5-2015

Pregenomic and Genomic Effects of 24,25-Dihydroxyvitamin D3

Yang Zhang
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd
Part of the Dietetics and Clinical Nutrition Commons, Food Science Commons, and the Nutrition Commons

Recommended Citation

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact rebecca.nelson@usu.edu.
PREGENOMIC AND GENOMIC EFFECTS OF 24,25-DIHYDROXYVITAMIN D₃

by

Yang Zhang

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Nutrition, Dietetics, and Food Sciences

Approved:

Dr. Korry Hintze
Major Professor

Dr. Heidi Wengreen
Committee Member

Dr. Marie Walsh
Committee Member

Dr. Mark R. McLellan
Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah
2015
ABSTRACT

Pregenomic and Genomic Effects of 24,25(OH)_2D_3

by

Yang Zhang, Master of Science

Utah State University, 2015

Major Professor: Dr. Korry Hintze
Department: Nutrition, Dietetics, and Food Sciences

Vitamin D is hydroxylated to form several active metabolites, of these, 1,25-dihydroxyvitamin D_3 [1,25(OH)_2D_3] is the most studied stimulatory product. It is now accepted that 1,25(OH)_2D_3 mediates its rapid actions on the control of phosphate homeostasis through its membrane receptor 1,25D_3-MARRS (membrane associated rapid response steroid binding) protein. Another metabolite, 24,25-dihydroxyvitamin D_3 [24,25(OH)_2D_3] has been reported to be inhibitory with respect to calcium and phosphate absorption in intestine. Previous work in this laboratory has indicated that 24,25(OH)_2D_3 inhibits phosphate uptake in isolated intestinal cells and perfused duodenal loops and in vivo. This thesis further tested the hypothesis that the actions of 24,25(OH)_2D_3 on phosphate homeostasis are physiologically important. Catalase has been identified as a binding protein for 24,25(OH)_2D_3. We determined the localization of catalase in the presence and absence of steroid, monitored catalase mRNA levels related to gene 24,25(OH)_2D_3 gene transcription regulation. We studied the effects of the two isomers of 24,25(OH)_2D_3 on localization of catalase in chicken enterocytes over a time course of 15 sec to 60 min. It was demonstrated that 24R,25(OH)_2D_3 is the effective metabolite for
catalase redistribution in vitro. We also studied the effects of vitamin D on catalase and phosphate uptake in chicken intestinal cells. It was once again demonstrated that 24R,25(OH)$_2$D$_3$ is the effective metabolite for decreasing phosphate uptake and catalase gene expression. These combined results lead us to conclude that 24,25(OH)$_2$D$_3$ is an important hormone in phosphate homeostasis in chick intestinal epithelial cells.
In the United States, the major dietary source of protein is poultry meat, particularly chicken. Phosphorus absorption in chickens is a critical problem in poultry production. It therefore is important to understand the cellular and molecular regulation of phosphorous absorption in intestine to increase efficiency of the process. This would provide the benefits of reducing feed costs and reducing phosphorous excretion, thereby contributing to the sustainability of agriculture in the United States. Therefore, the focus of this thesis is determining the effect of vitamin D metabolites involved in phosphate homeostasis using the chicken as a model.
To Xiubin Zhang and Yao Zhang
ACKNOWLEDGMENTS

I would like to express my gratitude to my major professors, Dr. Ilka Nemere and Dr. Korry Hintze, for teaching me their experienced research skills and giving me the valuable advice and comments. Without their assistance and understanding, I would not have been able to complete my studies. I would especially like to thank my committee members for their kindness and support and their helpful critical reviews and suggestions for improving my thesis.

I would like to dedicate this thesis to my beloved parents, Xiubin Zhang and Yao Zhang, who always love and understand and support me unconditionally.

I would also like to express my thanks to my friends Tremaine, Yu Meng and others, for their sincere friendship. Thank you all.

Yang Zhang
# CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT ........................................................................................................ iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT ............................................................................................... iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS ............................................................................................. vii</td>
</tr>
<tr>
<td>LIST OF FIGURES ................................................................................................. ix</td>
</tr>
<tr>
<td>LIST OF TABLES .................................................................................................. xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND DEFINITIONS ................................................ xii</td>
</tr>
</tbody>
</table>

## CHAPTER

1. LITERATURE REVIEW .................................................................................. 1

2. EFFECTS OF 24,25-DIHYDROXYVITAMIN D₃ ON LOCALIZATION OF CATALASE IN CHICK ENTEROCYTES .................................................. 36
   - Abstract ........................................................................................................ 36
   - Introduction ................................................................................................. 37
   - Experimental .............................................................................................. 39
   - Results ........................................................................................................ 42
   - Discussion ................................................................................................. 57
   - References .................................................................................................. 59

3. EFFECTS OF VITAMIN D ON CATALASE EXPRESSION AND PHOSPHATE UPTAKE IN CHICKEN INTESTINAL ............................................. 62
   - Abstract ........................................................................................................ 62
   - Introduction ................................................................................................. 62
   - Experimental .............................................................................................. 63
   - Results ........................................................................................................ 69
   - Discussion ................................................................................................. 80
   - References .................................................................................................. 82

