and actin (mouse monoclonal, Cell Signaling Technology, 3700S).

### Statistical Analysis

All statistical analyses were performed in GraphPad PRISM 6 software (GraphPad Software Inc., San Diego, CA, USA) by using One-way ANOVA with a Dunnett's post hoc test. A $p$ value of $<0.05$ was considered to be statistically significant.

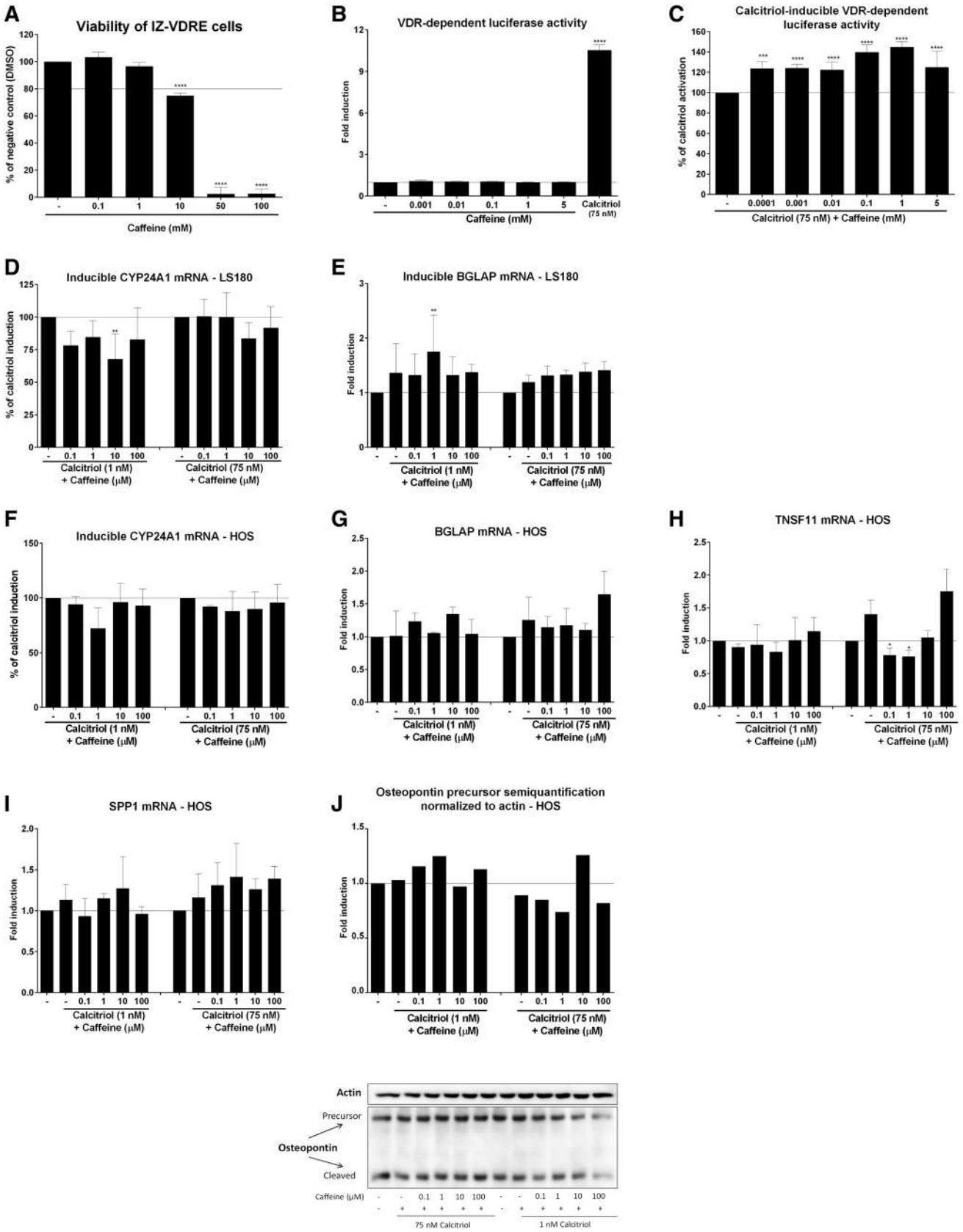
## Results

At the beginning, we asked a question if caffeine can affect the activity of VDR. For this purpose, we employed recently developed IZ-VDRE cell line, which contains stably transfected nanoluciferase reporter system responding to the change in VDR activity [20]. However, this cell line was first used to establish non-toxic caffeine concentrations. We found that caffeine concentrations higher than 1 mM resulted in the significant decrease of IZ-VDRE viability (Fig. 1a). Since the $IC_{20}$ was roughly around 5 mM, we further tested this concentration as the highest in reporter gene assay (RGA). Caffeine had no impact on VDR-dependent nanoluciferase activity, while positive control (calcitriol; 75 nM) induced nanoluciferase activity approx. 10-fold (Fig. 1b). However, when combined together, caffeine significantly potentiated calcitriol-inducible VDR-dependent nanoluciferase activity about 20–40% above calcitriol itself (Fig. 1c). This effect was observed from 0.1 µM to 5 mM of caffeine concentration. Moreover, due to the pharmacokinetic data (see "Introduction" section) and slight cytotoxicity of 5 mM caffeine, we further used caffeine up to 100 µM concentration only.

Since IZ-VDRE cells were derived from LS180, we wanted to confirm caffeine-potentiated calcitriol effect at mRNAs for target genes of VDR in this cell line. We

**Table 1** Primer sequences with appropriate UPL numbers

| Gene symbol | Forward primer | Reverse primer | UPL probe number |
|---|---|---|---|
| GAPDH | CTCTGCTCCTCCTGTTCGAC | ACGACCAAATCCGTTGACTC | 60 |
| CYP24A1 | TCATCATGGCCATCAAAACA | GCAGCTCGACTGGAGTGAC | 88 |
| BGLAP | CCAGCCCTATGGATGGG | TTTTCAGATTCCTCTTCTGGAGTT | 32 |
