Vitamin D Receptor Activators Induce an Anticalcific Paracrine Program in Macrophages
Requirement of Osteopontin

Xianwu Li, Mei Y. Speer, Hsueh Yang, Jamie Bergen, Cecilia M. Giachelli

Objectives—Vascular calcification is highly correlated with morbidity and mortality, and it is often associated with inflammation. Vitamin D may regulate vascular calcification and has been associated with cardiovascular survival benefits.

Methods and Results—We developed a macrophage/smooth muscle cell (SMC) coculture system and examined the effects of vitamin D receptor activators (VDRA), calcitriol and paricalcitol, on SMC matrix calcification. We found that treatment of SMC alone with VDRA had little effect on phosphate-induced SMC calcification in vitro. However, coculture with macrophages promoted SMC calcification, and this was strikingly inhibited by VDRA treatment. Several VDRA-induced genes, including bone morphogenetic protein-2 (BMP2), tumor necrosis factor-α, and osteopontin, were identified as candidate paracrine factors for the protective effect of VDRA. Of these, osteopontin was further investigated and found to contribute significantly to the inhibitory actions of VDRA on calcification in macrophage/SMC cocultures.

Conclusion—The ability of VDRA to direct a switch in the paracrine phenotype of macrophages from procalcific to anticalcific may contribute to their observed cardiovascular survival benefits. (Arterioscler Thromb Vasc Biol. 2010;30:321-326.)

Key Words: coculture ■ macrophage ■ osteopontin ■ smooth muscle ■ vascular calcification

Vascular calcification is a common finding in patients with cardiovascular disease, diabetes, and end-stage renal disease (ESRD). Vascular calcification has been positively correlated with coronary atherosclerotic plaque burden, increased risk of myocardial infarction, and plaque thickness and calcification score in children on dialysis. Although vitamin D overload-induced cardiovascular disease is usually secondary to hypercalcemia, hyperphosphatemia, and vascular calcification, the mechanisms medi-

The role of vitamin D in regulating vascular calcification appears to be complex. Hypervitaminosis D leads to ectopic calcification in people. Likewise, in experimental models, high levels of vitamin D, either in nonuremic or uremic animals, induce vascular calcification. These effects are in large part caused by the stimulatory effect of vitamin D on intestinal absorption of calcium and phosphate, thereby leading to elevated serum mineral levels that predispose to ectopic calcium deposition. However, clinical studies have shown that serum levels of calcitriol are inversely correlated with coronary artery disease and future cardiovascular events, including lower extremity amputation. In ESRD patients, vascular calcification is strongly correlated with coronary artery disease and future cardiovascular events, including lower extremity amputation. In ESRD patients, vascular calcification is a strong prognostic marker of cardiovascular disease mortality and is likely to be a major contributor to the 10- to 100-fold increase in cardiovascular mortality risk observed in these patients. Underscoring this problem, the American Heart Association has indicated that ESRD patients should be considered at the highest risk for cardiovascular disease.

Vitamin D is a steroid hormone that plays an essential role in mineral metabolism, skeletal health, and immunity. The active metabolite of vitamin D, calcitriol [1, 25 (OH)2 vitamin D], exerts the majority of its actions via the nuclear vitamin D receptor (VDR). The complex of calcitriol and the VDR binds to vitamin D response elements in the promoters of target genes and regulates gene expression. A broad spectrum of vitamin D-regulated genes have been identified, including those involved in bone and mineral metabolism, cell proliferation and differentiation, and immunomodulation.

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At the cardiovascular survival benefits of vitamin D remain obscure. The possibility that survival benefits of VDRA relate to regulation of vascular calcification was suggested by a recent study in uremic low-density lipoprotein-deficient mice. Lower doses of VDRA inhibited, whereas higher doses induced, atherosclerotic plaque calcification. In this regard, accumulating evidence indicates that vascular calcification is an actively regulated process involving several cell types, including vascular smooth muscle cells (SMC) and macrophages, 2 key components of atherosclerotic lesions. Numerous molecules that either promote or inhibit vascular calcification have been identified, including those with the ability to regulate the osteochondrogenic transdifferentiation of SMC. Accumulation of macrophages is associated with vascular calcification in human carotid and coronary arteries, and several in vitro studies have shown that macrophages regulate vascular calcification by promoting an osteogenic phenotypic transition of SMC. Thus, it is possible that the beneficial effects of VDRA on vascular calcification may be mediated via direct effects either on SMC, macrophages, or both. Thus, in the present study, we developed a macrophage/SMC coculture system to examine the effects of VDRA on SMC calcification in vitro.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement (available at http://atvb.ahajournals.org).

