Effect of 24,25-dihydroxyvitamin D$_3$ on localization of catalase in chick enterocytes

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Abstract

The vitamin D metabolite, 24,25(OH)$_2$D$_3$ has been reported to have hormonal activity. Catalase has been reported to be a binding protein for 24,25(OH)$_2$D$_3$, based on sequence analysis of the protein isolated on the basis of specific binding of the metabolite. In the current work, we report that 24R,25(OH)$_2$D$_3$, not 24S,25(OH)$_2$D$_3$ is the effective metabolite for catalase redistribution as judged by confocal microscopy. We have used male chick intestinal cells treated with either vehicle, 24S,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$ or 1,25(OH)$_2$D$_3$ to determine the localization of catalase. Confocal microscopy analyses showed punctate staining, on the cell surface and in the cytoplasm of cells treated with vehicle, 24R,25(OH)$_2$D$_3$, 24S,25(OH)$_2$D$_3$ or 1,25(OH)$_2$D$_3$ for all time points tested. Cells treated with 24R,25(OH)$_2$D$_3$ showed punctuate staining of catalase inside the nucleus. Western analysis confirmed that the punctuate staining in the nucleus arose from the redistribution of cell surface catalase. Western analysis also indicated 24S,25(OH)$_2$D$_3$ treatment resulted in redistribution of catalase to the nucleus, but to a lesser extent than treatment with 24R,25(OH)$_2$D$_3$. By understanding the molecular and cellular actions of 24,25(OH)$_2$D$_3$ in chick intestine, progress will be made in enhancing phosphate and calcium absorption in animals to supply the minerals for adequate bone growth, and phosphate in manure of production animals could be diminished.

Introduction

Vitamin D was discovered in 1922 and has been categorized as a pre-hormone ever since, based on the fact that the utilization of most vitamin D in higher animals undergoes a photochemical process. Activation of vitamin D starts in the liver with the production of major circulating metabolite 25-hydroxyvitamin D$_3$ [25(OH)D$_3$], followed by hydroxylation in the kidneys to yield two metabolites, either 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] made when phosphate and calcium levels are low or 24,25-dihydroxyvitamin D$_3$ [24,25(OH)$_2$D$_3$] made when phosphate and calcium levels are high.

24,25-dihydroxyvitamin D$_3$ is no longer considered as an inactive metabolite. Earlier studies showed that in order to reach normal chick hatchability [1] and bone formation [2], both 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ were necessary. While 1,25-dihydroxyvitamin D$_3$ stimulates the rapid transport of calcium and phosphate in both perfused chick duodenal loops and isolated enterocytes, 24,25-dihydroxyvitamin D$_3$ inhibits such stimulation [3,4]. In osteoblasts and osteosarcoma cells, 1,25(OH)$_2$D$_3$ has been found to have a rapid effect on calcium-channel-opening, while 24,25(OH)$_2$D$_3$ was found to inhibit this non-nuclear effect [5-7]. In chick intestinal cells, 1,25(OH)$_2$D$_3$ has been found to have an acute, non-nuclear effect on phosphate transport, and 24,25(OH)$_2$D$_3$ has been found to inhibit the reaction [8], with no effect on parathyroid hormone (PTH) stimulated phosphate transport [9]. In perfused chick duodenal loops, 24,25(OH)$_2$D$_3$ inhibits the rapid stimulation of phosphate transport [8] mediated by 1,25(OH)$_2$D$_3$, as well as calcium transport [10].