4. SUMMARY AND CONCLUSIONS .................................................................. 83
   - Summary ...................................................................................................... 83
   - Conclusions .............................................................................................. 85
   - References .................................................................................................. 87
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic presentation of phosphate homeostasis</td>
</tr>
<tr>
<td>1.2</td>
<td>Structure of vitamin D and its metabolites</td>
</tr>
<tr>
<td>1.3</td>
<td>Regulation of PTH and 1,25(OH)₂D₃ in calcium homeostasis</td>
</tr>
<tr>
<td>1.4</td>
<td>Model for interaction between 1,25D₃-MARRS receptor and catalase</td>
</tr>
<tr>
<td>2.1</td>
<td>Time course study of catalase localization in isolated intestinal cells: ethanol control treatment</td>
</tr>
<tr>
<td>2.2</td>
<td>Time course study of catalase localization in isolated intestinal cells: 6.5 nM 24R,25(OH)₂D₃ hormone treatment</td>
</tr>
<tr>
<td>2.3</td>
<td>Time course study of catalase localization in isolated intestinal cells: 200 pM 24S,25(OH)₂D₃ hormone treatment</td>
</tr>
<tr>
<td>2.4</td>
<td>Time course study of catalase localization in isolated intestinal cells: 360 pM 1,25(OH)₂D₃ hormone treatment</td>
</tr>
<tr>
<td>2.5</td>
<td>Time course study of catalase localization in isolated intestinal cells: 0.01% ethanol (final concentration) after prior incubation with antibody</td>
</tr>
<tr>
<td>2.6</td>
<td>Time course study of catalase localization in isolated intestinal cells: 6.5 nM 24R,25(OH)₂D₃ hormone treatment after prior incubation with antibody</td>
</tr>
<tr>
<td>2.7</td>
<td>Time course study of catalase localization in isolated intestinal cells: 200 pM 24S,25(OH)₂D₃ hormone treatment after prior incubation with antibody</td>
</tr>
<tr>
<td>2.8</td>
<td>Time course study of catalase localization in isolated intestinal cells: 360 pM 1,25(OH)₂D₃ hormone treatment after prior incubation with antibody</td>
</tr>
<tr>
<td>2.9</td>
<td>Western analyses on effect of vitamin D and time on catalase expression in the nucleus</td>
</tr>
<tr>
<td>2.10</td>
<td>Western blot of nuclear extract catalase band intensity quantified by ImageJ software</td>
</tr>
<tr>
<td>3.1</td>
<td>Time course of 1,25(OH)₂D₃, 24R,25(OH)₂D₃ or 24S,25(OH)₂D₃ – mediated ³²P uptake in chicken intestinal cell suspensions, relative to vehicle controls</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>3.2</td>
<td>Effect of vehicle, 1,25(OH)<em>{2}D</em>{3}, or 24,25(OH)<em>{2}D</em>{3} on $^{32}$P uptake in cultured chicken intestinal cells.</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of vitamin D on gene expression for catalase in cultures of chicken intestinal cells.</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of vehicle, 1,25(OH)<em>{2}D</em>{3}, or 24R,25(OH)<em>{2}D</em>{3} on $^{32}$P uptake in transfected cultured chicken enterocytes.</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of vitamin D on protein levels of catalase in cultured chicken enterocytes.</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Vitamin D metabolite action summary</td>
<td>15</td>
</tr>
</tbody>
</table>


LIST OF ABBREVIATIONS AND DEFINITIONS

- $\mu$Ci: Microcurie, a unit of radiation
- $\mu$g: Microgram
- $\mu$l: Microliter
- $1,25(OH)_{2}D_{3}$: 1,25-dihydroxyvitamin D$_{3}$
- $1,25D_{3}$-MARRS: 1,25-dihydroxyvitamin D$_{3}$ membrane associated, rapid response steroid-binding receptors
- $24,25(OH)_{2}D_{3}$: 24,25-dihydroxyvitamin D$_{3}$
- $^{32}$P: radioisotope for phosphorous
- BSA: Bovine serum albumin
- $P_1$ fraction: pellets contain nuclei, BBM and large cell debris
- $P_2$ fraction: Pellets contain BLM, Golgi, mitochondria, lysosomes and peroxisomes
- CPM: counts per minute, a measurement of radioactivity
- ECF substrate: enhanced luminescence substrate
- FBS: Fetal bovine serum
- GBSS: Gey’s balanced salt solution
- mM: Millimolar
- pM: Picomolar
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>P value</td>
<td>Probability to test the hypothesis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TED</td>
<td>Tris/EDTA/dithiothreitol</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
</tbody>
</table>
CHAPTER 1

LITERATURE REVIEW

Outline

This literature review will begin with an overview of vitamin D history and background. Next, metabolic mechanisms of vitamin D will be discussed and introduced. Then, a more focused look at different vitamin D metabolites and their receptors will be presented; phosphate and calcium homeostasis will also be addressed. Last, an overview of catalase metabolism and applications will be addressed.