Human SMC were from Clonetics Corporation. Human THP-1 and mouse P388D1 macrophages were from ATCC, Manassas, VA (CCL-46). Macrophage/SMC cocultures were performed in transwells in growth media (GM) containing 1.24 mmol/L phosphate or calcification media (CM) containing 2.6 mmol/L phosphate. P388D1 cells deficient in osteopontin (OPN) or VDR were generated using the pSUPER RNA interference system (Oligoengine).

**Results**

**Expression of VDR in THP-1, Human SMC, and Mouse Macrophages**

VDR expression profiles were examined in the various cell types used in our study, including differentiated and undifferentiated human THP-1 cells, human SMC, and mouse P388D1 macrophages. As shown in Figure IA (please see http://atvb.ahajournals.org), bands at 264 bp or 208 bp were amplified from human or mouse cells, respectively. These results indicate that VDR is expressed in human and mouse macrophages, as well as in human SMC.

**VDRA Have Minimal Direct Effects on SMC Calcification In Vitro**

Previous studies have provided contradictory results regarding vitamin D action on SMC calcification in vitro. To determine the effect of VDRA in our system, human SMC were incubated with CM in the presence of either calcitriol or paricalcitol at concentrations of 0.5, 5, and 50 nM for 10 days, and calcium content of the extracellular matrix was determined. As shown in Figure IB, there was a small (~20%) reduction in calcium content compared to vehicle-treated control in VDRA-treated SMC, but these effects were not dose-dependent, suggesting that they were not VDR-mediated. Incubation of SMC with GM did not induce matrix calcification (data not shown), consistent with our previous studies.

**Macrophages Promote SMC Calcification in Cocultures**

To determine the regulatory roles of macrophages in vascular calcification, macrophage/SMC cocultures were performed in 6-well transwells. SMC and macrophages were cocultured in either GM or CM for 10 days, and calcium content in the extracellular matrix of SMC layer was determined. As shown in Figure 1, macrophage coculture substantially increased SMC calcification (SMC vs macrophage/SMC: 18.65 vs 66.97 μg Ca/mg protein; \( P<0.05 \)). However, no mineralization was observed in either SMC culture alone or macrophage/SMC coculture under normal phosphate conditions. The enhanced calcification observed in macrophage/SMC cocultures suggested that macrophages might release soluble factors that modulate the calcification capacity of SMC.

**Inhibition of SMC Calcification in Macrophage/SMC Cocultures by VDRA**

To determine the effect of VDRA on SMC calcification in macrophage/SMC coculture, the 2 cell types were incubated with GM or CM in the presence or absence of various concentrations of calcitriol or paricalcitol for 10 days. As shown in Figure 2, both calcitriol and paricalcitol dose-dependently inhibited SMC calcification induced by CM with maximal ~7.5-fold inhibition observed with 50 nM paricalcitol.
vitamin D receptor (VDR). Macrophages treated with VDR-specific siRNA had extremely low levels of VDR mRNA compared to cells treated with a control (CT) siRNA, indicating robust knockdown efficiency (Figure IIA). Using these cells, the effect of VDRA on calcification in macrophage–SMC cocultures was again examined. As shown in Figure IIB, P388D1 macrophages deficient in VDR (VDR siRNA) did not inhibit calcium deposition in response to either calcitriol or paricalcitol treatment, whereas macrophages that expressed normal levels of VDR (CT siRNA) showed significant inhibition of calcification in response to VDRA treatment.

The findings of VDR-dependent inhibition of SMC calcification by macrophages in response to VDRA treatment were in striking contrast to the minimal effects on calcification observed with VDRA treatment of SMC alone and the robust induction of SMC calcification observed after coculture with untreated macrophage. Together, these data suggest that VDRA, acting through the VDR, induce a procalcific switch, we tested several candidate vitamin D-responsive genes that were known to generate secreted proteins and that have been implicated in biomineralization. Thus, macrophages were treated with 50 nM calcitriol or paricalcitol for 3 or 6 days, and the expression levels of BMP2, tumor necrosis factor (TNF)-α, OPN, and transforming growth factor-β were determined by quantitative polymerase chain reaction. Figure 3A shows that calcitriol and paricalcitol equivalently inhibited BMP2 expression, with >70% inhibition observed after 6 days of treatment. TNF-α mRNA levels were also reduced by VDRA treatment at day 3 (Figure 3B), although this effect disappeared by day 6 (data not shown).