| SPP1 | CGCAGACCTGACATCCAGTA | GGCTGTCCCAATCAGAAGG | 61 |
| TNSF11 | TTCAAGGAGCTGTGCAAAAG | GCTCCTCTTGGCCAGATCTAA | 158 |


A
Viability of IZ-VDRE cells
% of negative control (DMSO)
Caffeine (mM)
B
VDR-dependent luciferase activity
Fold induction
Caffeine (mM)
Calcitriol (75 nM)
C
Calcitriol-inducible VDR-dependent luciferase activity
% of calcitriol activation
Calcitriol (75 nM) + Caffeine (mM)
D
Inducible CYP24A1 mRNA - LS180
% of calcitriol induction
Calcitriol (1 nM) + Caffeine (μM)
Calcitriol (75 nM) + Caffeine (μM)
E
Inducible BGLAP mRNA - LS180
Fold induction
F
Inducible CYP24A1 mRNA - HOS
% of calcitriol induction
G
BGLAP mRNA - HOS
Fold induction
H
TNSF11 mRNA - HOS
Fold induction
I
SPP1 mRNA - HOS
Fold induction
J
Osteopontin precursor semiquantification normalized to actin - HOS
Fold induction
Actin
Precursor
Osteopontin
Cleaved
Caffeine (μM)
75 nM Calcitriol
1 nM Calcitriol

◂**Fig. 1** The impact of caffeine on inducible VDR activity in LS180 and HOS cells. **a** Caffeine decreases viability of IZ-VDRE cells (derived from LS180) after 24 h; **b** caffeine has no effect on VDR-driven luciferase activity in IZ-VDRE cells after 24 h; **c** caffeine potentiates calcitriol-inducible VDR-driven nanoluciferase activity in IZ-VDRE cells after 24 h. Modulatory effect of caffeine on calcitriol-inducible CYP24A1 mRNA (**d**) and BGLAP mRNA (**e**) in LS180 cells after 24 h. Caffeine modulates calcitriol stimulated expression of CYP24A1 (**f**), BGLAP (**g**), TNSF11 (**h**), and SPP1 (**i**) mRNAs in HOS cells after 24 h. **j** Semi-quantification of osteopontin precursor and representative western blot of precursor and cleaved product of osteopontin in HOS cells stimulated by combinations of caffeine and calcitriol—24 h. **a**–**i** Data represent mean ± SEM of five or more consecutive passages. *, **, ***, ****—value is significantly different from untreated cells (DMSO) (**a**, **b**) or calcitriol-treated cells (**c**, **d**, **h**) ($p < 0.05$, 0.01, 0.001, 0.0001)

