Modulation of Osteopontin Post-translational State by 1,25-(OH)₂-Vitamin D₃

DEPENDECE ON Ca²⁺ INFLUX*

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In osteoblastic ROS 17/2.8 cells, 1,25-(OH)₂-vitamin D₃ stimulates transcription of the extracellular matrix phosphoprotein osteopontin (OPN). We now show post-translational regulation of OPN production by 1,25-(OH)₂-D₃. Prior to transcriptional up-regulation of OPN, 1,25-(OH)₂-D₃ induces a shift in OPN isoelectric point (pl) from 4.6 to 5.1. Loading equal amounts of OPN recovered from ROS 17/2.8 cells exposed to 1,25-(OH)₂-D₃ or carrier for 3 h reveals that the pl shift represents reduced phosphorylation. Trypsin cleavage patterns of OPN produced after 1,25-(OH)₂-D₃ treatment indicates phosphorylation changes in the resulting peptides. Using structural analogs to 1,25-(OH)₂-D₃, we found that analog AT (25-(OH)-16-ene-23-yne-D₃), which triggers Ca²⁺ influx but does not bind to the vitamin D receptor, mimicked the OPN pl shift, whereas analog BT (1,25-(OH)₂-22-ene-24-cyclopropyl-D₃), which binds to the vitamin D receptor without triggering Ca²⁺ influx, did not. Likewise, inclusion of the Ca²⁺ channel blocker nifedipine blocks the charge conversion of OPN. Isolation of OPN from rat femurs and tibiae demonstrates the existence of two OPN charge forms in vivo. We conclude that 1,25-(OH)₂-D₃ regulates OPN not only at the transcriptional level, but also modulates OPN phosphorylation state. The latter involves a short term (<3 h) treatment and is associated with membrane-initiated Ca²⁺ influx.

Osteopontin (OPN) is a non-collagenous, glycosylated phosphoprotein originally found in bone matrix (1, 2), but now known to be expressed in many tissues including kidney, hypertrophic chondrocytes, placenta, T-lymphocytes, macrophages, secretory epithelia and ganglia of the inner ear, and smooth muscle of the vascular system (3, 4). OPN is also found in bone matrix (1, 2), but now recognized to be involved in the regulation of bone metabolism and cell adhesion (11). Potential roles for OPN function through the integrin receptor include the mediation of cell attachment (12). OPN can promote attachment of various cell types and can initiate signal transduction through integrin-associated kinases. Other proposed roles for OPN include chemotaxis (13, 14), inhibition of nitric oxide synthase expression (15), activation of pp60src (16), hydroxyapatite binding (17), and Ca²⁺ binding (18).

Hormone regulation plays an important role in OPN production. Our laboratory has examined the effect of the secosteroid hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂-D₃) on OPN expression and secretion. After uptake of 1,25-(OH)₂-D₃ into a cell, the hormone binds to the vitamin D receptor (VDR), translocates to the nucleus, dimerizes preferentially with the retinoid X receptor, then binds to the vitamin D response element (VDRE) located in the promoter region of 1,25-(OH)₂-D₃-responsive genes (19). In the case of OPN, this results in increased transcription as seen by higher OPN mRNA steady state levels at 24–48 h and, eventually, higher secreted protein levels (21). The OPN gene in rat is regulated by the additive action of two VDREs (3), which respond to both 1,25-(OH)₂-D₃ and to bioactive analogs of 1,25-(OH)₂-D₃ that bind to nuclear receptors (20).

1,25-(OH)₂-D₃ regulation also occurs through rapid plasma membrane-initiated responses, which have been well studied in osteoblasts. We previously reported the 1,25-(OH)₂-D₃ regulation of L-type voltage-sensitive calcium channels (VSCC) in ROS 17/2.8 osteosarcoma cells (22, 23). Using patch-clamp techniques, nanomolar concentrations of 1,25-(OH)₂-D₃ were shown to increase open times of VSCCs and shift the threshold of activation toward the resting potential of the plasma membrane (22). Ca²⁺ influx assays show this increase in VSCC open time leads to elevated Ca²⁺ influx into the cell (23). 1,25-(OH)₂-D₃ activates other osteoblast signaling pathways that are independent of transcription such as a rapid increase in phospholipase C activity (24), activation of protein kinase C (25), and regulation of whole cell chloride currents (26).