As shown in Figure 4A, both calcitriol and paricalcitol increased OPN mRNA levels with a maximal 2-fold increase observed with calcitriol. Consistent with mRNA results, both calcitriol and paricalcitol increased protein levels of OPN comparably (Figure 4B). Furthermore, OPN induction by VDRA was blocked by VDR siRNA (Figure III), indicating that the effect was mediated by VDR. VDRA treatment did not alter levels of transforming growth factor-β mRNA in macrophages (data not shown).

**Downregulation of OPN Production in Macrophages Prevents VDRA-Mediated Inhibition of SMC Calcification in Coculture**

To determine whether synthesis of OPN by VDRA-treated macrophages was required for their inhibitory effect on SMC calcification, we generated OPN-deficient macrophages via siRNA transduction of P388D1 mouse macrophages; ~80% reduction of OPN mRNA was achieved in OPN siRNA cells compared to CT siRNA cells, as determined by quantitative polymerase chain reaction (Figure IVA). To determine knockdown efficiency of siRNA on activated macrophages, CT siRNA and OPN siRNA macrophages were cultured in the presence of phorbol 12-myristate 13-acetate, a known inducer of OPN.43 Consistent with mRNA data (Figure IV), secreted OPN levels were 88% lower in OPN siRNA compared to CT siRNA cells in the absence of PMA (Figure IVB). PMA significantly increased OPN secretion in CT siRNA cells, and this effect was almost completely blocked in OPN siRNA cells (95% reduction in OPN siRNA vs CT siRNA; Figure IVB). Thus, OPN siRNA cells were deficient in both constitutive and inducible OPN expression.

To determine the specificity and identify potential off-target effects of OPN siRNA, mRNA levels of BMP2 and TNF-α were examined in both OPN siRNA and CT siRNA cells. BMP2 mRNA was not detected in either cell type in the presence or absence of VDRA (data not shown). TNF-α expression was similar in OPN siRNA and CT siRNA cells under basal conditions, and VDRA treatment inhibited TNF-α equivalently in the 2 cell types (Figure IVC). OPN siRNA and CT siRNA cells were then cocultured with SMC in CM in the presence of 50 nM calcitriol and paricalcitol for 10 days. Calcium content of the extracellular matrix of SMC layer was measured. As shown in Figure 5,
Discussion

Vascular calcification occurs in 2 different patterns in the arterial wall, depending on disease state. In atherosclerosis, calcification occurs predominantly in the arterial intima associated with inflamed and necrotic regions of the plaque. In arteriosclerosis, calcification occurs predominantly in the arterial media in the absence of inflammation. Both types of calcification are observed in ESRD patients and are thought to contribute to the increased cardiovascular disease risk in these patients.44,45

The majority of ESRD patients are vitamin D-deficient and are treated with VDRA to prevent secondary hyperparathyroidism. VDRA treatment has cardiovascular survival benefits in these patients. The mechanisms mediating the cardiovascular survival benefits of VDRA remain to be identified. SMC contain VDR and have been previously reported to respond to VDRA treatment with alterations in proliferation and changes in gene expression.17,18 However, past studies have provided contradictory results regarding VDRA action on SMC calcification in vitro. Several studies reported that VDRA treatment induced calcification in bovine and rat SMC.35–37 In contrast, Wolisi and Moe16 and Wu-Wong et al38 did not observe VDRA effect on mineralization in either bovine or human vascular SMC. Our studies are in agreement with the latter studies, because we found very little specific effect of VDRA treatment on calcification in human SMC culture. These disparate results are most likely attributable to different experimental conditions or cell sources, which are known to affect SMC susceptibility to calcification.39

Macrophages play a crucial role in the pathogenesis of various diseases and conditions, such as atherosclerosis, autoimmune diseases, and chronic inflammation. Macrophages accumulate in atherosclerotic lesions and are associated with various stages of calcification in human carotid arteries.22 In plaques, macrophages colocalize with SMC and may regulate their function via paracrine factors. Thus, we used macrophage/SMC cocultures to model cell interactions that exist in atherosclerotic plaques. We found that coculture with macrophages substantially increased SMC calcification compared to SMC cultured in the absence of macrophages. The procalcific action of macrophages was most likely mediated by the production of soluble paracrine factors, because macrophage/SMC cocultures were performed in transwells without cell–cell contact. Our studies are in agreement with previous studies demonstrating that monocyte/macrophage coculture enhanced SMC calcification in vitro.33,34

