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Mechanism of 24,25-Dihydroxyvitamin D_3-Mediated Inhibition Of Rapid, 1,25-Dihydroxyvitamin D_3-Induced Responses: Role of Reactive Oxygen Species

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Abstract In intestine, $24,25(OH)_2D_3$, which is made under conditions of calcium-, phosphate-, and $1,25(OH)_2D_3$ sufficiency, inhibits the stimulatory actions of $1,25(OH)_2D_3$ on phosphate and calcium absorption. In the current work, we provide evidence that $24,25(OH)_2D_3$-mediated signal transduction occurs mechanistically through increased H_2O_2 production which involves binding of $24,25(OH)_2D_3$ to catalase and resultant decreases in enzyme activity. Physiological levels of H_2O_2 mimicked the action of $24,25(OH)_2D_3$ on inhibiting $1,25(OH)_2D_3$-stimulated phosphate uptake in isolated enterocytes. Moreover, the molecular basis of such inhibition was suggested by the presence of two thioredoxin domains in the $1,25D_3$-MARRS protein/ERp57: Exposure of cells to either $24,25(OH)_2D_3$ or H_2O_2 gradually reduced $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS protein, between 10 and 20 min of incubation, but not to VDR. Feeding studies with diets enriched in the antioxidants vitamins C and E showed that net phosphate absorption in vivo nearly doubled relative to chicks on control diet. Antioxidant diets also resulted in increased $[^3H]1,25(OH)_2D_3$ binding to both $1,25D_3$-MARRS and VDR, suggesting benefits to both transcription- and membrane-initiated signaling pathways. Intriguingly, phosphorous content of bones from birds on antioxidant diets was reduced, suggesting increased osteoclast activity. Because mature osteoclasts lack VDR, we analyzed a clonal osteoclast cell line by RT-PCR and found it contained the $1,25D_3$-MARRS mRNA. The combined data provide mechanistic details for the $1,25(OH)_2D_3/24,25(OH)_2D_3$ endocrine system, and point to a role for the $1,25D_3$-MARRS protein as a redox-sensitive mediator of osteoclast activity and potential therapeutic target. J. Cell. Biochem. 99: 1572–1581, 2006. © 2006 Wiley-Liss, Inc.

Key words: phosphate absorption; antioxidants; 24,25-dihydroxyvitamin D; chickens; $1,25D_3$-MARRS/ERp57/GR58

Vitamin D is metabolized in the liver to produce 25-hydroxyvitamin D_3, and then further hydroxylated in the kidney to either 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] when serum levels of calcium and phosphate are low, or to 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] when mineral levels are normal or high. Initially, $24,25(OH)_2D_3$ was thought to be an inactivation product. However, many laboratories have contributed observations that indicate $24,25(OH)_2D_3$ has unique, physiologically relevant actions [see Farach-Carson and Nemere, 2003 for review]. In intestine, $24,25(OH)_2D_3$ inhibits phosphate and calcium transport [Nemere, 1996, 1999; Tryfonidou et al., 2002; Zhao and Nemere, 2002], thereby creating an endocrine feed back loop.

We recently reported that a binding protein for $24,25(OH)_2D_3$ has sequence similarity to the enzyme catalase [Larsson et al., 2006], suggesting that $24,25(OH)_2D_3$ may modulate the levels of hydrogen peroxide within the cell. Hydrogen peroxide, in turn, functions as a signaling molecule [Waypa et al., 2002; Yano and Yano,

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2002; Watanabe et al., 2003; Wood et al., 2003]. We therefore undertook the current studies to determine the potential role of reactive oxygen species in $24,25(OH)_2D_3$ signaling as it pertains to phosphate uptake in intestinal cells.

MATERIALS AND METHODS

Animals, Surgical Procedures, and Cell Isolation

All procedures were approved by the Institutional Animal Care and Use Committee at Utah State University. White Leghorn cockerels (Privett Hatchery, Portales, NM) were raised for 3–7 weeks on a standard vitamin D-supplemented diet (Nutrena Feeds, Murray, UT). Unless otherwise indicated, cells were isolated from one duodenum per experiment. On the day of use, animals were anesthetized with chloropent, the duodenal loop removed to ice-cold physiological saline, and chilled for 15 min. The pancreas was then excised, the loop everted, rinsed in fresh saline, and cells isolated by chelation in citrate media at pH 5.0 [Nemere et al., 2004].

The isolated cells were collected by centrifugation at 500*g*, 5 min (4°C), and resuspended in 40 ml of Gey's balanced salt solution (GBSS; Nemere et al., 2004; Sterling and Nemere, 2005) without bicarbonate.

Determination of Catalase Activity and H_2O_2 Production

For the determination of catalase activity, 3.2 ml of cell suspension were incubated for a 5 min basal period prior to the addition of the vehicle ethanol (0.02% final concentration), 6.5 nM $24,25(OH)_2D_3$, or 130 pM $1,25(OH)_2D_3$. Aliquots (100 μl per time point) were removed to tubes on ice during the basal incubation period and at 1, 3, 5, 7, and 10 min after addition of test substances. The tubes were centrifuged at 500*g*, 5 min (4°C) and the supernatant carefully separated from the cell pellet. Supernatants and cell homogenates were analyzed for catalase activity by the method of Aebi [1984].

For the determination of H_2O_2 production, isolated intestinal epithelial cells were resuspended in 30 ml of GBSS, and 5 ml aliquots placed in six, 50-ml conical tubes. The suspensions were treated with the vehicle ethanol or 6.5 nM $24,25(OH)_2D_3$ and incubated for 2, 5, or 10 min. The tubes were then placed on ice for 15 min prior to homogenization and determination of H_2O_2 by the procedure of Hill et al. [1988].