Background

In the past several years, vitamin D has received enormous attention, specifically at the public health level, mainly because of the discovery of its many unexpected health benefits. It is now realized that vitamin D is important not only for mineralization and skeletal growth but also in regulation of the immune system, the parathyroid gland, the skin, and in cancer prevention. Since the initial discovery of vitamin D in the early 20\textsuperscript{th} century, vitamin D was recognized not only for its major medical importance by eliminating rickets as a major medical problem, but also had received a bad reputation because of outbreak of idiopathic hypercalcemia after World War II attributed to food fortification with vitamin D [1]. In the United States and Canada, there is a campaign to increase the awareness of the benefits of vitamin D as well as to increase the recommended daily allowance of vitamin D [2]. However, vitamin D is potentially toxic when provided in large amounts [3]. A report in 2007 indicates 10,000 units of vitamin
D₃ per day is safe for 6 months [4] (NOAEL: no observed adverse effects level). In 2011, the Food and Nutrition Board set the upper limit (UL) for vitamin D₃ to 4,000 IU per day, simply calculated from the NOAEL by dividing by a safety factor (which is 2.5 in vitamin D’s case). This is an important start, and the recommendation probably will be changed in the years to come. It is important to note that vitamin D is fat-soluble so it is stored in the adipose tissue and accumulation of vitamin D could possibly result in toxicity.

**History and Metabolic Mechanisms**

Sir Edward Mellanby found the first evidence of the existence of vitamin D in 1919 [5]. He was able to produce rickets in dogs between 5 and 8 weeks by feeding them one of four natural diets and keeping them indoors, and cure the disease with cod liver oil, which is also rich in vitamin A. So there was uncertainty whether it was vitamin A that treated rickets or some new factor. In the same year, Huldshinsky discovered that rickets in chicken could be cured by ultraviolet light [6], which might be the first lead that vitamin D is not really a traditional vitamin. In 1922, McCollum [7] demonstrated that cod liver oil contained another vitamin that is responsible for bone mineralization, which he named “vitamin D”. Scientists have demonstrated that ultraviolet light is capable of converting an inactive substance into an active substance that could cure rickets [8, 9], thus led to the conclusion that the skin is of major importance in the production of vitamin D.

Increasing the amount of meat on chicken and turkeys, which requires sufficient support of bone structure, has always been a goal in poultry production. To date, strategies for improving bone strength have included increasing dietary calcium and
phosphate, as well as vitamin D (calciferol), which is not really a strict vitamin but the pre-hormone that serves as the parent compound for the production of three active metabolites: 25-hydroxyvitamin D₃, 1,25-dihydroxyvitamin D₃, and 24,25-dihydroxyvitamin D₃. Discovered in 1919-1924, vitamin D is a fat-soluble antirachitic factor, and it is naturally present in very few foods: a small amount in milk and eggs, and relatively large amounts in fatty fish. The actual vitamin D content of fortified milk is not the same as the stated 400 IU per liter [10]. Vitamin D is primarily produced when ultraviolet rays (action spectrum 280-320 nM or UVB) in sunlight triggers vitamin D synthesis endogenously under the skin. Today, many populations are still at risk of vitamin D inadequacy despite the fortification of vitamin D in foods and the ability of the body to synthesize the vitamin, these include breastfed infants [11], older adults [12], people with limited sun exposure, people with dark skin [13], people with fat malabsorption [14] and people who are obese [15] or who have undergone gastric bypass surgery [16]. In the past, vitamin D was thought to only influence only bone diseases such as rickets and osteoporosis through regulation of active transport of calcium in enterocytes [17]. However, recently vitamin D has been accepted as a major player in overall human health [18], playing a role in chronic diseases such as arthritis, obesity, cancer, auto-immune diseases, cardiovascular diseases, and diabetes [19]. Vitamin D has been shown to influence virtually every cell in the human body, and receptors that respond to vitamin D have been found in nearly every cell type [19]. One recent study has shown that a large cohort of women age 65 and older experience low vitamin D levels, which possibly contributed to mild weight gain [20]. An association between vitamin D and cancer was first proposed in 1980 based on an ecological study of colon
cancer mortality rates and annual sunlight levels in the United States [21]. Currently, researchers have shown that higher vitamin D levels decrease the risk of 15 different types of cancer [22]. In an effort to understand how to use vitamin D as a weapon to combat chronic diseases, the underlying mechanisms involved with calcium and phosphate homeostasis that is at the core of cell signaling, growth and proliferation must be understood.

Our understanding of how vitamin D mediates biological responses has entered a new era. Phosphorus and calcium are two of the most abundant trace elements in the body and must be regulated in order to maintain proper cellular functions. Vitamin D promotes calcium absorption and maintains adequate serum calcium and phosphate concentrations to enable normal bone mineralization, growth and remodeling [23]. Vitamin D modulates cell growth, neuromuscular and immune function, and reduces inflammation. It also regulates expression of many genes encoding proteins that regulate cell proliferation, differentiation, and apoptosis [23].