In chick intestinal cells, hormone-stimulated phosphate uptake is initiated by ligand binding to the 1,25D$_3$-membrane associated, rapid response steroid-binding receptor – 1,25D$_3$-MARRS [11], also known as ERp57/GR58/PDIA3. The ability of 24,25(OH)$_2$D$_3$ to inhibit 1,25D$_3$-MARRS receptor activation of protein kinase A and C activities was found in chick intestine [12] and kidney [4], which indicates the existence of a specific binding protein for 24,25(OH)$_2$D$_3$. Percoll gradient analysis revealed lysosomal fractions to have the highest [³H]24,25(OH)$_2$D$_3$ binding activity [10]. In order to explain how 24,25(OH)$_2$D$_3$ works to effect inhibition, a cellular binding protein (66 kDa) was isolated, purified [10] and sequenced [13]. It was found to have a binding constant of 7 nM for 24,25(OH)$_2$D$_3$, and identified as catalase using Edman degradation techniques [13]. The enzyme catalase is sensitive to cell signaling molecules [14]. It was found that the inhibitory action of 24,25(OH)$_2$D$_3$ is caused by a decrease in catalase activity in both chick intestine [15,16] and kidney [12], accompanied by an increase in H$_2$O$_2$ production [13]. Thus, one possible mechanism for the inhibitory action would be the oxidation of thioredoxin domains in 1,25D$_3$-MARRS receptor that occurred after 10 min of exposure [15], accompanied by a loss of binding activity. However, studies [15] showed a time-dependent decrease with either 24,25(OH)$_2$D$_3$ or H$_2$O$_2$ treatments after 5 minutes of incubation, indicating another mechanism because 24,25(OH)$_2$D$_3$ does not compete with 1,25(OH)$_2$D$_3$ for MARRS binding.

In intestinal cells, studies showed 1,25D$_3$-stimulated phosphate uptake is mediated by PKC signaling [3], and 24,25(OH)$_2$D$_3$ seems to abolish that effect [10]. Thus, another possible mechanism would be the affecting the signal transduction pathway [17].

In this study, chick enterocytes were used to determine the localization of catalase in response to different vitamin D steroid hormones. Western analysis was performed to verify the results from confocal microscopy.

Experimental

Animals

All surgical procedures were approved by Utah State University Institutional Animal Use and Care Committee. White leghorn cockerels were obtained on the day of hatch (Privett Hatchery, Portales, NM) and raised on a commercially available vitamin D-replete diet (Nutrena Feeds, Murray, UT) generally for 3-7 weeks prior to experimentation. On the day of use, chicks were anesthetized with anhydrous ethyl ether (Fisher Scientific, Fair Lawn, NJ). The abdominal cavity was surgically opened and the duodenal loop was removed to ice-cold 0.9% saline solution and chilled for 15 min. The pancreas was excised from the duodenal loop and discarded. The duodenal loop was everted and rinsed three times in chilled saline solution.

The chick intestinal cells were isolated with citrate chelation media (96 mM NaCl, 27 mM Citrate Anhydrous, 1.5 mM KCl, 8 mM KH$_2$PO$_4$, 5.6 mM Na$_2$HPO$_4$, pH 5.0 - the acidic pH promotes viability and retention of morphology) [3,18]. The cells were collected by low speed centrifugation (500 x g, 5 min, 4ºC), and cell pellets were resuspended in a small volume of Gey's balanced salt solution (GBSS, containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH$_2$PO$_4$, 0.84 mM NaHPO$_4$, 1.03 mM MgCl$_2$•6H$_2$O, 0.28 mM MgSO$_4$•7H$_2$O, 0.9 mM CaCl$_2$, pH 7.3). Aliquots of the cell suspension (0.4 ml) were pipetted into 35 mm-plastic Petri dishes (Falcon, Scientific Products; Franklin Lakes, NJ) containing 1.5 ml of RPMI 1640 medium and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin, Sigma Chemical Co, St. Louis, Mo). The cells were incubated overnight in the absence of serum at 37ºC with 5% CO$_2$/95% air to promote cell adhesion.