Phosphate Uptake

Isolated intestinal cells resuspended in GBSS were combined with 2 μl/ml $H_3{}^{32}PO_4$ (PerkinElmer, Boston, MA) and then further pipetted into tubes containing 50 μM H_2O_2 (final concentration) or an equivalent volume of water at $T = -10$ min. Samples (100 μl each) were removed at $T = -5$ and $T = -1$ min and pipetted into 900 μl ice-cold GBSS. Hormone (130- or 300 pM $1,25(OH)_2D_3$) or vehicle was then added at $T = 0$ min and additional samples taken at $T = 5$, 10, 15, and 20 min, and pipetted into ice-cold GBSS. After centrifugation (1,000*g*, 5 min, 4 C), the supernatants were decanted and the insides of the tubes swabbed with a Kimwipe. Cell pellets were resuspended in water and analyzed for radioactivity by liquid scintillation spectrophotometry.

Determination of Specific $[^3H]1,25(OH)_2D_3$ Binding to $1,25D_3$-MARRS Protein and the VDR

Isolated intestinal epithelial cells were resuspended in 30–40 ml of GBSS and 5 ml of cell suspension placed in each of 6–8 tubes. At $T = 0$ min, cells were treated as controls, or exposed to 6.5 nM $24,25(OH)_2D_3$, and in parallel experiments, cells were treated as controls or exposed to 50 μM H_2O_2. At $T = 5$, 10, 20, and 30 min, the suspensions were centrifuged, and the pellets homogenized in 250 mM sucrose, 5 mM histidine-imidazole, 2 mM EGTA, pH 7.0 (30 strokes in a Potter-Elvehjem homogenizer).

For the $1,25D_3$-MARRS protein, approximately 50 μg of cell protein was incubated overnight on ice in triplicate either with 1 nM $[^3H]1,25(OH)_2D_3$ (PerkinElmer; adjusted to 82 Ci/mmol) alone (total binding) or in triplicate in the presence of a 200-fold molar excess of unlabeled steroid (non-specific binding). The buffer was 10 mM Tris, 1.5 mM EDTA, pH 7.4, deliberately omitting dithiothreitol which regenerates free thiol moieties. The following morning, bound and free radioactivity was separated using the perchloric acid procedure [Larsson and Nemere, 2003a,b].

Equivalent incubations were performed for the VDR, but bound and free radioactivity were separated by the hydroxylapatite (HAP) method [Larsson and Nemere, 2003a,b].

Protein Determinations

Protein was analyzed with the Bradford reagent (BioRad, Hercules, CA) with bovine γ-globulin (Sigma Chemical Co, St. Louis, MO) as standard.

Feeding Studies

White Leghorn cockerels were maintained for 3 days on chick grower diet (TD.04258, Harlan-Teklad, Madison, WI) with vitamin D reduced to 200 IU/kg diet in order to avoid over production of 24,25$(OH)_2D_3$. Chicks were then separated into four groups (five chicks per group). The control group was maintained on the same diet, while the other groups were fed diet either enriched with 800 ppm ascorbic acid (+C diet; TD.04259), 100 IU/kg of supplemental vitamin E ($2 \times$ E; TD.04260), or containing both 800 ppm ascorbic acid and 100 IU/kg of supplemental vitamin E (+C, $2 \times$ E; TD.04261). Each group was maintained for 3.5 weeks, and housed separately for the last 3 days. During the final 3 days, feed consumption was monitored and manure collected for determination of phosphorous. At the end of this time, duodenal mucosa was collected from one chick per group for determination of specific $[^3H]$1,25$(OH)_2D_3$ binding to either the 1,25D_3-MARRS protein (in the post nuclear pellet obtained at 20,000*g*, 10 min) or the VDR (in the nuclear pellet obtained at 1,000*g*, 20 min) as described above. The remaining four chicks per group were used to determine phosphate absorption after 1 min in vivo following introduction of luminal $H_3{}^{32}PO_4$ (2 μCi/lumen). Blood was collected following decapitation, and serum analyzed for radioactivity. Wings and femurs were collected for the determination of tensile strength and phosphorous content of bone ash.

Statistical Analyses

For experiments comparing two groups, Student's *t*-test for unpaired observations was used; all others were analyzed by ANOVA.

Analysis of Osteoclasts for 1,25D_3-MARRS

RNA was isolated from clonal HD-11 EM cells grown as previously described [Steinbeck et al., 1998; Daumer et al., 2002] and stored in 75% ethanol. Samples were centrifuged at 10,000 rpm for 10 min to pellet RNA. The ethanol supernatant was removed and the pellet was allowed to dry for 10 min. RNA was resuspended in 500 μl nuclease-free water and the concentration was determined by spectrophotometry. Reverse transcription reaction was carried out using Omniscript RT kit using the protocol supplied by Qiagen (Valencia, CA). The PCR reaction was performed using HotStar Taq PCR kit (Qiagen) using 5′TACTATGATGTGTATGA3′ as the forward primer and 5′TATGGAGAAGGCACATCATT3′ as the reverse primer to yield a product of 545 base pairs.