**Vitamin D Synthesis**

The following steps occur in vitamin D synthesis [17]: in the epidermis there is abundant 7-dehydrocholesterol, when the skin is exposed to the UVR, 7-dehydrocholesterol is photolyzed to pre vitamin D₃. Vitamin D₃ (cholecalciferol, from animal sources) is a secosteroid since the B ring of 7-dehydrocholesterol is broken from photolysis, which in turn gives it a cis-triene structure [24]. The cis-triene structure gives vitamin D₃ its unique ultraviolet absorption at 265 nm with a minimum at 228 nm. With continued UVR exposure, vitamin D₃ is converted to biologically inactive lumisterol and tachysterol [25]. The formation of lumisterol and tachysterol is reversible and can be
converted back to vitamin D₃ if needed. This explains the lack of vitamin D₃ toxicity through prolonged sun exposure. After formation, vitamin D₃ is transported to the liver and is enzymatically hydroxylated to 25-hydroxyvitamin D₃ [calcidiol, 25(OH)D₃, the major circulating form of the vitamin] after binding to its carrier protein-vitamin D-binding protein (DBP) which does not bind pre vitamin D₃ [26]. Hydroxylation is catalyzed by a microsomal cytochrome P450 enzyme, type I mitochondrial cytochrome P450 oxidases, CYP2R1 or the mitochondrial cytochrome P450 CYP27A1, which utilizes NADPH and molecular oxygen to catalyze the hydroxylation reaction [27]. 25(OH)D₃ quickly enters the circulation with normal circulating levels between 25 nmol/L to 200 nmol/L, and it has a half-life of about 15 days [28]. 25(OH)D₃, bound to DBP, is then transported to the kidneys and is finally hydroxylated by 1α-hydroxylase (also termed CYP27B1 or P450C1, an enzyme that is highly expressed in renal proximal tubules [29]) at the C1 position to hormonally active 1,25(OH)₂D₃. This occurs when serum levels of calcium and phosphate are low. The serum levels of 1,25(OH)₂D₃ range from 75 pmol/L to 200 pmol/L and it has a serum half-life of 10-24 hours [30]. Regulation of CYP27B1 is controlled by PTH, and fibroblast growth factor (FGF)-23 which inhibits expression respectively [25]. By contrast, when serum levels of calcium and phosphate are high, suppression of 1α-hydroxylase by high levels of circulating calcium or phosphate stimulates another hydroxylase (CYP24 or P450C24) that converts 25(OH)D₃ to 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃). Both positive (PTH, estrogen, and growth hormone) and negative (1,25(OH)₂D₃, calcium) regulators of the renal hydroxylases have been identified, and acute regulation of renal 1,25(OH)₂D₃ production is mediated through the adenylate cyclase and protein kinase C pathways [31, 32].
Vitamin D and Calcium Transport

When intestinal calcium transport was measured in vitamin D-deficient rats given a dose of vitamin D₃, a 12-hour period was required for a response [33]. This coupled with the observation that it took 24h before intestinal calcium absorption reached a maximum level led to the belief that vitamin D₃ needed to be activated before it could carry out its physiological functions on calcium metabolism [34, 35]. In a study that provided radiolabeled vitamin D₃ to vitamin D-deficient rats caused the disappearance of radiolabeled vitamin D₃ and the appearance of polar metabolite fractions [34]. Researchers observed that the polar metabolite fraction stimulated intestinal calcium transport much more rapidly than vitamin D₃. In a subsequent study, pigs were given vitamin D₃, blood was collected and a lipid extraction followed by several chromatography steps yielded a vitamin D₃ metabolite that was about fourfold more potent than vitamin D₃ in stimulating intestinal calcium transport. It was structurally identified as 25-hydroxyvitamin D₃ [36]. This observation and the finding that the hydroxylated metabolite took 12h to maximize intestinal calcium absorption in vitamin D deficient rats [17] and supported the belief that 25(OH)D₃ is the circulating active form of vitamin D₃. It was then reported [37] that a polar metabolite of vitamin D₃ in the intestine that was distinct from 25(OH)D₃ also existed. The further modified metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃, also known as calcitriol] was isolated from chicken intestines, and the molecular attributes were revealed by mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions [38]. Once the structure was discovered, it took 2 years and a 21 step synthesis to chemically synthesize 1,25(OH)₂D₃, yielding about 1mg final product out of 1kg starting materials [17]. The
configuration of the hydroxyl group on position 1 was discovered-1α,25(OH)₂D₃ (the natural metabolite) and 1β,25(OH)₂D₃ [39]. The hydroxylation of the 1α position is strongly feedback-regulated and is one of the major endocrine systems regulating plasma calcium and phosphorus concentrations.

**Phosphate and Calcium Homeostasis**

Homeostatic regulation of phosphate and calcium and their effects on the intestine, kidneys and bone are maintained by several key hormones, which includes the vitamin D metabolites, calcitonin, fibroblast growth factor-23 (FGF-23), and parathyroid hormone (PTH) [40-42]. When serum phosphate or calcium levels are low, the parathyroid glands synthesize PTH, which acts on the kidneys to decrease phosphate reabsorption, to increase calcium reabsorption, and to stimulate synthesis of 1α-hydroxylase to convert circulating 25(OH)D₃ into 1,25(OH)₂D₃. Then 1,25(OH)₂D₃ travels through the bloodstream to enhance phosphate and calcium absorption in the intestine. Both PTH and 1,25(OH)₂D₃ simultaneously act on osteoclasts to induce bone resorption thereby increasing circulating phosphate and calcium levels to normal ranges.