Confocal Microscopy

The following morning, media were replaced with 0.1% BSA in GBSS (GBSS-BSA, Bovine Serum Albumin, Sigma, St. Louis, MO) and cells treated either with vehicle (0.01% ethanol) or hormone for 15 sec to 60 min (15 sec, 30 sec, 7 min, 10 min, 15 min, 25 min, 30 min, 40 min, 50 min, 60 min). At the end of each time point, media were replaced with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), 3% sucrose in PBS (phosphate buffered saline) and fixed for 30 min. After washing with 0.1% PBS-BSA, cells were incubated with 0.05% NaBH$_4$ in PBS for 5 min to eliminate auto-fluorescence in the brush border, then washed and permeabilized with 0.15% Triton X-100 in PBS for 5 min for intracellular catalase activity, or without Triton X-100 for surface catalase activity. After washing with 0.1% PBS-BSA, cells were overlaid with normal rabbit serum (JacksonImmuno Research, West Grove, PA) for 5 min and then washed. Coverslips were then overlaid with primary antibody Ab365 (a highly specific polyclonal antibody, Center for Integrated BioSystems, Logan, UT) for 30 min (1/1000 in 0.1% PBS-BSA), and then incubated for another 30 min (after addition of more PBS-BSA to prevent drying), washed, and then overlaid with fluorescently-tagged secondary antibody Alexa Fluor 594 (Jackson Immuno Research, West Grove, PA), excitation at 591 nm and emission at 614 nm; and Phalloidin (Sigma-Aldrich, St. Louis, MO) labeled with fluorescein isothiocyanate (with excitation at 495 nm and emission at 513 nm) for 30 min. Coverslips were then washed three times. After the final wash, the coverslips were placed over mounting media (10% 1 M Tris, 80% glycerol) on a microscope slide, and sealed for subsequent confocal microscopy analysis. A Nikon TE-200 microscope (BioRad) was used for confocal imaging. Images were collected with ZEN software, using a 60x oil immersion objective and further processed with ImageJ and Adobe Photoshop CS5.

Western Blots

The isolated intestinal cells described above were collected by centrifugation at 500×g, 5 min (4ºC), and resuspended in 30 ml of GBSS. 5 ml of the cell suspensions were combined with test substance in GBSS to give a final concentration of 0.01% ethanol, 6.5 nM 24R,25(OH)$_2$D$_3$ and 200 pM 24S,25(OH)$_2$D$_3$. The cells were incubated for 10 minutes and 25 minutes, then 1 ml were removed to 10 ml of ice-cold PBS for cytoplasmic and nuclear extraction. The extraction procedure involves mixing with a series of detergents in the presence of protease inhibitors.

SDS-PAGE and Western blot analyses were used to determine immunoreactive levels of catalase in control and vitamin D treated cells. Protein was determined with the Bradford reagent (Bio-Rad, Hercules, CA) and then samples (5-30 μg/well) were separated on 8% (wt/vol) SDS-PAGE gels with 5% stacking gels. After separation on SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Fisher Scientific) by the use of a TransBlot SD Semidry transfer cell (Bio-Rad.) and Western analyses were performed. To avoid nonspecific binding, the membrane was soaked for 1 hr at 37ºC in blocking solution (0.5% nonfat dry milk in phosphate buffered saline (PBS; 0.9% NaCl and 10 mM Na$_2$HPO$_4$, pH 7.4), followed by washing three times for 5 min each time with washing solution (0.1%(wt/vol) BSA in Tris-buffered saline (TBS; 0.9% NaCl in 20 mM Tris-HCl, pH 7.4), and incubation with primary antibody (Abcam Inc., Cambridge, MA) overnight at 4°C. After three additional washes, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) in 1% BSA and 0.05% Tween 20 in TBS for 2 hr at room temperature and then washed as previously indicated. Immunoreactive bands were visualized with the chromogens 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, and relative amounts of catalase were quantitated using Adobe Photoshop.