Interestingly, we found that treatment of macrophage/SMC cocultures with VDRA converted the stimulatory effect of macrophages on SMC calcification to a striking inhibitory effect. VDRA-mediated effects were abolished when VDR-deficient macrophages were used in the macrophage/SMC cocultures. These findings indicate a crucial role of macrophage in regulation of SMC calcification by VDRA acting through the macrophage VDR. Our studies also provide a potential mechanism for the recent findings of Mathew et al,30 who showed that physiological doses of VDRA inhibited aortic calcification as well as osteogenic gene expression in uremic, high-fat-fed low-density lipoprotein-deficient mice.

Most of the biological actions of VDRA are mediated by transcriptional regulation of VDRA-responsive genes. In the present study, we found that BMP2 and TNF-α were highly
expressed by macrophages, but that VDRA treatment strikingly inhibited their expression. BMP2 is a bone morphogenetic protein that promotes bone formation and mineralization in vivo and SMC calcification in vitro.46 TNF-α is an important inflammatory mediator that is expressed at high levels in classically activated macrophages that has been implicated in SMC calcification in vitro and in vivo.54,47,48 Thus, VDRA treatment reduces production of procalcifying molecules by macrophages, and this likely contributes to their ability to attenuate SMC calcification in coculture. In contrast, Wu-Wong et al49 used DNA microarray covering 22,000 different human genes to characterize the VDRA-mediated gene expression profile in human SMC treated with calcitriol and paricalcitol. A total of 181 VDRA target genes were identified. However, BMP2, TNF-α, and OPN were not among these target genes. Whether VDRA treatment alters circulating levels of these factors is not known but would be predicted from our studies.

In addition to inhibition of procalcifying gene expression, VDRA treatment induced OPN levels in macrophages. OPN is a secreted protein that has been shown to inhibit SMC calcification in vitro and in vivo.42,50–52 Inhibition of calcification by OPN is highly dependent on its level of phosphorylation, with nonphosphorylated OPN showing little anticalcific activity.53–55 Vehicle-treated macrophages expressed some OPN constitutively, but both OPN mRNA and secreted protein levels were increased after VDRA treatment. Interestingly, we observed that 50 nM paricalcitol was more potent at inhibiting SMC calcification compared with calcitriol, although paricalcitol had less effect on OPN mRNA and protein than calcitriol. There may be several explanations for this observation. First, it is possible that paricalcitol preferentially induced a more highly phosphorylated form of osteopontin. Extent of phosphorylation is known to dramatically affect the potency of osteopontin as an inhibitor of calcification.54 Because the enzyme-linked immunosorbsent assay and Western blots do not distinguish between specific phosphorylated forms or extent of phosphorylation of OPN, we cannot rule out this possibility. Second, it is possible that paricalcitol inhibited other procalcifying molecules or induced other anticalcific proteins that contribute to the overall effect. BMP2 and TNF-α do not appear to explain this effect, because both were inhibited equivalently by calcitriol and paricalcitol.

The functional importance of OPN induction was revealed by treatment of macrophages with OPN siRNA. OPN-deficient macrophages no longer suppressed SMC calcification after VDRA treatment as compared to OPN-sufficient macrophages. These results suggest a potentially important role of macrophage-derived OPN in the regulation of vascular calcification by VDRA. Whereas it is likely that a secreted form of OPN that could bind apatite and inhibit crystal growth was responsible for this inhibitory activity based on previous mechanistic studies,52–54 we cannot exclude the possibility that an intracellular form of OPN might also be important, because the siRNA strategy used in our studies would inhibit both extracellular and intracellular forms of OPN. An intracellular form of OPN arising from an internal translation initiation site has recently been described that controls dendritic cell function and interferon-α production.56,57

**Conclusion**

In conclusion, our studies are the first to our knowledge to demonstrate an anticalcific effect of VDRA in vitro. Our studies suggest that classically activated macrophages promote vascular calcification by releasing procalcifying factors, such as BMP2 and TNF-α and low levels of the anticalcific molecules OPN. VDRA treatment, however, induces a procalcific to anticalcific paracrine switch in macrophages, whereby levels of BMP2 and TNF-α are reduced and levels of OPN are elevated. These findings suggest a novel mechanism for the survival benefit of VDRA observed in the general population, ESRD patients, and experimental animal models.