Abnormally high serum phosphate or calcium levels also trigger hormonal regulation to maintain normal circulating ranges. Elevated phosphate levels stimulate FGF-23 synthesis in bone [43, 44] which act on the kidneys to decrease 1,25(OH)₂D₃ synthesis by inhibiting 1α-hydroxylase activity, increasing phosphate excretion, decreasing phosphate reabsorption which inhibits synthesis of phosphate transporters [45]FGF-23 also acts by decreasing intestinal phosphate absorption which acts to internalize phosphate transporters, and stimulate 24-hydroxylase activity to synthesize 24,25(OH)₂D₃ [46-49]. Elevated calcium levels stimulate calcitonin synthesis in the
thyroid glands [50, 51] which acts on bone to inhibit bone resorption [52, 53] and inhibit PTH synthesis in the parathyroid glands. Inhibition of PTH synthesis allows increased synthesis of 24-hydroxylase in the kidneys to convert 25(OH)D$_3$ into 24,25(OH)$_2$D$_3$ which inhibits calcium transport in the intestine [40, 54]. Directly or indirectly, 25(OH)D$_3$, calcitonin, FGF-23, and PTH act on 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ synthesis to regulate calcium or phosphate reabsorption and excretion in the kidneys, resorption from bone, or absorption in the intestine to maintain homeostatic levels of both microminerals.
Figure 1.1 Schematic presentation of phosphate homeostasis. The kidney converts 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃ when the organism is deficient in vitamin D, phosphate, and/or calcium. Hypocalcemia also signals the parathyroid glands to release parathyroid hormone (PTH), which stimulates 1α-hydroxylation of 25(OH)D₃. 1,25(OH)₂D₃ stimulates absorption of minerals from the intestine and reabsorption from the kidney. Fibroblast growth factor 23 (FGF-23) inhibits the formation of 1,25(OH)₂D₃ and promotes phosphaturia. 24,25-dihydroxyvitamin D₃, which blocks stimulated uptake of minerals from the intestine and PTH release from the parathyroid glands.
Figure 1. Structure of vitamin D and its metabolites
Figure 1. 2 Regulation of PTH and 1,25(OH)₂D₃ in calcium homeostasis
25(OH)D₃

25-hydroxyvitamin D₃, a pre-hormone activated through the action of 25-hydroxyvitamin D₃ 1 hydroxylase, was once thought to have no biological activity of its own. However, evidence has been found to prove the opposite. Early reports suggested a possible physiological role in egg hatchability [55, 56]. The metabolite is 1.4 times more active than vitamin D₃ in curing rickets in rats and chicks determined by the bone ash assay [57]. It stimulates calcium transport in the intestine and bone resorption in vivo more rapidly than cholecalciferol does [57]. A stimulation of bone resorption in tissue culture in vitro was observed with 25(OH)D₃ [58]. An enhancement of calcium transport in isolated duodenal loops was observed after perfusion with 100 nM 25(OH)D₃ compared with controls [59, 60]. 25(OH)D₃ has also been observed to suppress parathyroid hormone synthesis [61]. Gene expression analysis indicated that 25(OH)D₃ induced 25-hydroxyvitamin D₃ 24-hydroxylase mRNA in a dose and time dependent manner [62]. 25(OH)D₃ has been shown to lower the risk of colorectal cancer [63], and has been associated with increasing overall survival in early stage non-small cell lung cancer (NSCLC) [64].

Effect of 1,25(OH)₂D₃ on Phosphate Absorption

1,25(OH)₂D₃, also known as calcitriol, stimulates the active transport of phosphorus in the small intestine when plasma or serum phosphate levels are low. When there is a vitamin D deficiency, phosphate transport is impaired and bone resorption occurs. A continued deficiency results in hypophosphatemia, rickets or osteomalacia [45]. Studies [46, 65] have demonstrated that chick intestinal cells can be rapidly stimulated to take up phosphate through a signal transduction pathway mediated by protein kinase C
This mechanism can be activated by the 1,25D₃-MARRS receptor, which is identical to protein disulfide-isomerase A₃ (PDIA₃) [65]. In rats [66], membrane initiated signaling relies on both cell surface VDR and the 1,25D₃-MARRS receptor. 1,25(OH)₂D₃ was shown to enhance phosphate uptake within 1 min suggesting that stimulation of the uptake process occurs through a pre-genomic pathway [67-70], not involving gene regulation which usually occurs over a period of several hours to days. That led researchers to identify a plasma membrane receptor to explain the rapid effects [71].

**Membrane Receptors for 1,25(OH)₂D₃**

In rat enterocytes, Lieberherr et al. [72] were the first to demonstrate the need for a membrane receptor in mediating phosphoinositide metabolism. Traditionally, 1,25(OH)₂D₃ has been shown to act through a nuclear vitamin D receptor [73] (VDR). Now, a membrane receptor called the 1,25(OH)₂D₃ membrane associated, rapid response steroid-binding receptor (1,25D₃-MARRS) has also been identified [40, 41, 71, 73]. The role of this protein is to facilitate the rapid effects of 1,25(OH)₂D₃-mediated calcium and phosphate absorption and uptake in isolated chick enterocytes and transport in perfused duodenal loops [46, 74, 75]. The 1,25D₃-MARRS receptor was subsequently reported to be identical to ERp57/GR58/PDIA3 and has been characterized through prosite analysis [74, 75] as containing several PKC and casein kinase phosphorylation sites, a Rel homology domain (RHD), and two thioredoxin folds. The latter binding sites allow it to interact with other proteins including catalase [65, 71].
Figure 1.4 Model for interaction between 1,25D₃-MARRS receptor and catalase. Upon binding 1,25(OH)₂D₃, the 1,25(OH)₂D₃-MARRS receptor dimerizes and initiates signal transduction through PKA for enhanced calcium uptake and PKC for enhanced phosphate uptake. Feedback inhibition of this stimulatory pathway is initiated when 24,25(OH)₂D₃ binds to catalase, thereby decreasing enzymatic activity. The increased H₂O₂ levels are known to inhibit stimulated ion uptake. Mechanistically, this occurs through oxidation of the 1,25(OH)₂D₃-MARRS receptor to decrease 1,25(OH)₂D₃ binding, and subsequent signal transduction, as well as directly inhibiting hormone-stimulated PKC activity.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Site of activation</th>
<th>Proposed action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D\textsubscript{3}</td>
<td>Liver</td>
<td>Increased Ca\textsuperscript{2+} uptake in rat and chick duodena</td>
<td>[70, 76]</td>
</tr>
<tr>
<td>1,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>Kidney</td>
<td>Increased phosphate uptake and transport in intestine</td>
<td>[46]</td>
</tr>
<tr>
<td>24,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>Kidney</td>
<td>Decreased phosphate uptake in presence of 1,25(OH)\textsubscript{2}D\textsubscript{3} in intestine</td>
<td>[40]</td>
</tr>
</tbody>
</table>