performed these measurements under two calcitriol concentrations, 1 and 75 nM, lower representing a physiologically deficient state with sub-saturating VDR activation and higher representing physiologically saturating state of VDR activation. As a marker of functional VDR-dependent pathway, we always measured CYP24A1 mRNA first. Lower calcitriol concentration (1 nM) induced CYP24A1 weakly (approx. 13–839 over DMSO-treated cells) and the presence of caffeine decreased mostly insignificantly mRNA level (Fig. 1d, *left*). Saturating concentration of calcitriol (75 nM) induced CYP24A1 robustly (approx. 27000–47000 over DMSO-treated cells) but the CYP24A1 mRNA expression was not affected by caffeine (Fig. 1d, *right*). Among expected genes associated with bone matrix formation, we were able to detect and measure BGLAP (osteocalcin) expression in LS180 only. The induction of BGLAP by calcitriol was insignificant with no additional impact of caffeine (Fig. 1e).

In the next step, we evaluated the effect of caffeine on inducible expression of VDR target genes in human osteosarcoma cells (HOS). In this cell line, we were able to detect all desired transcripts. A marker of VDR activation, CYP24A1 was induced in average 1.2- and 16-fold over control for 1 and 75 nM calcitriol, respectively. However, no significant effect of caffeine was observed (Fig. 1f). Similarly, there was no effect of caffeine on expression of BGLAP or SPP1 (Fig. 1g, i). However, two lowest concentrations of caffeine decreased calcitriol-inducible TNSF11 mRNA in HOS cells significantly with remaining concentrations having rather opposite effect (Fig. 1h). In addition, we tested if this cell line expresses osteocalcin or osteopontin at protein level. While we were not able to detect osteocalcin, we detected two discrete bands for osteopontin (Fig. 1j; *lower panel*). These should belong to osteopontin precursor (approx. 66 kDa) and cleaved products (25–55 kDa), as antibody manufacturer claims. However, in general, no concentration-dependent effect of caffeine was observed.

At the end of the study, we used probably the most relevant in vitro model, normal human osteoblasts (NHOst). A marker of VDR activity, CYP24A1 was induced by calcitriol at 1 nM (range 13–41-fold induction over DMSO-treated cells) and 75 nM (range 2100–93000-fold induction over DMSO-treated cells) concentration. For 1 nM calcitriol, caffeine displayed J-shaped dose–response curve as 0.1 and 1 μM concentrations decreased CYP24A1 mRNA but 10 and 100 μM had rather opposite effect (Fig. 2a, *left*). Similar but less intensive and insignificant pattern was observed for saturating calcitriol concentration (Fig. 2a, *right*). The expression of TNSF11 mRNA was induced by 1 nM calcitriol only weakly (approx. 1.5-fold induction over DMSO-treated cells) but much strongly with 75 nM calcitriol (approx. 7.3-fold induction over DMSO-treated cells). In the latter case, caffeine displayed U-shaped dose–response pattern (Fig. 2b, *right*). The expression of BGLAP mRNA was induced by 1 nM (range 3–7-fold induction over DMSO-treated cells) as well as by 75 nM (range 20–60-fold induction over DMSO-treated cells) calcitriol but the presence of caffeine had no effect (Fig. 2c). Last investigated gene, SPP1 (osteopontin) was not induced by 1 nM but 75 nM (range 2.5–10.5-fold induction over DMSO-treated cells) calcitriol. However, only the lowest concentration of caffeine decreased significantly calcitriol-inducible SPP1 mRNA (Fig. 2d, *right*). In the culture of human osteoblasts, we were able to detect proteins, osteocalcin, and osteopontin. Osteocalcin was not induced by 1 nM calcitriol, and caffeine had no additional effect (Fig. 2e). Saturating concentration of calcitriol (75 nM) induced osteocalcin in average 2.5-fold and caffeine stimulated further increase of the protein by 50–100% above calcitriol itself (Fig. 2e). In the case of osteopontin, we clearly detected the precursor product, which was induced for both 1 and 75 nM concentrations of calcitriol (approx. 1.9- and 2.3-fold induction over DMSO-treated cells, respectively). While caffeine weakly potentiated low calcitriol-inducible osteopontin protein, it had biphasic effect on high calcitriol-inducible osteopontin protein level (Fig. 2e; *right upper panel*).