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**Disclosure**

None.

**References**


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Supplementary Figure I. VDR levels and effects of VDRAs on calcification in SMC cultured alone. (A) Total RNA extracted from the indicated cell types was transcribed into cDNA and used for PCR reactions with primers specific to human or mouse VDR. Differ. THP-1: THP-1 cell treated with PMA; Undiff. THP-1: THP-1 cell without PMA treatment; VSMC: Human aortic smooth muscle cell; CT siRNA: P388D1 cell transduced with control nucleotide sequence; OPN siRNA: P388D1 cell transduced with OPN RNAi target sequence. Sequence identity of these bands as VDR was confirmed by DNA sequencing. (B) Human SMC were treated with various concentrations of calcitriol or paricalcitol in CM for 10 days. Calcium content of cultures was measured and presented as mean ± S.D. (n = 3). * Significant decrease compared with vehicle (P <0.05).
Supplementary Figure II. The VDR mediates the inhibitory effects of calcitriol and paricalcitol treated macrophages on SMC calcification. (A) VDR mRNA levels in non-transduced (WT), control siRNA transduced (CT siRNA) and VDR siRNA transduced P388D1 macrophages were determined by QPCR and normalized to 18s rRNA. (*P <0.05). (B) Cocultures of SMC with either CT siRNA or VDR siRNA P388D1 macrophages were treated with 50 nM calcitriol or paricalcitol in CM for 10 days. Calcium content of the SMC culture was measured and presented as mean ± S.D. (n = 3). * Significant decrease compared with vehicle (P <0.05).
Supplementary Figure III. VDR is required for VDRA-induced OPN expression in macrophages. Total RNA was obtained from CT siRNA and VDR siRNA P388D1 macrophages treated with 50 nM calcitriol or paricalcitol for 3 days. The levels of OPN mRNA were determined by QPCR and normalized to 18s rRNA.* Significant increase compared with vehicle ($P < 0.05$).
Supplementary Figure IV. Downregulation of OPN production in P388D1 macrophages by siRNA (A) OPN mRNA levels in OPN siRNA and CT siRNA P388D1 macrophages were determined by QPCR and normalized to 18s rRNA. (*P < 0.05). (B) OPN siRNA and CT siRNA P388D1 macrophages were cultured in the presence or absence of 10 ng/mL PMA for 48 hours. Conditioned media were collected for OPN detection by Western blot analysis. Data shown are representative of two independent experiments. (C) OPN siRNA and CT siRNA P388D1 macrophages were treated with 50 nM calcitriol for 3 days. mRNA levels of TNFα in both cell types were determined by QPCR and normalized to 18s rRNA. (* P < 0.05).
Supplemental Materials and Methods

Materials
Calcitriol and paricalcitol were provided by Abbott Laboratories (Abbott Park, IL) and were dissolved in ethanol.

Cell culture
Human primary aortic SMC were purchased from Clonetics Corporation (Palo Alto, CA). Cells were cultured in growth medium (GM) containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 15% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Human monocytic cell line, THP-1, was obtained from ATCC and cultured with RPMI 1640 supplemented with 5% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Mouse monocyte/macrophage cell line, P388D1, was purchased from ATCC (CCL-46) and maintained in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.

Generation of OPN- or VDR deficient mouse macrophages
P388D1 cells deficient in OPN (OPN siRNA) or vitamin D receptor (VDR siRNA) were generated using the pSUPER RNA interference system (Oligoengine, Seattle, WA) as described previously. In brief, the 19 nucleotide targeting sequences 5'-GCAAGAAACTCTTCCAAGC-3' or 5'-GCGAGAGATGATCATGAAG-3' from the coding sequences of mouse OPN (NM_009263) or VDR (NM_009504) were selected, respectively. The control cell lines with mutations of the 19 nucleotide targeting sequences (CT siRNA) were also generated. The transduced cell lines were selected by puromycin. OPN mRNA and protein levels were evaluated by quantitative real-time reverse transcription polymerase chain reaction (QPCR) and Western blot analysis. VDR expression was evaluated by QPCR.

Calcification assay
SMC calcification was induced by incubation with calcification media (CM) that consisted of GM supplemented with NaH₂PO₄/Na₂HPO₄ to 2.6 mM final concentration of
phosphate for 10 days. Calcium content of the SMC extracellular matrix was determined using the \(O\)-cresolphthalein complexone method as previously described\(^2\). The calcium content in individual wells was normalized to protein content.