Table 1. 1 Vitamin D metabolite action summary
Mechanism of action for 1,25(OH)$_2$D$_3$

The effects of 1,25(OH)$_2$D$_3$ are mediated through two mechanisms:

1.) Genomic actions – the cellular actions of this hormone are due to the binding of the hormone to a nuclear vitamin D receptor (nVDR), and represents a slow mechanism since it is involved with gene transcription and protein synthesis, for example in bone matrix [77]. This ligand-receptor complex interacts with the retinoid X receptor, and regulates the expression of approximately 500 genes [18]. The 1,25D$_3$-MARRS receptor has also been found to translocate to the nucleus upon binding hormone and associate with heterochromatin, suggesting gene regulation [78].

2.) Pre-genomic or rapid response actions – there are some cellular responses that are rapidly stimulated by 1,25(OH)$_2$D$_3$ and cannot be explained by the slow mechanism via VDR. These actions are mediated through the 1,25D$_3$-MARRS receptor. The signal transduction pathway involved in the rapid stimulation of mineral transport can be activated through protein kinase C, phospholipase C, protein kinase A and mitogen activated protein kinase [79, 80].

Effect of 24,25(OH)$_2$D$_3$ on phosphate uptake

While 1,25(OH)$_2$D$_3$ stimulates the rapid transport of calcium and phosphate as illustrated in Table 1, 24,25(OH)$_2$D$_3$ inhibits such stimulation [40, 46, 54]. Hormone-stimulated phosphate uptake in chick intestinal cells is initiated by ligand binding to the 1,25D$_3$-MARRS receptor and is inhibited by 24,25(OH)$_2$D$_3$. Since 24,25(OH)$_2$D$_3$ does not compete for binding the 1,25D$_3$-MARRS receptor [81], in order to elucidate how 24,25(OH)$_2$D$_3$ effects inhibition, a cellular binding protein with Kd = 7 nM for
24,25(OH)₂D₃ was purified [82] and sequenced and subsequently identified as catalase [83]. The majority of binding material was vesicular [82] while specific binding was found at the cell surface [84]. A functional consequence of 24,25(OH)₂D₃ binding to catalase was found to be a decrease in enzyme activity, accompanied with an increase in H₂O₂ production [85], beyond basal levels [86]. Oxidation of the 1,25D₃-MARRS receptor and inhibition of binding to [³H]1,25(OH)₂D₃ was found to be one possible mechanism of inhibition [85]. Antioxidants were found to block the inhibitory actions of 24,25(OH)₂D₃, whereas pro-oxidants were found to mimic the inhibitory actions of the steroid [48].

**Membrane receptor for 24,25(OH)₂D₃**

As stated previously, the metabolite 24,25(OH)₂D₃ is produced when an animal is calcium, phosphate, and vitamin D replete, and represents an important feedback loop to provide a protective mechanism against excess 1,25(OH)₂D₃ [40, 46, 87, 88]. Sequence analysis of a 24,25(OH)₂D₃ binding protein was performed and five regions of homology was found with enzyme catalase [83]. Studies were then undertaken to determine whether 24,25(OH)₂D₃ treatment would affect catalase activity. The results showed a time-dependent decrease in catalase activity in isolated intestinal epithelial cells after the addition of physiological levels of 24,25(OH)₂D₃ (6.5 nM), whereas 1,25(OH)₂D₃ did not have a similar effect [85]. Another study was then performed to test whether pre-incubation of cell suspensions with low concentration of hydrogen peroxide would inhibit the rapid 1,25(OH)₂D₃-stimulated uptake of ³²P. The results showed that after the addition of 130 pM 1,25(OH)₂D₃, ³²P uptake reached 135% of controls; whereas a 10-min pre-incubation of cells with 50 µM H₂O₂ abolished the increase [85]. The effect of
hydrogen peroxide was shown to be reversible. The catalase inhibitor 3-amino-1,2,4-triazole [89] mimicked the effects of 24,25(OH)\textsubscript{2}D\textsubscript{3} since it blocked 1,25(OH)\textsubscript{2}D\textsubscript{3}-stimulated phosphate uptake [90]. Pre-incubation of chicken enterocytes with either a commercially available anti-catalase antibody [83] prevented the inhibitory actions of 24,25(OH)\textsubscript{2}D\textsubscript{3} [48].