Results and discussion

Time course study of catalase localization after hormone *in vitro*

In initial experiments, catalase localization by confocal microscopy was determined by adding hormones before primary antibody Ab365. Chick intestinal cells were cultured in petri dishes, treated with 0.01% ethanol, 6.5 nM 24R,25(OH)$_2$D$_3$, 200 pM 24S,25(OH)$_2$D$_3$, or 300 pM 1,25(OH)$_2$D$_3$, concentrations that have been shown to be equivalent to circulating levels [19], for selected times (15 sec, 30 sec, 7 min, 10min, 15 min, 25 min, 30 min, 40 min, 50 min, 60 min), and fixed for confocal microscopy. Red staining depicts Alexa Fluor 594 fluorescence, which is indicative of catalase localization on the cell surface. Figure 1A shows shorter time points and Figure 1B shows longer time points. Control cells treated with 0.01% ethanol for selected times revealed obvious surface staining, with a low level inside the nucleus. These observations in this and subsequent experiments were reproduced in triplicate experiments. Figure 2 depicts results from experiments in which cells were treated with 24R,25(OH)$_2$D$_3$ for shorter (Figure 2A) and longer (Figure 2B) time points, in which there were obvious increases in nuclear staining (indicated by yellow arrows) as well as surface staining relative to controls. Figure 3 depicts results from experiments in which cells were treated with 24S, 25(OH)$_2$D$_3$ for shorter (Figure 3A) and
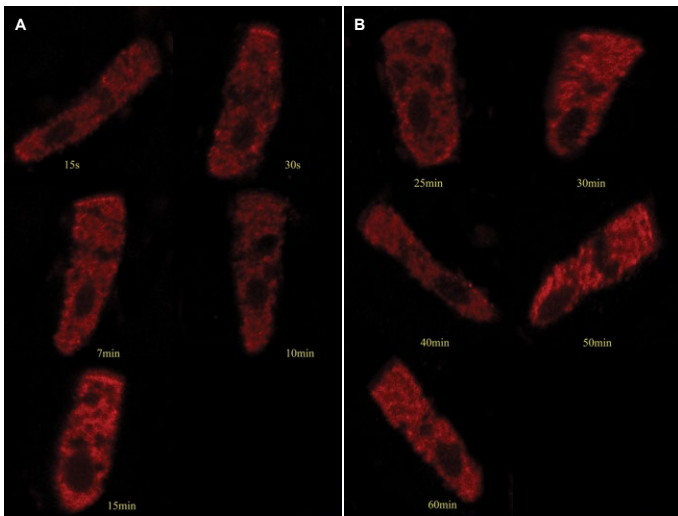
Figure 1. Time course study of catalase localization in isolated intestinal cells of control treatment group. A: shorter time points (15 sec-15 min); B: longer time points (25 min-60 min). Cells were isolated by citrate chelation, resuspended in Gey's balanced salt solution, and pipeted into petri dishes containing a coverslip and RPMI 1640, allowing cells to adhere overnight. The following day, media were replaced with GBSS-0.1% BSA. Vehicle was then added for the indicated times, after which cells were fixed, either permeabilized or not, and stained with anti-catalase antibody. In permeabilized, vehicle-treated cells, punctate staining was visible; catalase redistribution to the nucleus was negligible.
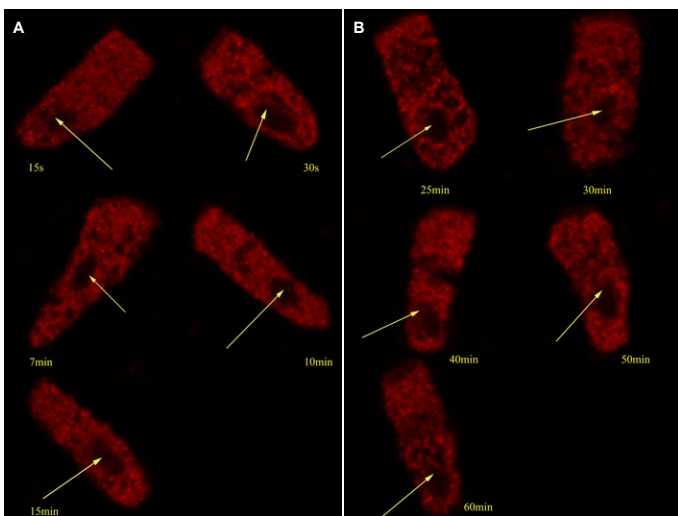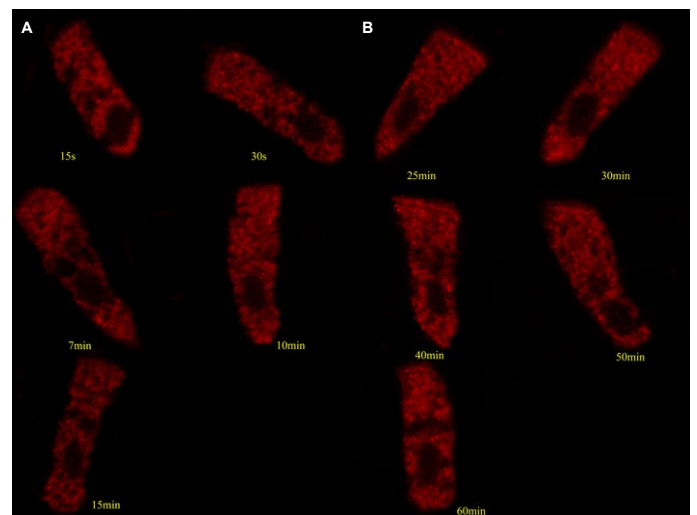