RESULTS

Effect of Seco-Steroids on Catalase Activity

The effect of 6.5 nM 24,25$(OH)_2D_3$ on cellular catalase activity is depicted in Figure 1A. While cellular levels of the enzyme in vehicle controls (open circles) remained at approximately 250 μm/min/mg protein, preparations treated with 6.5 nM 24,25$(OH)_2D_3$ (closed circles) exhibited a decrease in specific activity to 64% of controls 1 min after seco-steroid ($P < 0.05$). At 10 min after seco-steroid, specific catalase activity was 72% of controls ($P < 0.05$). In contrast, treatment of cells with 130 pM 1,25 $(OH)_2D_3$ resulted in no significant changes in catalase specific activity relative to controls (Fig. 1B). Supernatant levels of catalase, normalized to cellular protein, showed no significant changes in response to either steroid under the conditions used (data not shown). Commercially available bovine catalase activity was assessed with 0.0325 μg of protein in the absence or presence of 6.5 nM 24,25$(OH)_2D_3$, 300 pM 1,25$(OH)_2D_3$, or 100 nM 25$(OH)_2D_3$. Only 24,25 $(OH)_2D_3$ reduced catalase levels to 66% of vehicle controls (0.16% ethanol, final concentration; $P < 0.05$, for eight determinations).

The logical outcome of a decrease in catalase specific activity would be an increase in H_2O_2 in response to 24,25$(OH)_2D_3$. The data presented in Figure 2 indicate that this indeed was the case. While cell suspensions treated with vehicle control maintained approximately 40 nm H_2O_2/mg protein, cells treated with 6.5 nM 24,25$(OH)_2D_3$ exhibited an increase that was significant ($P < 0.05$) at 5 and 10 min of incubation, relative to corresponding controls (Fig. 2). Indeed, the 30% decrease in catalase specific activity in response to a 10-min exposure to 24,25$(OH)_2D_3$ was paralleled by a 30% increase in H_2O_2 elicited by the steroid.

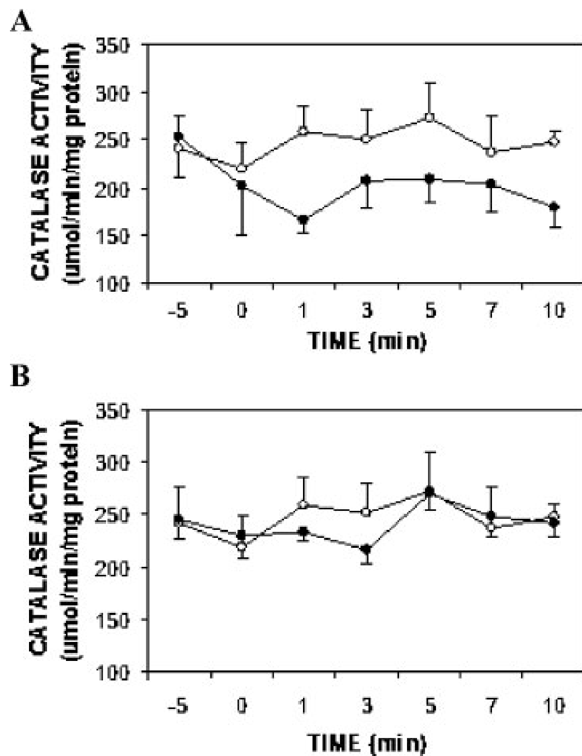

Fig. 1. Rapid inhibition of catalase activity in isolated intestinal cells treated with $24,25(OH)_2D_3$. Chick duodenal cells were isolated by a chelation procedure, collected by centrifugation at 500*g*, 5 min, and resuspended in 40 ml of Gey's Balanced Salt Solution (GBSS). Two, 3.2-ml aliquots were placed in separate 50-ml conical centrifuge tubes, and 100 µl samples removed to microfuge tubes on ice at $T = -5$ and 0 min. Immediately thereafter, control incubations (open circles) received vehicle (ethanol, 0.05% final concentration, and either 6.5 nM $24,25(OH)_2D_3$ (**A**; closed circles) or 130 pM $1,25(OH)_2D_3$ (**B**; closed circles). Additional samples were removed at the indicated times, centrifuged at 1,000*g*, 5 min, the supernatants decanted, and the inside of the tubes swabbed with a Kimwipe while inverted. Cell pellets were homogenized in 0.5 ml double distilled water and analyzed for decreasing absorbance at 240 nm following addition of 40 µM H_2O_2. Change in absorbance was related to cell protein. Values represent mean ± SEM for n = 8 independent experiments (A) and n = 5 independent experiments (B).

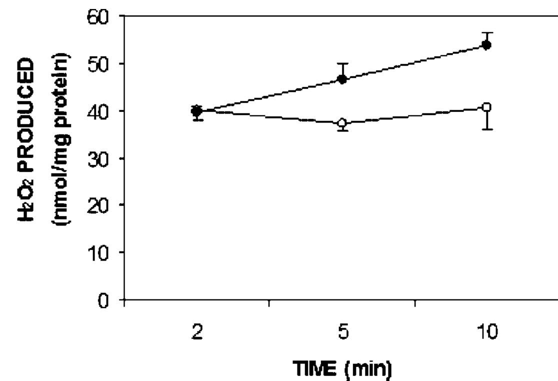

Fig. 2. Production of H_2O_2 in cells treated with $24,25(OH)_2D_3$. Cells were isolated as described in the legend to Figure 1 and resuspended in 30 ml GBSS. Six, 5-ml aliquots were placed into 50 ml conical centrifuge tubes and either treated with the vehicle ethanol (0.05%, final concentration) or 6.5 nM $24,25(OH)_2D_3$. At 2-, 5-, and 10-min, one control and one treated sample were placed on ice and chilled for 15 min prior to homogenization. One ml of each whole homogenate was analyzed for H_2O_2 with a colorimetric procedure. Values represent mean ± SEM for n = 5 independent experiments.