**Mechanism of actions for 24,25(OH)\textsubscript{2}D\textsubscript{3}**

One mechanism for the inhibitory effects of 24,25(OH)\textsubscript{2}D\textsubscript{3} could be through oxidation of the thioredoxin domains of the 1,25D\textsubscript{3}-MARRS receptor. The 24,25(OH)\textsubscript{2}D\textsubscript{3}-mediated decrease in \[^{3}\text{H}]1,25(OH)\textsubscript{2}D\textsubscript{3} binding to MARRS was not observed when incubated with the strong reducing agent dithiothreitol [65]. Nemere et al. have previously reported that 24,25(OH)\textsubscript{2}D\textsubscript{3} does not bind to the 1,25D\textsubscript{3}-MARRS receptor [81]. Chicken enterocytes were incubated for 5, 10, 20 or 30 min with the addition of a physiological level (6.5 nM) 24,25(OH)\textsubscript{2}D\textsubscript{3}. Cells were collected by centrifugation, then homogenized, then incubated with \[^{3}\text{H}]1,25(OH)\textsubscript{2}D\textsubscript{3} in the absence (total binding) or presence (nonspecific binding) of a 200-fold molar excess of unlabeled steroid. The result indicated no classical VDR binding detection [71]. Since hydrogen peroxide could also influence the signal transduction pathway, isolated intestinal cells were incubated with phorbol ester after pre-incubation with hydrogen peroxide. The results showed that hydrogen peroxide inhibited the phorbol stimulation of \(^{32}\text{P}\) uptake and PKC signaling in response to 1,25(OH)\textsubscript{2}D\textsubscript{3} [91]. The ability of 24,25(OH)\textsubscript{2}D\textsubscript{3} to decrease catalase activity was determined by follow-up studies. A decrease in catalase specific activity and catalase protein levels were observed after primary cultures of chick
enterocytes after exposure to 24,25(OH)_{2}D_{3} for 5 h [92]. The model depicted in Figure 1.3 was formulated based on these observations and data.

**Catalase**

The discovery of catalase (H_{2}O_{2}: H_{2}O_{2}-oxidoreductase, EC 1.11.1.6) was first hinted at 1811 when the French chemist who discovered hydrogen peroxide (H_{2}O_{2}), Louis Jacques Thenard [93], suggested its breakdown is caused by an unknown substance. In 1900, the German chemist Oscar Loew found catalase in both plants and animals, and was the first to name it catalase [94]. In 1937, catalase from bovine liver was crystallized by James B. Sumner and Alexander Dounce, achieving one of the first successful crystallizations of an intracellular enzyme [95], and the molecular weight was determined in 1938, catalase is a tetramer with a total molecular weight of approximately 24kDa. [96].

**Physiological importance and molecular mechanism**

Oxygen is necessary for life but also produces free radicals that can damage cell membranes, proteins and DNA. Hydrogen peroxide is a naturally occurring but destructive waste product of all oxygen-dependent organisms. It is produced in the human body when fatty acids are converted to energy, and when white blood cells attack and kill bacteria. The optimum pH for human catalase is approximately 7.0 [97], and can dispose of five times more H_{2}O_{2} than glutathione peroxidase [98]. Hydrogen peroxide can freely cross membranes and generate the most reactive free radicals, hydroxyl radicals [99]. Hydrogen peroxide produced by the glucose oxidation system and can damage nuclear and mitochondrial DNAs [100]. Catalase, which is normally found in the peroxisomes [101] of nearly all aerobic cells, prevents this naturally occurring and
constantly generating hydrogen peroxide from harming the cells during these processes [102-104]. Catalase is a biological bi-functional catalyst, like superoxide dismutase (SOD) and glutathione peroxidase, is produced naturally within the body. Catalase works flawlessly with superoxide dismutase system to prevent free radical damages to cells, preventing cell death. SOD converts the dangerous superoxide radical to hydrogen peroxide, which catalase then rapidly converts to harmless water and oxygen [98].

Catalase is a tetrametric enzyme consisting of four identical subunits of 60 kDa, each containing a heme group and a NADPH binding site [105, 106]. The ferric oxidation state allows the enzyme to react with H₂O₂, and four tightly bound NADPH molecules to prevent the enzyme from inactivation by H₂O₂ [106] [107]. Catalase exerts its functions in two ways depending on the concentration of H₂O₂: 1) catalytic reaction 2H₂O₂ → 2H₂O + O₂: decomposition of H₂O₂ to give H₂O and O₂ if the concentration of H₂O₂ is high; 2) peroxidatic reaction ROOH + AH₂ → H₂O + ROH + A: oxidization of substrate if the concentration of H₂O₂ is low and in the presence of a suitable hydrogen donor (i.e., ethanol, methanol, phenol) [108]. The peroxidase function is probably the result of gene duplication of an ancestral peroxidase gene [109] and is found in bacteria and fungi [110]. The concentration of catalase varies in different tissues, and differs from species to species. The highest concentration from tissues is found in the liver. A higher level of a catalase substrate in the cell leads to higher levels of catalase. This gene is conserved in human, chimpanzee, dog, mouse, rat, chicken, fruit fly, C. elegans and rice. It has been theorized that one of the primary reasons cells age is the damage to DNA caused by free radicals and oxidizing agents [111-114] such as hydrogen peroxide, and that elevating
levels of the body’s endogenous antioxidants – SOD, catalase, and glutathione peroxidase – could both improve human health and increase human lifespan [115-118].

**Hypothesis and Objectives**

The overall hypothesis is 24R,25(OH)\(_2\)D\(_3\) is the active metabolite in phosphate homeostasis by using immunological techniques to determine the localization of catalase and by monitoring catalase mRNA levels to determine if 24,25(OH)\(_2\)D\(_3\) regulates gene transcription.