Figure 2. Time course study of catalase localization in isolated intestinal cells of the 24R,25(OH)$_2$D$_3$ treatment group. Procedures were as described in the legend to Figure 1, but cells were treated with 6.5 nM hormone at the indicated times. Punctate staining was visible; catalase redistribution to nucleus was evident as soon as 15 sec after 24R,25(OH)$_2$D$_3$ treatment.

longer (Figure 3B) time points, in which there were slight increases in nuclear staining relative to controls, but to a much lesser extent compared to 24,25(OH)$_2$D$_3$ treatment. Figure 4 depicts results from experiments in which cells were treated with 1, 25(OH)$_2$D$_3$ for shorter (Figure 4A) and longer (Figure 4B) time points, in which there were no increases in nuclear staining nor surface staining relative to controls, since 1,25(OH)$_2$D$_3$ does not compete with 24R,25(OH)$_2$D$_3$ for binding to catalase. In a previous study, it was found that 24R,25(OH)$_2$D$_3$ is capable of decreasing phosphate absorption after a 1-h injection in vivo and 24S,25(OH)$_2$D$_3$ is capable of increasing phosphate absorption after a 5-h injection in vivo, suggesting that the inhibitory effect might be mainly performed by 24R,25(OH)$_2$D$_3$ [20]. One possible gene

regulatory mechanism could be catalase binding to STAT3 [21]. Further study is required to see if STAT3 is a binding partner of catalase.

The question was raised as to the origin of the nuclear staining. In order to answer that, additional confocal microscopy experiments were undertaken to determine whether cell surface catalase was the source of steroid-mediated nuclear redistribution. In these experiments, primary antibody was first added to cells for 30 min, and subsequently incubated with vehicle, 1,25(OH)$_2$D$_3$, 24S,25(OH)$_2$D$_3$, or 24R,25(OH)$_2$D$_3$. Figure 5 depicts control cells treated with 0.01% ethanol for shorter (Figure 5A) and longer (Figure 5B) time points revealed obvious surface staining, but little inside the nucleus. As shown in Figure 6A, B, nuclear redistribution after 24R,25(OH)$_2$D$_3$ treatment did not occur, suggesting that ligand binding to cell surface catalase induced redistribution to the nucleus, but was blocked by the antibody. Similarly, treatment of cells with antibody first inhibited the effects of 24S,25(OH)$_2$D$_3$ (Figure 7A,
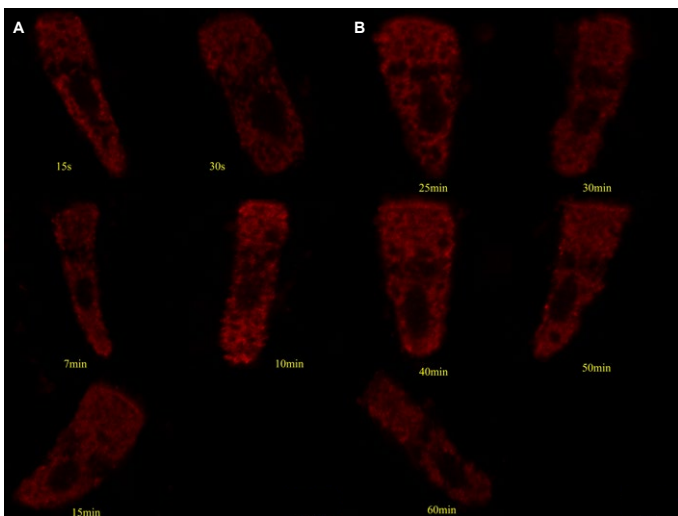