In this publication, we report the effect of 1,25-(OH)₂-D₃ on osteoblast-like ROS 17/2.8 cells at a time period between the classic genomic response and the rapid membrane-associated responses. Examination of OPN secreted by ROS 17/2.8 cells after a 3-h exposure to 1,25-(OH)₂-D₃ reveals the production of a form of OPN with a higher isoelectric point (pl) than the original. Structural analogs of 1,25-(OH)₂-D₃ and Ca²⁺ channel blockers were used to determine if a nuclear receptor-mediated response or a membrane-initiated response was responsible for the OPN pl shift. Our results provide evidence for short term...
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regulation by 1,25-(OH)₂D₃ of post-translational modification of OPN, a phenomenon that may modify its functional properties in bone matrix.

EXPERIMENTAL PROCEDURES

Cell Culture—ROS 17/2.8 cells were grown in Dulbecco’s modified Eagle’s medium/1% F12 medium (Atlanta Biologicals, Norcross, GA) containing 10% fetal bovine serum (Life Technologies, Inc.). RO 17/2.8 osteoblast-like osteosarcoma cells were chosen because of their responsiveness to 1,25-(OH)₂D₃ and because of their ability to synthesize and secrete high levels of OPN mRNA and protein. Cells were seeded at low density (30,000 cells/ml) and allowed to grow until they reached 60–70% confluence. For protein isolation, cells were transferred to serum-free medium overnight, then treated with 2.5 mM 1,25-(OH)₂D₃ (Bioitmol, Plymouth Meeting, PA), 2.5 mM analog AT (25–OH-16-ene-23-yne-D₃), 25 mM analog BT (1,25-(OH)₂-22-ene-24-cyclopentyl-D₃) (gifts of Dr. Anthony Norman), or carrier (ethanol), and cultured for the indicated times prior to harvest. Concentrations of the analogs used were based on previous studies (23). 50 mM nifedipine (Calbiochem, La Jolla, CA) was used to block L-type Ca²⁺ channels.

For radiolabeling, cells were transferred to serum-free Dulbecco’s modified Eagle’s medium/F12 medium without Na₃PO₄ or methionine, respectively for 18 h. Phosphate in the form of [³²P]orthophosphate or [³⁵S]methionine in the form of [³⁵S]methionine (NEN Life Science Products) was added to the medium 30 min prior to the addition of reagents (167 μCi/20 ml medium). Experiments were performed in serum-free medium to prevent binding of 1,25-(OH)₂D₃ to the vitamin D-binding protein. Previous studies have shown that cells remain viable under these conditions for 48–72 h.

OPN Purification from ROS 17/2.8 Cell Medium by Barium Citrate Precipitation—Medium was collected from ROS 17/2.8 cells that had been exposed to 1,25-(OH)₂D₃ with or without Ca²⁺ channel blockers, analog AT, analog BT, or carrier (ethanol) alone. OPN was isolated using the barium citrate procedure (5). Briefly, 3.8% sodium citrate and 15% BaCl₂ were added to the medium at 1:10 of the total volume. After shaking for 10 min at 4 °C, the mixture was centrifuged for 10 min at 3800 × g. Supernatant was displaced by centrifugation for 10 min at 3800 rpm, the supernatant was again dissected, and the pellets were washed with H₂O for 10 min at 4 °C. After another round of centrifugation, the supernatant was removed, and OPN was eluted from the pellet by dissolution in 0.2 mM sodium citrate, pH 6.8. Excess salts were removed with a desalting column (Pierce). Further purification was accomplished by passing the sample through an affinity column (described below).

Immunoaffinity Chromatography—IGW was immobilized with cyanogen bromide-activated Sepharose 4B (Sigma) and coupled as described (27). Briefly, 1 g of cyanogen bromide-activated Sepharose 4B with 5 mg of α-rat OPN IgG (purified using ImmunoPure (A/G) IgG purification kit as per manufacturer’s instructions (Pierce)) in 0.1 M Bicine-HCl, pH 8.5, 0.5 % NaCl was agitated continuously for 2 h at room temperature, then washed for 10 min with 15% BaCl₂ at 4 °C. After centrifugation for 10 min at 3800 rpm, the supernatant was again discarded, and the pellet was washed with H₂O for 10 min at 4 °C. After another round of centrifugation, the supernatant was removed, and OPN was eluted from the pellet by dissolution in 0.2 mM sodium citrate, pH 6.8. Excess salts were removed with a desalting column (Pierce). Further purification was accomplished by passing the sample through an affinity column (described below).