Effect of H_2O_2 on $1,25(OH)_2D_3$-Stimulated Phosphate Uptake

We have previously reported that 24,25 $(OH)_2D_3$ inhibits $1,25(OH)_2D_3$-stimulated phosphate uptake both in the perfused duodenal loop, as well as in isolated intestinal cells [Zhao and Nemere, 2002]. We, therefore, tested the effect of H_2O_2 as a potential signal transduction agent for its effect on hormone-stimulated phosphate uptake in enterocytes. Depending on cell protein, incubations with physiological levels of $24,25(OH)_2D_3$ produced 40–100 µM H_2O_2, comparable to what was reported in other systems [Dhar-Mascareno et al., 2003]. We, therefore, selected a concentration at the lower end of this range. Figure 3A,B illustrates the results of these experiments using two different concentrations of $1,25(OH)_2D_3$ (closed circles) relative to cells exposed to 50 µM H_2O_2 prior to addition of hormone (closed triangles), and vehicle controls (open circles). While H_2O_2 had no effect on phosphate uptake by controls (data not shown), the freshly prepared reactive oxygen species completely abolished the stimulatory effect of 130 pM $1,25(OH)_2D_3$. After 20 min of incubation, steroid stimulated ^{32}P uptake rose to 153% of equivalent preparations exposed to 50 µM H_2O_2 ($P < 0.02$, Fig. 3A). In another series of incubations, in which the H_2O_2 was diluted 1 h prior to use, rather than immediately prior to addition, inhibition of $1,25(OH)_2D_3$ stimulated phosphate uptake was transient, occurring for the first 7 min of the time course, with a return to stimulated levels by 10 min of incubation. Thus, inhibition was reversible.

In additional experiments, 300 pM $1,25(OH)_2$ D_3, equivalent to circulating levels in young cockerels [Sedrani, 1984] was tested. As shown in Figure 3B 50 µM H_2O_2 also inhibited this higher level of steroid hormone. Addition of hormone to cell suspensions resulted in a significant increase in ^{32}P levels at 5 min ($P < 0.05$,

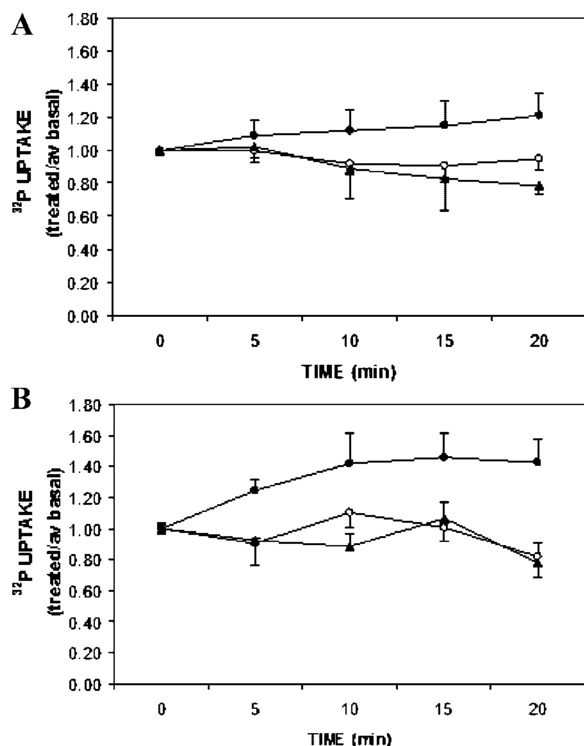

Fig. 3. Hydrogen peroxide blocks the rapid stimulatory effect of $1,25(OH)_2D_3$ on phosphate uptake in isolated intestinal cells. Cells were isolated and resuspended as described in the legend to Figure 1. Ten ml of cell suspension was combined with 20 μCi of $H_3{}^{32}PO_4$ at $T = -10$, and then 3.2 ml pipetted into each of three 50-ml conical centrifuge tubes, one of which contained 10 μl of H_2O_2 diluted to give 50 μM oxidant, final concentration (closed triangles). Samples of 100 μl were removed at $T = -5$ and -1 min, and at $T = 0$ one suspension was treated with vehicle (0.05% ethanol, final concentration, open circles) and the other two with (**A**) 130 pM $1,25(OH)_2D_3$ ($n = 6$) or (**B**) 300 pM $1,25(OH)_2D_3$ ($n = 6$). Additional samples were removed at the indicated times. All samples were pipetted into 900 μl of ice-cold GBSS, centrifuged, the supernatant decanted, and the inside of the tubes swabbed with a Kimwipe while still in the inverted position. Cell pellets were homogenized in 500 μl of reagent grade water, and analyzed for protein and radioactivity. Each value of specific radioactivity was normalized to average basal uptake values.

relative to corresponding H_2O_2-treated cells), which rose to 183% of levels in H_2O_2-pretreated cells by 20 min of incubation.

Effect of Cellular Treatment With $24,25(OH)_2D_3$ or H_2O_2 on Specific Binding of $[^3H]1,25(OH)_2D_3$

We have reported that the $1,25D_3$-MARRS protein, which is identical to glucose regulated protein GR58 and ERp57, contains two thioredoxin domains available for protein–protein interactions [Nemere et al., 2004]. Thus, one potential mechanism of inhibition would be oxidation of thiols in the $1,25D_3$-MARRS protein to diminish ligand binding.