**Von Kossa Staining**

Calcium phosphate deposits in the extracellular matrix of SMC cultures were visualized by Von Kossa staining. In brief, after fixing with 4 % paraformaldehyde, calcium mineral was stained with 5 % silver nitrate, and then 5 % sodium thiosulfate. Counterstaining was performed with 1 % toluidine blue. Calcified crystals were visualized as dark brown under microscope.

**Macrophage/SMC coculture**

PMA-differentiated THP-1 or P388D1 cells were used for these studies. For THP-1, cells were seeded in polyester transwell inserts with 0.4 \(\mu\)M pore size (Corning Inc., Corning, NY) and incubated with 100 nM phorbol myristate acetate (PMA) for 24 hours to induce differentiation. Transwell inserts containing differentiated THP-1 or P388D1 macrophages were transferred to a 6 well plate containing SMC. The cocultures were incubated in GM or CM for 10 days.

**VDRA treatment of macrophages**

PMA-differentiated THP-1 cells or P388D1 macrophages were treated with 50 nM calcitriol or paricalcitol in growth medium (GM) for 3 or 6 days. RNA was extracted for measurement of gene expression by QPCR as detailed below.

**Reverse transcription PCR and quantitative real-time PCR**

Total RNA was isolated from cells using RNeasy kit (Qiagen, Chatsworth, CA). cDNA synthesis was performed using Omniscript Reverse Transcriptase from Qiagen. The PCR primers for human VDR were: Forward 5'- GTTACTCTCAACCTGGACAACAAG-3' and reverse 5'-AGTCTCCTTCTCTTCTCTCTGAT-3', the amplified fragment corresponds to base pairs 3671-3933 of human VDR. The PCR primers for mouse VDR were: Forward 5'-GACCGCCTATCCCAACAC-3' and reverse 5'-
ATCTCATTGCGGAACACCTC-3', the amplified fragment corresponds to base pairs 1194-1402 of mouse VDR. The identities of the amplicons as human or mouse VDR were confirmed by DNA sequence analysis.

Levels of BMP2, TNFα, VDR and OPN mRNAs were assessed by QPCR using the ABI Prim 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All of the Taq probes have a FAM (fluorochrome reporter) tag at the 5'-end and an MGB quencher at the 3'-end. The sequences are: 1) Human BMP2 forward 5’- TGCGCAGCTTCCACCAT -3’ and reverse 5’- GGTGTTTTCCCACCTCGTTTCT -3’, the probe 5’- AGAATCTTTGAAGAAGACT-3’. 2) Human OPN forward 5’- TGTCCTCTGAAGAAAACAAATGACTT -3' and reverse 5'- TCATGGCTTTTCGTGACTTACT -3’, the probe 5’- AAACAAGAGACCCTTCC-3’. 3) Mouse OPN forward 5’-TGAGGTCAAGATCTAGGACTTTCC-3’ and reverse 5’- TTAGACTCACCCTCTTCTGTG-3’, and probe 5’-TTCTGATGAACAGTATCCTG-3’. 4) Mouse VDR forward 5’- GGAATGCCTCCGCCATAAG-3’ and reverse 5’- TGAGGAATGGACTCTCTTCTTG-3’, and probe 5’- CACTGCTTGTGTCAAGG-3’. mRNA levels of human and mouse TNFα were assessed using TaqMan Gene Expression Assay Kits (Assay ID Hs00174128_m1 and Mm99999068_ml, respectively, Applied Biosystems). All PCR reactions were performed in triplicate. Quantification of gene expression was calculated by the standard curve method according to the manufacturer’s protocol and normalized to 18S rRNA.

**Western blot analysis of secreted OPN**

Macrophages were cultured in the presence or absence of 10 ng/mL of PMA for 24 hours. The cells were then washed with PBS and treated with PMA for another 24 hours in 800 µl of the culture media without FBS, and collected for OPN detection by Western blot as described 3. Parallel gels were stained with Coomassie blue as loading controls (data not shown).
**OPN ELISA assay**

Conditioned media from macrophages with various treatments were collected for measurement of secreted OPN using human OPN ELISA Kit (Assay Designs, Ann Arbor, MI).

**Statistical analyses**

Results are expressed as mean ± SD. Significance between groups was determined by ANOVA followed by Fisher's PLSD, *P*-values less than 0.05 were considered significant.

**References:**