Two-dimensional Gel Electrophoresis and Isoelectric Focusing Gel Electrophoresis—Proteins were suspended in a minimum volume of lysis buffer containing 9.5 μl urea, 2% Triton X-100, 2% ampholines (1.6% pH 5–7, 0.4% pH 3–10 or 1.0% pH 2–4, 1.0% pH 3–10), and 5% β-mercaptoethanol. Isoelectric focusing was performed in tube gels overnight at 200 V or for 3.5 h at 750 V. Gels were removed from the tubes and soaked for 20 min in sample buffer (0.06 μl Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol), then overlayed onto 8.75% or 12.5% polyacrylamide gels. The system was electrophoresed to completion using SDS-PAGE (29). Two-dimensional gels were stained with Coomassie G, then dried and exposed to autoradiographic film.

Isoelectric gel electrophoresis was performed using IsoGel agarose IEF plates (FMC Bioproducts, Rockland, ME). Proteins were lyophilized and resuspended in deionized H₂O. Proteins were focused on pH range 3–10 gels following the manufacturer’s instructions. Gels were then stained and exposed to autoradiographic film or transferred to nitrocellulose using a press blot procedure as described by the manufacturer.

Immunoblot Analysis—SDS-polyacrylamide gels were transferred to nitrocellulose at 15 V in transfer buffer (0.025 μl Tris, 0.2 μl glycine, 20% methanol) overnight. Blots were dried and then blocked in PBS containing 3% BSA and 0.15% Tween 20 at room temperature for 2 h. Blots were then incubated in PBS containing 3% BSA and 0.15% Tween 20 with a 1:10,000 dilution of primary antibody (goat α-rat OPN) at room temperature for 1 h. Blots were then washed five times for 10 min each in PBS containing 1.5% BSA and 0.075% Tween 20. Next, blots were incubated in PBS containing 3% BSA and 0.15% Tween 20 with a 1:100,000 dilution of peroxidase-conjugated donkey α-goat IgG (Jackson Immunoresearch, West Grove, PA) or a 0.150,000 dilution of alkaline phosphatase-conjugated swine α-goat IgG secondary antibody (Boehringer Mannheim). Blots were again washed 5 times for 10 min each in PBS containing 1.5% BSA and 0.075% Tween 20. For alkaline phosphatase detection, blots were incubated in carbonate buffer with detection substrate (0.35 mM nitro blue tetrazolium, 0.35 mM 5-bromo-4-choloro-3-indolyl phosphate, 0.1 mM NaH₂CO₃, 1.0 mM MgCl₂, pH 9.8) until appearance of coloration. Peroxidase was detected using a chemiluminescent procedure. Blots were incubated in 50% Lumino/enhancer solution, 50% stable peroxide solution (Pierce) for 5 min and exposed to autoradiographic film for 1 min.

RESULTS

Effect of 1,25-(OH)₂D₃ on OPN pl—We examined the effect of 1,25-(OH)₂D₃ on OPN production during the first 3 h of treatment, prior to nuclear receptor-mediated transcriptional upregulation. Proteins from the cell fraction were isolated as described “Experimental Procedures” and visualized by two-dimensional gel electrophoresis. OPN from ROS 17/2.8 cells treated with vehicle was found to focus in two discrete peaks...
spots at pI 4.6 and 5.1 (Fig. 1A). The identity of the spots focusing at these locations as OPN was confirmed by Western blotting (data not shown) using goat α-rat OPN. The ratio of protein found at pI 5.1 compared with pI 4.6 was 0.26:1 assessed by densitometry. In Fig. 1B, OPN from ROS 17/2.8 cells exposed to 2.5 nM 1,25-(OH)\(_2\)D\(_3\) for 3 h focuses almost completely at pI 5.1, with only a small fraction (12:1 ratio) at pI 4.6. Western blotting confirmed the focused spots as OPN (data not shown). These experiments were repeated with cells labeled with \([^{35}\text{S}]\)methionine to visualize total protein from the cell fraction. Most spots were unchanged in location, but the spot corresponding to OPN shifted to a more basic pI after 3 h 1,25-(OH)\(_2\)D\(_3\) treatment (data not shown). The identity of the spot which disappears altogether (small arrow in Fig. 1, A and B) is presently unknown.