As a first step in testing this hypothesis, isolated enterocytes were treated with vehicle or 6.5 nM $24,25(OH)_2D_3$ at $T = 0$ min, and incubated for 5, 10, 20, or 30 min prior to chilling, harvesting of the cells by centrifugation, and disruption in homogenization medium. As depicted in Figure 4A, cells incubated for 5 min in the presence of $24,25(OH)_2D_3$ or vehicle had essentially equivalent levels of specific $[^3H]1,25(OH)_2D_3$ binding to the $1,25D_3$-MARRS protein ($P > 0.05$), indicating no competition of one steroid for the binding protein of another [see also Nemere et al., 2002]. However,

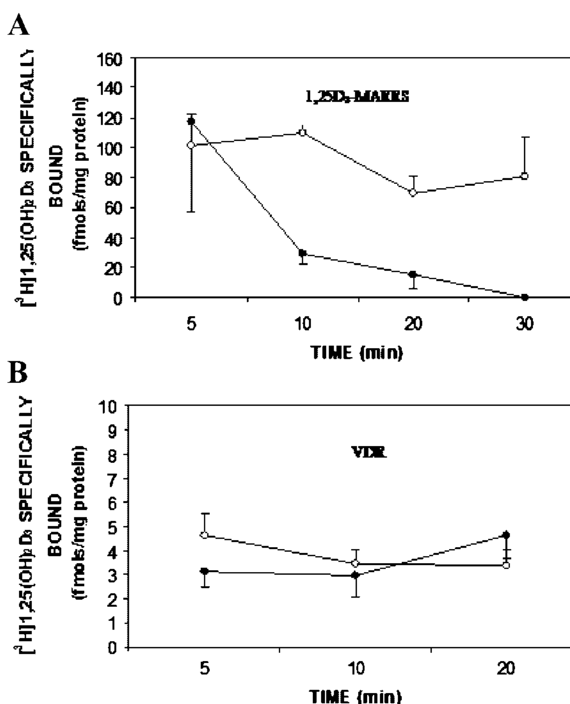

Fig. 4. Incubation of cells with $24,25(OH)_2D_3$ gradually inhibits $[^3H]1,25(OH)_2D_3$ binding to the $1,25D_3$-MARRS protein, but not to the VDR. Isolated intestinal epithelial cells were resuspended in GBSS as described in the legend to Figure 1 and divided into 5-ml aliquots. Half of the aliquots received 6.5 nM $24,25(OH)_2D_3$ at $T = 0$, and the other half received the vehicle ethanol (0.05%, final concentration). At the indicated times, aliquots were removed to ice. Cells were collected by centrifugation and homogenized in 250 mM sucrose, 5 mm histidine-imidazole, 2 mM EGTA, pH 7.0. Samples (50 μg of protein in 10 mM Tris, 2 mM EDTA, pH 7.4) were incubated (overnight, on ice) in triplicate with 1 nM $[^3H]1,25(OH)_2D_3$ alone (total binding), or in triplicate in the presence of a 200-fold molar excess of unlabeled steroid (nonspecific binding). The following morning, bound and free radioactivity were separated by either the perchloric acid precipitation technique for $1,25D_3$-MARRS (**A**; $n = 3$) or by binding the hydroxylapatite for the VDR (**B**; $n = 2$). Values represent mean ± SEM (A) or range (B).

10–20 min of incubation with $24,25(OH)_2D_3$ reduced specific binding of $[^3H]1,25(OH)_2D_3$ by approximately 80% ($P < 0.05$. relative to corresponding controls). Equivalent incubations in the presence of dithiothreitol restored specific binding, supporting the involvement of thiols.

In several of the cell preparations, specific binding to the VDR was also determined (Fig. 4B). Specific binding of $[^3H]1,25(OH)_2D_3$ to the VDR was not affected by incubation of cells with $24,25(OH)_2D_3$, relative to corresponding controls, at the times tested.

We then determined whether incubation of isolated intestinal epithelial cells with 50 μM H_2O_2 affected binding to either protein. Figure 5 illustrates the results of these experiments, and indicates that specific binding of $[^3H]1,25(OH)_2D_3$ to the $1,25D_3$-MARRS protein was inhibited at 10–20 min of exposure to oxidant ($P < 0.05$, relative to corresponding controls)—but not at 5 min—whereas specific binding to the VDR was undiminished. Indeed, after 20 min of exposure to H_2O_2, an increase in specific ligand binding to the VDR was apparent.