Figure 3. Time course study of catalase localization in isolated intestinal cells of the 24S,25(OH)$_2$D$_3$ treatment group. Procedures were described as in the legend to Figure 1. Punctate staining was visible; catalase redistribution to nucleus was detected but to a much lesser extent compared to 24R,25(OH)$_2$D$_3$ treatment.
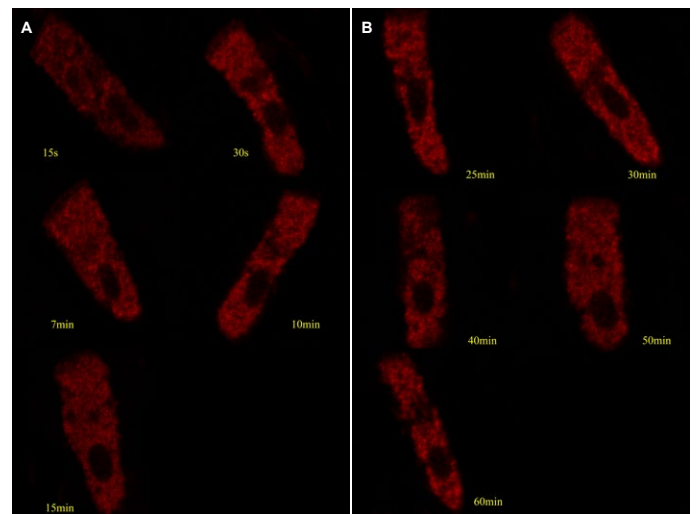
Figure 4. Time course study of catalase localization in isolated intestinal cells of the 1,25(OH)$_2$D$_3$ treatment group. Procedures were described as in the legend to Figure 1. Punctate staining was visible; catalase redistribution to nucleus was not evident.
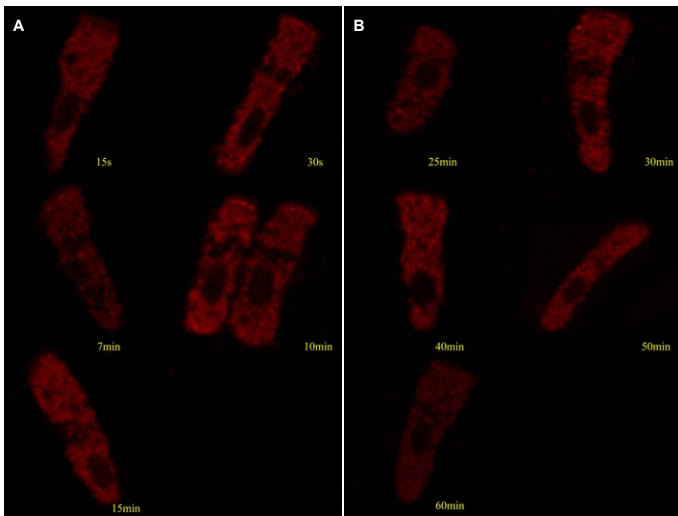
Figure 5. Time course study of catalase localization in isolated intestinal cells treated with antibody prior to vehicle. Cells were isolated by citrate chelation, resuspended in Gey's balanced salt solution, and pipeted into petri dishes containing a coverslip and RPMI 1640, allowing cells to adhere overnight The following day, media were replaced with primary antibody for 30 min. Vehicle or hormone was then added for the indicated times, after which cells were fixed, either permeabilized or not, and stained with anti-catalase antibody. Punctate staining was visible; catalase redistribution to the nucleus was not evident.
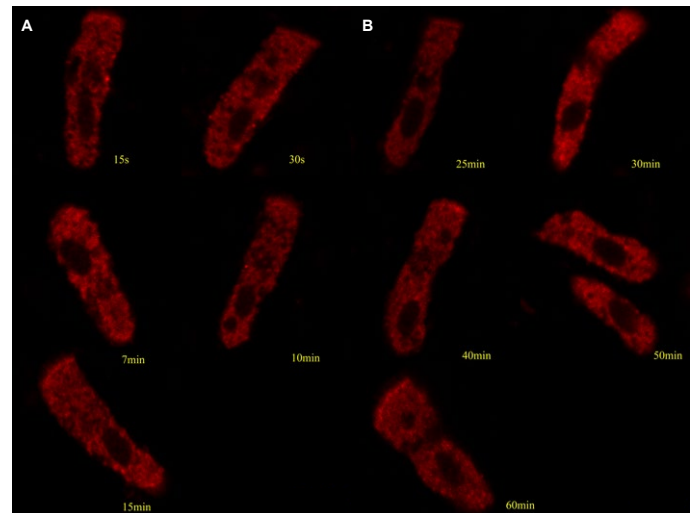

Figure 6. Time course study of catalase localization in isolated intestinal cells treated with antibody prior to 24R,25(OH)$_2$D$_3$. Procedures were described as in the legend to Figure 5. Punctate staining was visible; catalase redistribution to nucleus was not evident.

and 7B), and as expected, no effect of 1,25(OH)$_2$D$_3$ treatment was found (Figure 8A and 8B).