For further verification of the pI shift, OPN was isolated from ROS 17/2.8 cell medium by immunoprecipitation. ROS 17/2.8 cells were cultured as described above, with the exception that the time of exposure to carrier or 1,25-(OH)\(_2\)D\(_3\) was increased to 18 h. This was done to permit the secretion of measurable levels of OPN protein prior to immunoprecipitation. Visualization of equal counts/min of immunoprecipitated OPN protein was accomplished by two-dimensional gel electrophoresis and autoradiography. Immunoprecipitated OPN from ROS 17/2.8 cells exposed to vehicle alone focused at pI 4.6 (Fig. 1C). OPN immunoprecipitated from ROS 17/2.8 cells treated with 2.5 nM 1,25-(OH)\(_2\)D\(_3\) focused at pI 5.1 and, to a much lesser extent, pI 4.6 (Fig. 1D). Medium from ROS 17/2.8 cells treated with vehicle or 1,25-(OH)\(_2\)D\(_3\) and passaged over a preimmune IgG column produced no spots when analyzed by two-dimensional gel electrophoresis (data not shown). These results show that OPN secreted into the medium undergoes a charge shift similar to that of OPN in the cell fraction (Fig. 1, A and B).

**Effect of 1,25-(OH)\(_2\)D\(_3\) on OPN Phosphorylation Levels**—Because the 1,25-(OH)\(_2\)D\(_3\)-induced pI change was over a short range (0.5 units), we hypothesized that this shift was the result of a reduced phosphorylation of OPN. An altered level of phosphorylation was a likely possibility because of the number of potential OPN phosphorylation sites. Radiolabeled medium was collected and proteins were precipitated using sodium citrate and BaCl\(_2\) as described under “Experimental Procedures.” OPN was isolated from the group of precipitated proteins by immunoaffinity chromatography as described above. Equal amounts of OPN (50 μg) from vehicle-treated and 1,25-

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**Fig. 1. Two-dimensional analysis of OPN from vehicle and 1,25-(OH)\(_2\)D\(_3\)-treated cells.** A, total protein from ROS 17/2.8 cells labeled with \([^{32}\text{P}]\text{Na}_3\text{PO}_4\) and treated with vehicle (ethanol) for 3 h was precipitated as described under “Experimental Procedures.” Visualization of the precipitated proteins was accomplished using two-dimensional gel electrophoresis. Molecular weight is indicated on the left; isoelectric point is indicated on the bottom. Large arrows indicate two-dimensional focusing of OPN at pI 4.6 and 5.1 in the first dimension and 66 kDa in the second dimension. Small arrow indicates the presence of unknown phosphorylated protein. pI was determined by comparison to standards and by measurement of first dimension gel slices at set intervals. B, total protein from ROS 17/2.8 cells labeled with \([^{32}\text{P}]\text{Na}_3\text{PO}_4\) and treated with vehicle (ethanol) for 3 h was precipitated and visualized as in A. Large arrows indicate two-dimensional focusing of OPN at pI 4.6 and pI 5.1, both at 66 kDa in the second dimension. Small arrow indicates absence of phosphorylated protein found in A. C, OPN was immunoprecipitated from medium isolated from ROS 17/2.8 cells labeled with \([^{32}\text{P}]\text{Na}_3\text{PO}_4\) and treated with vehicle (ethanol) for 18 h. Immunoprecipitation was performed as described under “Experimental Procedures.” Visualization of equal counts/min of immunoprecipitated OPN was performed as in A. Arrow indicates two-dimensional focusing of immunoprecipitated OPN at pI 4.6 and 66 kDa. D, OPN was immunoprecipitated from medium isolated from ROS 17/2.8 cells labeled with \([^{32}\text{P}]\text{Na}_3\text{PO}_4\) and treated with 2.5 nM 1,25-(OH)\(_2\)D\(_3\) for 18 h. Visualization of equal counts/min of immunoprecipitated OPN was performed as in A. Arrows indicate two-dimensional focusing of immunoprecipitated OPN at pI 5.1 and 4.6 in the first dimension and 66 kDa in the second dimension.
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Effects of Structural Analogs to 1,25-(OH)2D3 on OPN Phosphorylation and Charge Shift—To determine whether 1,25-(OH)2D3 treatment and consequent reduced phosphorylation, could alter sensitivity to proteolytic cleavage, OPN from vehicle-treated and 1,25-(OH)2D3-treated cultures was subjected to trypsin digestion (Fig. 3). ROS 17/2.8 cells were treated with 1,25-(OH)2D3 or carrier with or without 50 mM nifedipine. ROS 17/2.8 cells were labeled with [32P]orthophosphate and treated with 1,25-(OH)2D3 for 3 h and trypically digested for 3 h at 37 °C. OPN isolated from peak D4a migrated at 73.4 kDa whereas the peak D4b migrated at 68.0 kDa. The largest peak migrating at 28 kDa appeared to be resistant to further proteolysis in OPN from vehicle-treated cultures (lane 3). The streaking found in lane 4 is likely the result of microheterogeneity of the protein.