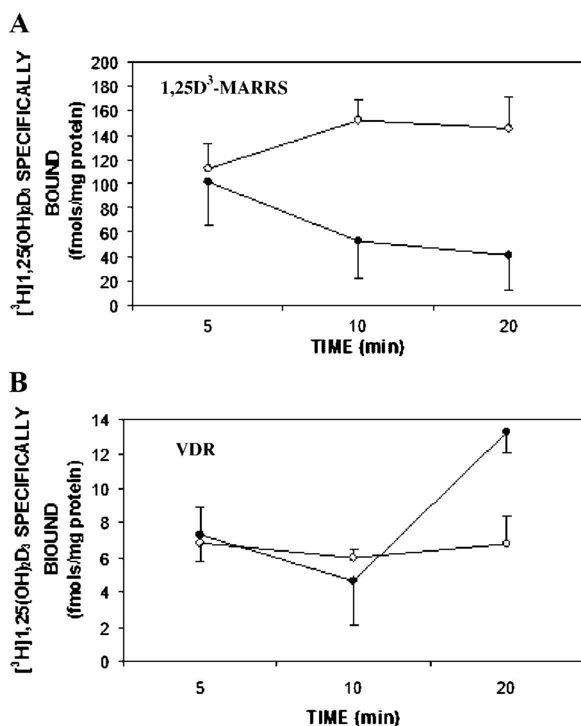

Fig. 5. Incubation of cells with H_2O_2 mimics the effects of $24,25(OH_2)D_3$ on $[^3H]1,25(OH)_2D_3$ binding. Intestinal epithelial cells were isolated, resuspended, and incubated as described in the legend to Figure 4, with the exception that 50 μM H_2O_2 was added at T = 0 min, rather than steroid. Procedures for binding of $[^3H]1,25(OH)_2D_3$ were as described in the legend to Figure 4. Values represent mean ± SEM for binding to $1,25D_3$-MARRS protein (**A**; n = 3) and mean ± range for VDR (**B**; n = 2).

Effect of Antioxidant Diets on Phosphate Absorption In Vivo

The biochemical data presented above suggested that addition of antioxidants to standard chick feed would increase phosphate absorption mediated by $1,25(OH)_2D_3$. In this regard, it should be noted that the primary effect of $1,25(OH)_2D_3$ in growing animals is on phosphate absorption, while in adults, the emphasis is on calcium absorption [Larsson and Nemere, 2003a,b]. The results of the feeding study, shown in Figure 6 indicate that supplementation of diets with either antioxidant vitamin alone (+C or 2 × E) or in combination (+C, 2 × E) significantly increased phosphate absorption ($P < 0.05$). Indeed, addition of ascorbic acid (which is not a vitamin requirement for chickens) nearly doubled phosphate absorption (Fig. 6).

Mucosal homogenates prepared from one bird per group were centrifuged at 1,000*g*, 20 min to yield a nuclear pellet [P_1, Nemere, 1996], and the resulting supernatant fraction centrifuged at 20,000*g*, 10 min to yield a pellet (P_2) containing basal lateral membranes and intracellular

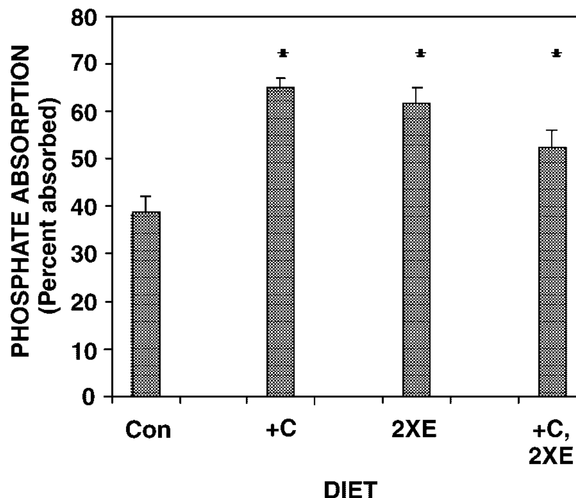

Fig. 6. Dietary vitamins C and E stimulate phosphate absorption in vivo. Chicks (five per group) were raised for 3 weeks on grower diets modified to reduce vitamin D to 200 IU/kg diet alone (Con), or supplemented with 800 ppm ascorbic acid/kg diet (+C), additional vitamin E to 100 IU/kg diet (2 × E) or both antioxidants (+C, 2 × E). On the final 3 days of feeding, chicks were housed individually, the amount of feed consumed weighed by difference, and manure collected for phosphate determination. Values represent mean ± SEM for 15 determinations. *$P < 0.05$, relative to control diet.

organelles [Nemere, 1996]. Analyses of the P_2 fractions indicated that all of the antioxidant diets increased specific [^{3}H]1,25(OH)$_2$D$_3$ binding to the 1,25D$_3$-MARRS protein (Fig. 7A). A very similar result was obtained for binding to the VDR in the P_1 fraction (Fig. 7B). Western analysis of P_2 fractions with Ab 099 against the 1,25D$_3$-MARRS receptor indicated no difference in protein levels due to dietary anti-oxidants (data not shown).

Analyses of the remaining four chicks per group for basal ^{32}P absorption failed to detect an influence of diet. Serum values (dpm/100 μl) were 185 ± 19, 140 ± 47, 169 ± 48, 168 ± 36 for Con, +C, $2 \times$ E, and +C, $2 \times$ E diets, respectively. In addition, wings and femurs were analyzed for tensile strength and phosphorous content as indicated in Table I. No significant differences were found in tensile strength as a result of dietary manipulation. However, phosphorous content was decreased in wing bones of birds on the +C, $2 \times$ E diet, and in femurs of chicks on the +C diet ($P < 0.05$, relative to corresponding control diet values). At first consideration, this might be attributed to activation of osteoclasts due to increased PTH release. However, PTH also enhances phosphate excretion in the kidneys. Since chicken manure contains both renal and fecal excretion, and the antioxidant diets clearly decreased phosphate in the manure, this did not seem to be a viable explanation.