## Discussion

In the current paper, we investigated if caffeine affects VDR-driven inducible expression of genes involved particularly in bone mineralization process. In general, for physiologically relevant concentration, we found weak but significant stimulation of VDR-driven nanoluciferase activity by caffeine in IZ-VDRE cells but relatively weak non-linear biphasic effect on calcitriol-inducible genes, particularly in normal human osteoblasts (NHOst). The simplified overview of obtained results is shown in Table 2.

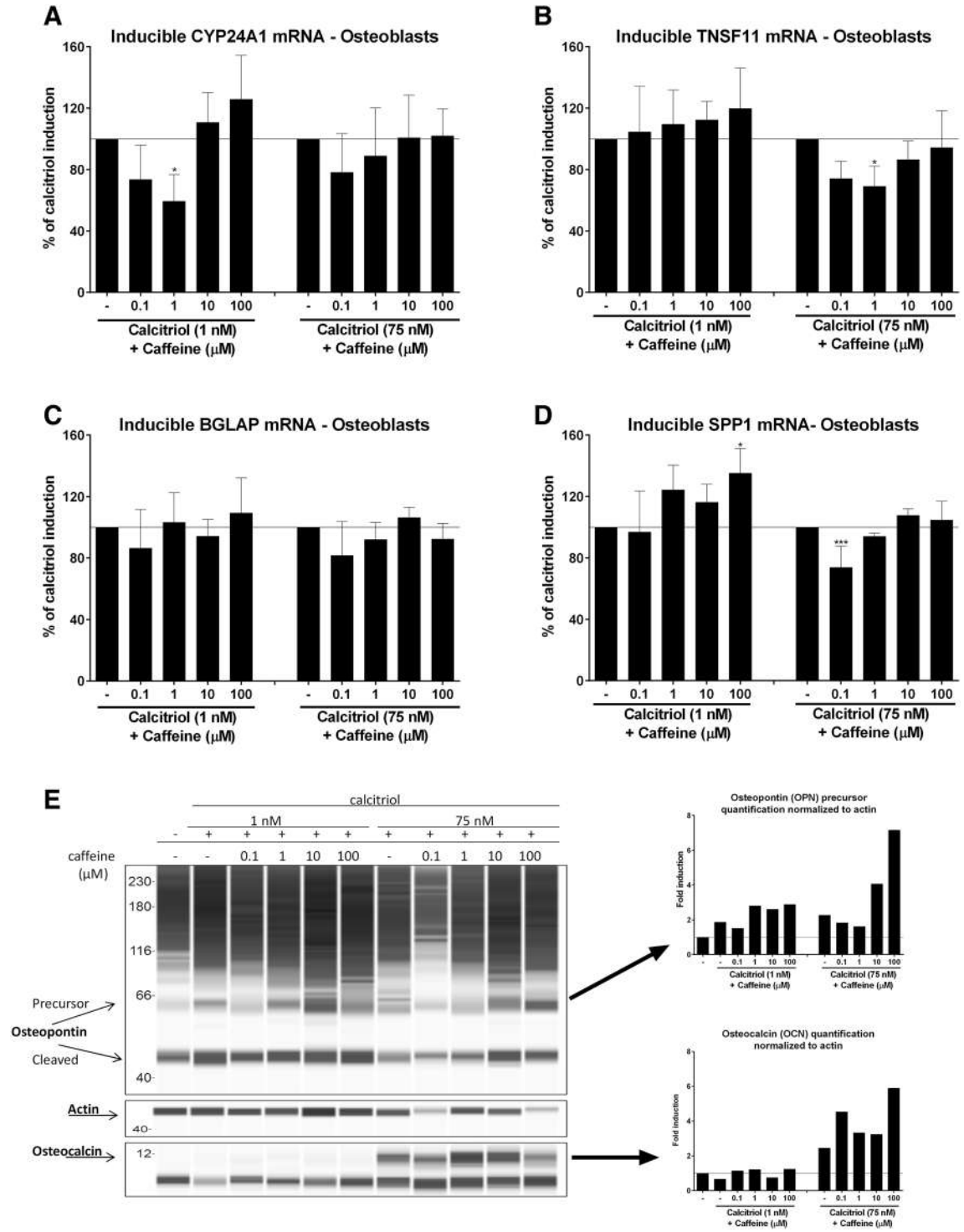



**Fig. 2** The impact of caffeine of inducible expression of VDR target genes in human osteoblasts. Caffeine modulates calcitriol-inducible expression of CYP24A1 (**a**), TNSF11 (**b**), BGLAP (**c**), and SPP1 (**d**) mRNAs in human osteoblasts. **e** Representative virtual western blots of osteocalcin, actin, and osteopontin after 24 h and quantification analysis from human osteoblasts is shown. **a**–**d** Data represent mean ± SEM of four consecutive passages. *, ***—value is significantly different from calcitriol-treated cells (**a**, **b**, **d**) ($p < 0.05$, 0.001)

First of all, there is a striking difference between effects of caffeine in calcitriol-inducible VDR-dependent nanoluciferase activity and calcitriol-inducible VDR-dependent gene expression. While significant reproducible potentiation was observed at the level of nanoluciferase activity, it was not observed at the level of mRNA for typical marker

**Table 2** Summary of caffeine on calcitriol-inducible VDR activity and mRNAs for VDR target genes

| | | Caffeine | Calcitriol (nM) | | Caffeine + calcitriol (nM) | |
|---|---|---|---|---|---|---|
| | | | 1 | 75 | 1 | 75 |
| IZ-VDR | VDR activity | – | N.D. | ↑ | N.D. | ↑↑ |
| LS180 | CYP24A1 | ND | ↑ | ↑↑ | ↓ | – |