Effect of Structural Analogos to 1,25-(OH)2D3 on OPN pI—To determine the existence of similar multiple charge forms in vivo, OPN was isolated from rat femurs and tibiae. Analysis of OPN from peak D4a and D4b of the DEAE-Sephacel column was accomplished by SDS-PAGE and by isoelectric gel electrophoresis (Fig. 6). OPN isolated from peak D4a migrated at 37.4 kDa (Fig. 6A, lane D4a; Fig. 6B, lane D4a). The streaking found in lane D4a is likely the result of microheterogeneity associated with the aggregation of OPN or partial degradation during the bone extraction procedure. A minor band of OPN was found at 28.8 kDa, possibly the product of thrombin cleavage. OPN from peak D4b migrated at 91.3 kDa (Fig. 6A, lane D4b; Fig. 6B, lane D4b). After isoelectric focusing, the gel was transferred to nitrocellulose via a press blot procedure. Western analysis and detection by alkaline phosphatase (Fig. 6C) was performed as described under “Experimental Procedures.” OPN1 from peak D4b (lane OPN1) was found to focus at pI 4.6.
analogous to OPN from control cells. Conversely, OPN2 from peak D4a (lane OPN2) was found to focus at pI 5.1, similar to OPN from ROS 17/2.8 cells treated with 1,25-(OH)\textsubscript{2}D\textsubscript{3} or analog AT. Streaking can be found in both lanes OPN1 and OPN2 of Fig. 6C, indicating the presence of multiple charge forms in addition to the two major bands or the presence of degradation products in bone extracts.

**DISCUSSION**

Increasing evidence supports a role for 1,25-(OH)\textsubscript{2}D\textsubscript{3} in cellular responses that either do not include the VDR or are completely non-genomic. One of the most studied responses is Ca\textsuperscript{2+} signaling initiated by 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Patch-clamp and Ca\textsuperscript{2+} influx assays from our laboratory have shown that physiological concentrations of 1,25-(OH)\textsubscript{2}D\textsubscript{3} increase VSCC open time in ROS 17/2.8 cells, thereby increasing Ca\textsuperscript{2+} influx into the cells (22, 23). 1,25-(OH)\textsubscript{2}D\textsubscript{3} can also stimulate Ca\textsuperscript{2+} influx through a similar mechanism in skeletal muscle cells (30). Although the changes in phosphorylation level of OPN from ROS 17/2.8 cells treated with 1,25-(OH)\textsubscript{2}D\textsubscript{3} appear to be post-translational, involvement of the VDR was possible. Even though up-regulation of OPN at the transcriptional level by 1,25-(OH)\textsubscript{2}D\textsubscript{3} through the VDR is unlikely in this time frame, previous data have shown up-regulation of other transcripts by 1,25-(OH)\textsubscript{2}D\textsubscript{3} in less than 3 h (25, 31–33). Analog BT has been shown to bind to the VDR as well or better than 1,25-(OH)\textsubscript{2}D\textsubscript{3} and can up-regulate OPN mRNA steady state levels in ROS 17/2.8 cells comparable to 1,25-(OH)\textsubscript{2}D\textsubscript{3} (20, 23). Analog AT increases Ca\textsuperscript{2+} influx through VSCCs in ROS 17/2.8 cells similarly to 1,25-(OH)\textsubscript{2}D\textsubscript{3} (22, 23). Analog BT, however, does not increase VSCC activity, and analog AT does not bind to the VDR or increase OPN mRNA steady state levels. Other analogs...
24-h exposure to 1,25-(OH)_2D_3.