As a further analysis, catalase staining intensity inside the nucleus was quantified with ImageJ software. Figure 9 depicts comparisons among 'hormone first treatments'—in which cells were treated with hormone prior to antibody. There was a significant increase in the intensity the 24R,25(OH)$_2$D$_3$ treated group, while the other groups stayed the same. Figure 10 depicts a comparison among cells treated with antibody prior to hormone, or 'antibody first treatments'. There was no obvious increase in the intensity among the four groups, indicating that the nuclear redistribution mediated by 24R,25(OH)$_2$D$_3$ is indeed from cell surface catalase.

An independent approach was taken to verify these findings. In

these experiments, cells were treated with either vehicle, 24S,25(OH)$_2$D$_3$, or 24R,25(OH)$_2$D$_3$ for 10 and 25 min, collected by centrifugation, and resuspended in homogenization buffer for subcellular fractionation. Aliquots of P1 (nuclei, brush borders, and unbroken cells), P2 (peroxisomes, lysosomes, mitochondria, Golgi, and basal lateral membranes, and S2 (microsomes and cytosol), were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting. The results reproducibly showed more nuclear redistribution after 24R,25(OH)$_2$D$_3$ treatment compared to 24S,25(OH)$_2$D$_3$ (Figure 11). One possible explanation might be there was a higher concentration of 24R,25(OH)$_2$D$_3$ in the incubations than 24S,25(OH)$_2$D$_3$. However, the fact that redistribution from cell surface and organelles to the nucleus following 24S,25(OH)$_2$D$_3$ was detected by Western analysis indicates


Figure 7. Time course study of catalase localization in isolated intestinal cells treated with antibody prior to 24S,25(OH)$_2$D$_3$. Procedures were described as in the legend to Figure 5. Punctate staining was visible; catalase redistribution to nucleus was not evident.
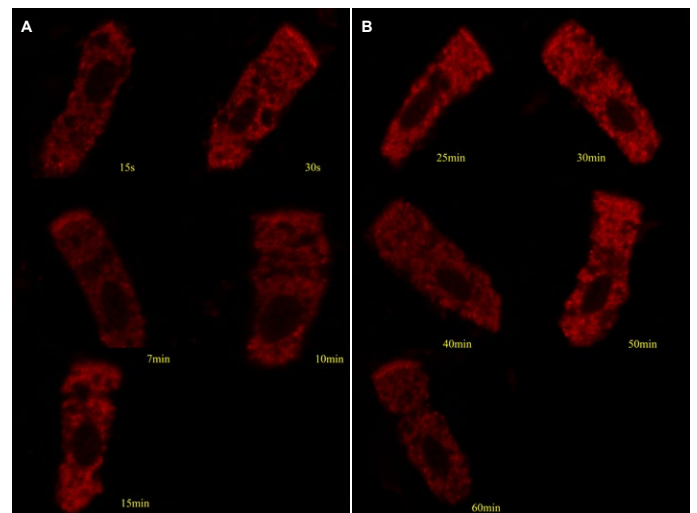
Figure 8. Time course study of catalase localization in isolated intestinal cells treated with antibody prior to 1,25(OH)$_2$D$_3$. Procedures were described as in the legend to Figure 5. Punctate staining was visible; catalase redistribution to nucleus was not evident.
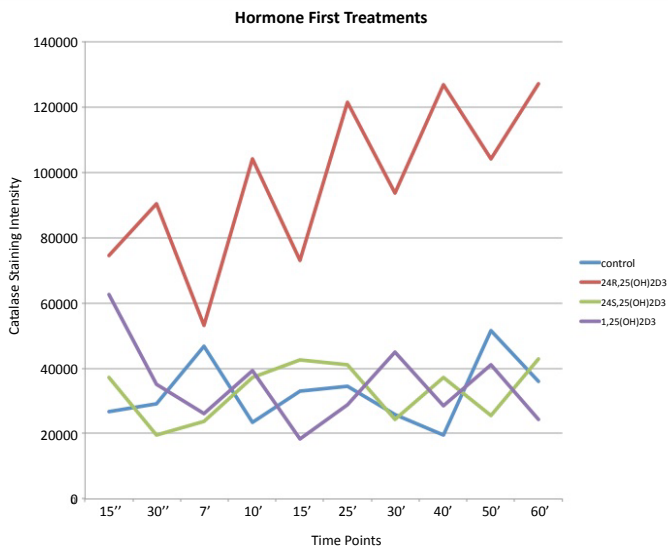
Figure 9. Quantitation of catalase staining intensity in isolated intestinal cell nuclei among vehicle, 24R,25(OH)$_2$D$_3$, 24S,25(OH)$_2$D$_3$, and 1,25(OH)$_2$D$_3$ treatment groups, hormone before antibody. A significant increase in catalase staining intensity was observed in the 24R,25(OH)$_2$D$_3$ treatment group indicating the nuclear redistribution of catalase.
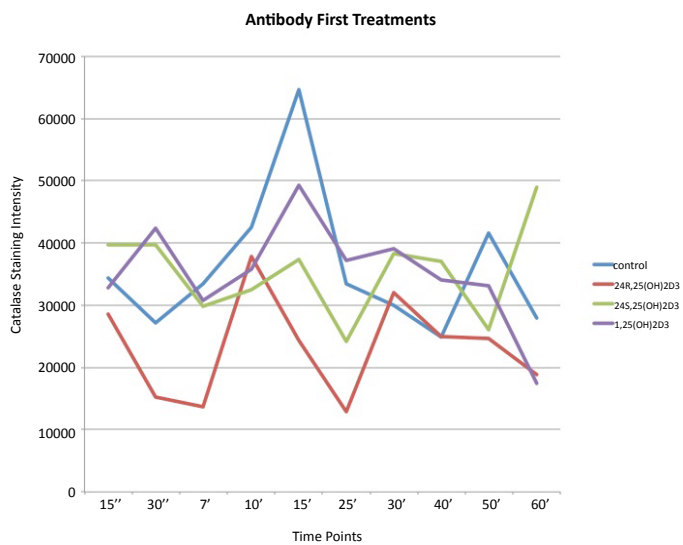