| | BGLAP | | – | – | ↑ | – |
| HOS | CYP24A1 | ND | ↑ | ↑↑ | – | – |
| | BGLAP | | – | – | – | – |
| | SPP1 | | – | – | – | – |
| | TNSF11 | | – | – | – | ↓ |
| Osteoblasts | CYP24A1 | ND | ↑ | ↑↑ | ↓ | – |
| | BGLAP | | ↑ | ↑↑ | – | – |
| | SPP1 | | – | ↑ | ↑ | ↓ |
| | TNSF11 | | ↑ | ↑↑ | – | ↓ |

– = No effect, one arrow = significant effect (in case of calcitriol next to DMSO-treated cells; in case of caffeine next to calcitriol-treated cells for at least one caffeine concentration); two arrows = stronger effect in comparison with adequate counterpart (e.g., calcitriol 1 vs. 75 nM)

*ND* not determined

of VDR activity, CYP24A1. This is surprising, especially when IZ-VDRE cells, which were used for monitoring VDR-dependent nanoluciferase activity, are derived from LS180 and contain three copies of vitamin D responsive element-I (VDRE-I) from CYP24A1 gene [20]. This difference suggests that other components of transcription machinery controlling the CYP24A1 expression are affected by caffeine more than VDR itself. This disturbance is more prevalent for sub-saturating calcitriol concentration both in LS180 and NHOst. Similar profile, as for CYP24A1, was observed at TNSF11 mRNA for VDR-saturating calcitriol concentration (Fig. 2b, *right*). While inducible by both calcitriol concentrations, caffeine displayed biphasic effect for the higher one, both in HOS as well as NHOst (Fig. 1h, 2b). This approximately 30% decrease of VDR-saturating calcitriol-inducible TNSF11 mRNA in the presence of caffeine also suggests that there exists caffeine concentration window which might have protective effect against osteoporosis. This statement is based on the fact that the decrease in RANKL (TNSF11 gene product) leads to osteopetrosis (denser bones), as it was observed in RANKL-deficient mice [23].