From ROS 17/2.8 cells stimulated by 1,25-(OH)_2D_3 and in OPN responding to OPN with two distinct isoelectric points. The function of NaCl, we hypothesized that these peaks might correspond to OPN eluting at two different concentrations. Since OPN from these peaks elutes at two different charge states. The 4.6 pI form apparently is equivalent to this 44-kDa form, and the 5.1 pI form of OPN is equivalent to the 55-kDa form. Contrary to their conclusions, however, is our finding that both forms can be isolated and do exist in rat long bone. OPN from rat long bone migrates differently than these 44- and 55-kDa forms. We find that the more acidic form, the more phosphorylated form, migrates slower on SDS-PAGE (Fig. 6B), compared with faster migration of the highly phosphorylated, more acidic 44-kDa OPN. These results are similar to the findings of Nemir et al. (48), where the phosphorylated OPN migrated slower than the non-phosphorylated form. In fact, the discrepancy in migration on SDS-PAGE between OPN from peaks D4a and D4b might be attributed to decreased binding of SDS to OPN from peak D4b. This decreased binding to SDS could result in slower migration on SDS-PAGE.

Currently, our laboratory is investigating the phosphorylation sites found on the two OPN isoforms isolated from rat long bone (D4a and D4b). Analysis of phosphorylation from bovine OPN revealed the existence of 28 sites of phosphorylation (51). Phosphorylation motifs matched the recognition sequence utilized by CKI and CKII coinciding with the evidence that a CKII-like activity phosphorylates OPN in the Golgi (52). In rat long bone, OPN from peak D4a has been analyzed for post-translational modifications (53). Phosphorylations were found on equivalent sites as those in bovine OPN, but some of the phosphorylations were partial (not found in every analysis). Partial phosphorylation of residues might play a role in the unusual streaking found when OPN is detected by Western blot procedures (Fig. 6). Although staining reveals the presence of distinct bands (Fig. 6A), Western blot analysis of OPN from rat long bone reveals not only the distinct bands, but also a broad range of forms (Fig. 6B). This is especially evident in the isoelectric gel (Fig. 6C), where OPN from D4a and D4b focused at major bands of pI 5.1 and 4.6, respectively, but was also detected in a broad band at higher isoelectric points. Notice that the D4a fraction does not appear to contain any of the lower pI form of OPN. Another interesting result with antibody detection is that the antibody to OPN utilized in this study apparently binds to the lower phosphorylated form of OPN more efficiently than to the higher phosphorylated form. This can be seen in OPN isolated from ROS 17/2.8 cells (Fig. 2) and in OPN from rat long bone (Fig. 6). A possible explanation is that this antibody was developed using OPN purified from peak D4a, and, therefore, might be more specific for the lower phosphorylated form.

OPN contains a GRGDS sequence that allows the protein to bind to cells containing the α_vβ_3 integrin. As previously mentioned, OPN was found in both phosphorylated and non-phosphorylated forms in normal rat kidney cells (48). Phosphorylated OPN showed cell surface association, apparently GRGDS-dependent, while the non-phosphorylated form was not found on the cell surface. Instead, the non-phosphorylated OPN was associated with fibronectin (via co-precipitation). Another study of OPN phosphorylation demonstrated that partially dephosphorylated OPN with tartrate-resistant acid phosphatase created a form that could no longer bind to osteoblasts (54). It may be that the OPN phosphorylation loss after 1,25-(OH)_2D_3 treatment results in decreased binding to cells containing the α_vβ_3 integrin.
In conclusion, we have isolated two charge forms of the phosphoprotein OPN from ROS 17/2.8 cells exposed to vehicle or 1,25-(OH)\(_2\)D\(_3\) that have uniquely different isoelectric points. This pI shift appears to be the result of reduced phosphorylation in cells treated with 1,25-(OH)\(_2\)D\(_3\). The loss in phosphorylation does not occur through a VDR-mediated genomic event, but is associated with Ca\(^{2+}\) influx. Finally, we report that the two charge isoforms of differing pI can be isolated from rat long bone.

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