Figure 10. Quantitation of catalase staining intensity in isolated intestinal cell nuclei among vehicle, 24R,25(OH)$_2$D$_3$, 24S,25(OH)$_2$D$_3$, and 1,25(OH)$_2$D$_3$ treatment groups, antibody before hormone. After blocking the cell surface catalase, no significant change was detected among the four treatments indicating the nuclear redistribution of catalase regulated by 24R,25(OH)$_2$D$_3$ is indeed from cell surface.

that confocal microscopy is the less sensitive method. The results shown in Figure 11 also indicate that the commercially available antibody is less specific than Ab 365 [17].

Acknowledgement
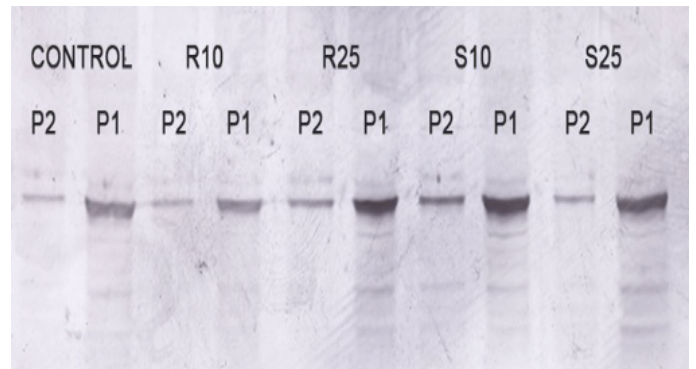
Figure 11. Western blot analysis further confirmed the redistribution of catalase from cell surface to nucleus. Cells were incubated with vehicle or steroid (24R or 24S) for the indicated lengths of time. The cells were then collected by centrifugation and the pellets resuspended in homogenization medium. After disruption in a homogenizer, the cells were subjected to centrifugation at 1000 x g for 20 min to yield pellet 1 (P1 containing nuclei, brush borders, and unbroken cells). And the supernatant again centrifuged to yield pellet 2 (P2 containing basal lateral membranes, golgi, mitochondria, and lysosomes. Western analyses were performed with primary antibody from Abcam.

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