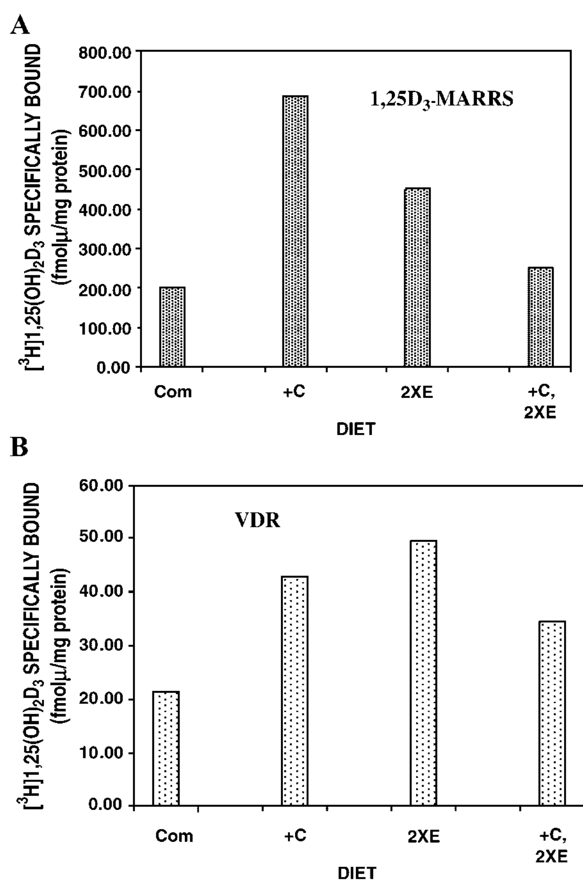

Fig. 7. Antioxidant diets enhance [^{3}H]1,25(OH)$_2$D$_3$ binding to both the 1,25D$_3$-MARRS protein and VDR. For one chick per group in the feeding study (legend to Fig. 6), duodena were removed from the anesthetized animal and chilled on ice for 15 min. At the end of this time, the pancreas was excised, the loop slit longitudinally, rinsed in ice-cold saline, and the mucosa collected by scraping. Mucosae were homogenized in 250 mM sucrose, 5 mM histidine-imidazole, 1 mM EGTA, pH 7.0, and subjected to differential centrifugation to yield a nuclear pellet (P_1, 1,000*g*, 20 min) for binding to the VDR and a post nuclear pellet (P_2, 20,000*g*, 10 min) for binding to the 1,25D$_3$-MARRS protein. Binding assays were as described in the legend to Figure 4.

A direct effect of 1,25(OH)$_2$D$_3$ on bone is to cause resorption. We subsequently investigated whether osteoclasts express transcripts for the 1,25D$_3$-MARRS protein.

Analysis of Osteoclasts for 1,25D$_3$-MARRS

RNA was extracted from HD-11EM clonal osteoclast cells and used for RT-PCR with primers designed against the avian 1,25D$_3$-MARRS transcript. Figure 8 demonstrates the presence of the transcript in replicate cultures of these cells.

A New Model of the Vitamin D Endocrine System

Figure 9 depicts our current understanding of the stimulatory actions of 1,25(OH)$_2$D$_3$ on phosphate and calcium transport, and how the metabolite 24,25(OH)$_2$D$_3$ inhibits that activity. The role of the 1,25D$_3$-MARRS redox state is also indicated, as diet may play a central role in modulating endocrine homeostasis.

DISCUSSION

The current work significantly increases our understanding of the vitamin D endocrine system through delineation of the signal transduction pathway that allows 24,25(OH)$_2$D$_3$ to inhibit the actions of 1,25(OH)$_2$D$_3$. In a previous report, we found that a cellular binding protein for 24,25(OH)$_2$D$_3$ is the enzyme catalase [Larsson et al., 2006]. In the present report, we found that the functional consequence of such binding was to decrease catalase activity, with a concomitant increase in H_2O_2, beyond basal levels [Ha et al., 2005]. The micromolar levels of

TABLE I. Bone Measurements as a Function of Diet

	Tensile Strength (kg)		Phosphorous (% of bone ash)	
Diet	Wings	Femurs	Wings	Femurs
Con	1.27 ± 0.17	1.74 ± 0.20	9.98 ± 0.78	9.72 ± 0.3
+C	1.20 ± 0.03	1.76 ± 0.18	8.67 ± 0.52	$6.63 \pm 1.26^*$
$2 \times E$	1.01 ± 0.06	1.55 ± 0.11	11.40 ± 0.90	8.88 ± 0.84
+C, $2 \times E$	1.17 ± 0.07	1.75 ± 0.12	$7.20 \pm 0.12^*$	9.61 ± 0.94

Values represent mean ± SEM for 10 determinations.
$^*P < 0.05$.

H_2O_2 thus produced (per approximately 10^9 cells) can inhibit $1,25(OH)_2D_3$-stimulated phosphate uptake in isolated intestinal cells. Intriguingly, estradiol, progesterone, and testosterone have also been reported to bind to "non-classical" proteins: Estradiol and progesterone have been reported to alter glyceraldehyde-3-phosphate dehydrogenase activity directly [Joe and Ramirez, 2001], while estradiol and testosterone have been reported to have opposite effects on microtubule polymerization [Kipp and Ramirez, 2003].

In the present study, the findings suggested that a target of inhibitory oxidation might be the $1,25D_3$-MARRS protein, which contains two thioredoxin folds [Nemere et al., 2004]. The reduced thiols are most likely responsible for protein–protein interactions that allow for positive cooperativity of binding [Larsson and Nemere, 2003a,b], and may facilitate movement of the $1,25D_3$-MARRS protein to the nucleus following ligand binding [Nemere et al., 2000; Nemere, 2005; Rohe et al., 2005].