Two remaining genes, the expression of which is controlled by VDR, we were able to detect at both mRNA and protein levels in human osteoblasts. The least affected was the expression of BGLAP that was not induced in any cancer cell lines by calcitriol but the induction was observed in human osteoblasts. However, caffeine had no effect on calcitriol-inducible BGLAP mRNA. This is not completely true for protein of BGLAP, osteocalcin (OCN), where some synergistic action was observed for caffeine and calcitriol at OCN protein level in comparison to calcitriol alone (Fig. 2e). This might suggest that caffeine affects protein stability rather than expression. This observation is in good agreement with the effect of caffeine on bone stromal cell line (M2-10B4), where OCN expression was increased above control with 100 μM but decreased with 300 μM and 1000 μM caffeine [24]. The same pattern was observed for calcitriol-induced alkaline phosphatase (ALP) activity in human osteosarcoma cells, where 200 μM caffeine stimulated but higher concentrations suppressed the effect of calcitriol [18]. However, due to the use of toxic and physiologically unreachable caffeine concentrations in both mentioned studies, the relevance of this finding for assessing the effects of caffeine on bone mineralization or remodeling process via VDR-controlled genes is uncertain.

The expression of SPP1 gene and its protein product osteopontin (OPN) had similar expression pattern with other studied VDR-controlled genes. However, the detection of OPN expression at protein level is complicated by the fact that OPN has a variable molecular mass between 44 and 75 kDa [25] which is likely a consequence of cell-/tissue-specific alternate splicings and post-translational modifications. This may also be the reason why there were only two discrete bands in HOS cells (Fig. 1j) but 2 bands and a smear in human osteoblasts (Fig. 2e).

In this study, we did not find a definite effect of caffeine on calcitriol-inducible expression of VDR-regulated genes involved in bone matrix formation. However, bone matrix formation, bone mineral density, or fracture risk might be affected by certain VDR polymorphisms, as it was suggested in some epidemiological studies [26, 27]. Therefore, caffeine might interact differently with signaling of VDR polymorphic variants. Among most studied VDR polymorphisms belong *BsmI*, *ApaI*, *TaqI*, and *FokI* polymorphisms. While

the first trio can be found close to the 3′ terminus of the VDR gene, they do not determine structural modifications of the receptor. Thus, the impact of caffeine on VDR activity is not likely, even though some authors hypothesized the effects of these polymorphisms on mRNA stability [28] and Rapuri et al. [17] found accelerated bone loss at the spine of elderly postmenopausal women with *tt* genotype. The only meaningful polymorphism, *FokI* results in the shorter version of VDR, which was reported to have different transcription rates than the longer one [29]. Thus, homozygous expression of *FokI* variant might provide slightly different results and it represents a future research challenge.

In conclusion, physiologically reachable caffeine concentrations modulate calcitriol-inducible expression of some VDR-driven genes, particularly in normal human osteoblasts in vitro. These non-linear biphasic dose-dependencies, which were observed in other cellular systems for caffeine, may at least partially contribute to explanation of contradictions from epidemiological studies, where other factors might be involved. These factors involve coordination mainly but not exclusively of osteoblasts and osteoclasts together with other bone matrix-forming cells and solid particles, the bone is made of. Therefore, the lack of any component of bones in cell culture makes difficult to do any conclusion on the impact of caffeine on bone health in human population. Moreover, based on comprehensive literature search of published human studies, it was concluded that moderate daily intake of caffeine at the dose up to 400 mg daily is not associated with adverse outcomes, like bone status and calcium balance (with consumption of adequate calcium) [30, 31]. Our data join this conclusion with statement that there may exist concentration window for caffeine that might act protectively against osteoporosis.

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## Compliance with Ethical Standards

**Conflict of interest** Ondrej Zenata, Adela Marcalikova, and Radim Vrzal declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human subjects performed by any of the authors.

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