Incubation of isolated intestinal cells with $24,25(OH)_2D_3$ for 5 min did not affect the binding of $[^3H]1,25(OH)_2D_3$, relative to controls, confirming yet again the specificity of the $1,25D_3$-MARRS protein. At 10 and 20 min of incubation with $24,25(OH)_2D_3$, however, the specific binding of $[^3H]1,25(OH)_2D_3$ to the $1,25D_3$-MARRS protein was progressively diminished. These results were paralleled by observations with exogenous addition of H_2O_2. In contrast, the VDR was either unaffected (incubations with $24,25(OH)_2D_3$), or exhibited enhanced binding of $[^3H]1,25(OH)_2D_3$ 20 min after exposure to low levels of H_2O_2). These results, in addition to others [Nemere et al., 2000, 2004; Larsson and Nemere, 2003a,b; Nemere, 2005] provide further evidence that the VDR is not involved in mediating the rapid response to $1,25(OH)_2D_3$ in intestine. However, the enhanced binding after exposure to reactive oxygen species suggests the existence of a mechanism to preserve transcriptional effects mediated by the VDR.

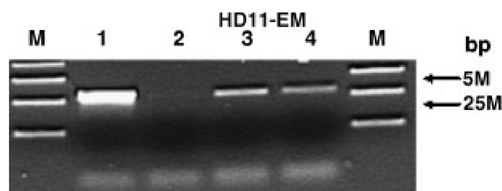

Fig. 8. $1,25D_3$ MARRS transcripts present in HD-11 EM pre-osteoclasts detected by RT-PCR. M = size marker; **lane 1** = plasmid positive control for RT-PCR; **lane 2** = negative control (no reverse transcriptase included in RT reaction); **lanes 3** and **4** are two replicate HD11-EM RNA samples.

The central importance of the redox state of the $1,25D_3$-MARRS protein suggested that phosphate uptake in vivo would respond positively to diets enriched in antioxidants. This was found to be the case. Diets formulated to contain 200 IU/kg of vitamin D reduced the likely overproduction of $24,25(OH)_2D_3$ [Tryfonidou et al., 2002], while antioxidants were anticipated to maintain the $1,25D_3$-MARRS protein in an active state. Indeed, inclusion of ascorbic acid (which is not a vitamin requirement in the chicken), doubled phosphate absorption, as did increasing the vitamin E levels of the diet. Intriguingly, specific binding increased for both the $1,25D_3$-MARRS protein and the VDR. This is understandable in light of the observation that transcriptional regulation by $1,25(OH)_2D_3$ is necessary to provide components of the mineral transport pathway, which then can be rapidly activated without transcription. The beneficial effects of antioxidant diets on intestinal mineral absorption may be a contributing factor to maintaining overall bone health [Kanatani et al., 2003; Lean et al., 2003; Magne et al., 2003] over prolonged periods. For the short duration of the feeding studies employed in the current work, however, no difference in tensile strength was observed. We

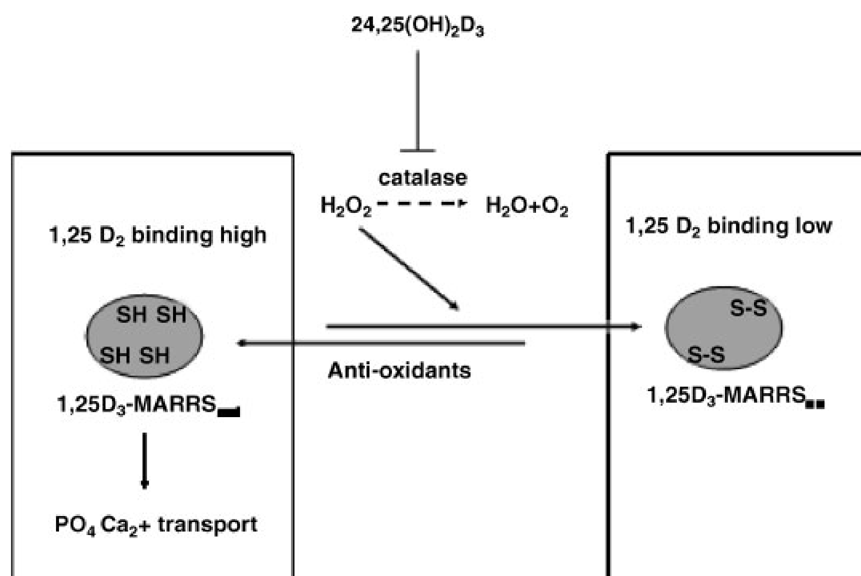

Fig. 9. A new model of interaction of vitamin D seco-steroids. When $1,25D_3$-MARRS protein is in the reduced state, ligand binding initiates signal transduction events that promote the rapid absorption of phosphate and calcium from intestine. $24,25(OH)_2D_3$ promotes a pro-oxidant state through decreased catalase activity, that functionally, but reversibly inactivates the $1,25D_3$-MARRS protein.

did, however, note a trend toward decreased phosphate in bone ash that was significantly different in two cases. In light of the enhanced phosphate absorption, the data suggested a direct activation of osteoclasts. We therefore analyzed avian cells for the $1,25D_3$-MARRS mRNA, and found it to be present. Clearly, this finding provides a potential explanation for the observations that the direct effect of $1,25(OH)_2D_3$ on bone is to promote resorption. Additional work will be undertaken to assess $1,25D_3$-MARRS protein levels and functions in osteoclasts.

In conclusion, we have elucidated a novel mechanism of $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2D_3$ action, and further established the physiological significance of the $1,25D_3$-MARRS protein in mineral and ion homeostasis.

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