**Part I – Effect of 24,25-dihydroxyvitamin D\(_3\) on localization of catalase in chick enterocytes.**

Hypothesis: 24R,25(OH)\(_2\)D\(_3\) is the effective metabolite for catalase redistribution as judged by confocal microscopy.

Objectives:

1.) To determine the localization of catalase;

2.) To evaluate whether punctuate staining in the nucleus arose from the redistribution of cell surface catalase.

**Part II – Effects of vitamin D on catalase expression and phosphate uptake in chicken intestinal cells.**

Hypothesis: 24R,25(OH)\(_2\)D\(_3\) is the effective metabolite for decreasing \(^{32}\)P uptake and catalase gene expression.

Objectives:

1.) To determine the effects of 24R,25(OH)\(_2\)D\(_3\) on \(^{32}\)P uptake in chick enterocytes;

2.) To determine whether 24R,25(OH)\(_2\)D\(_3\) has any effect on catalase gene expression;

3.) To evaluate whether 24R,25(OH)\(_2\)D\(_3\) has effect on catalase protein expression.
References


87. Sundell, K. and B.T. Bjornsson, Effects of vitamin D3, 25(OH) vitamin D3, 24,25(OH)2 vitamin D3, and 1,25(OH)2 vitamin D3 on the in vitro intestinal


CHAPTER 2

EFFECT OF 24,25-DIHYDROXYVITAMIN D₃ ON LOCALIZATION OF CATALASE IN CHICK ENTEROCYTES

Abstract

The vitamin D metabolite, 24,25(OH)₂D₃ has been reported to have hormonal activity. Catalase has been reported to be a binding protein for 24,25(OH)₂D₃, 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)₂D₃, not 24S,25(OH)₂D₃ is the effective metabolite for catalase redistribution as judged by confocal microscopy. We have used male chick intestinal cells treated with either vehicle, 24R,25(OH)₂D₃, 24S,25(OH)₂D₃ or 1,25(OH)₂D₃ 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)₂D₃, 24S,25(OH)₂D₃ or 1,25(OH)₂D₃ for all time points tested. Cells treated with 24R,25(OH)₂D₃ 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)₂D₃ treatment resulted in redistribution of catalase to the nucleus, but to a lesser extent than treatment with 24R,25(OH)₂D₃. By understanding the molecular and cellular actions of 24,25(OH)₂D₃ 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 D₃ [25(OH)D₃], followed by hydroxylation in the kidneys to yield two metabolites, either 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] made when phosphate and calcium levels are low or 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] made when phosphate and calcium levels are high. 24,25-dihydroxyvitamin D₃ is no longer considered an inactive hormone. Earlier studies showed that in order to reach normal chick hatchability [1] and bone formation [2], both 1,25(OH)₂D₃ and 24,25(OH)₂D₃ were necessary. While 1,25-dihydroxyvitamin D₃ stimulates the rapid transport of calcium and phosphate in both perfused chick duodenal loops and isolated enterocytes, 24,25-dihydroxyvitamin D₃ inhibits such stimulation [3, 4]. In osteoblasts and osteosarcoma cells, 1,25(OH)₂D₃ has been found to have a rapid effect on calcium-channel-opening, while 24,25(OH)₂D₃ was found to inhibit this non-nuclear effect [5-7]. In chick intestinal cells, 1,25(OH)₂D₃ has been found to have an acute, non-nuclear effect on phosphate transport, and 24,25(OH)₂D₃ 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)₂D₃ inhibits the rapid stimulation of phosphate transport [8] mediated by 1,25(OH)₂D₃, as well as calcium transport [10].

In chick intestinal cells, hormone-stimulated phosphate uptake is initiated by ligand binding to the 1,25D₃-membrane associated, rapid response steroid-binding
receptor – 1,25D₃-MARRS [11], also known as ERp57/GR58/PDIA3. The ability of 24,25(OH)₂D₃ to inhibit 1,25D₃-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)₂D₃. Percoll gradient analysis revealed lysosomal fractions to have the highest [³H]24,25(OH)₂D₃ binding activity [10]. In order to explain how 24,25(OH)₂D₃ works to affect 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)₂D₃, 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)₂D₃ is caused by a decrease in catalase activity in both chick intestine [15, 16] and kidney [12], accompanied by an increase in H₂O₂ production [13]. Thus, one possible mechanism for the inhibitory action would be the oxidation of thioredoxin domains in 1,25D₃-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)₂D₃ or H₂O₂ treatments after 5 minutes of incubation, indicating another mechanism because 24,25(OH)₂D₃ does not compete with 1,25(OH)₂D₃ for MARRS binding.

In intestinal cells, studies showed 1,25D₃-stimulated phosphate uptake is mediated by PKC signaling [3], and 24,25(OH)₂D₃ 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 KH₂PO₄, 5.6 mM Na₂HPO₄, 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 KH₂PO₄, 0.84 mM NaHPO₄, 1.03 mM MgCl₂•6H₂O, 0.28 mM MgSO₄•7H₂O, 0.9 mM CaCl₂, 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 37°C 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 (15s, 30s, 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 immunoreactivity, or without Triton X-100 for surface catalase immunoreactivity. 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 PBS-0.1% 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 (with excitation at 591 nm and emission at 614 nm) (JacksonImmuno Research, West Grove, PA) 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 x 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)2D3 or 200 pM 24S,25(OH)2D3. 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 used a nuclear extract kit (Active Motif, Carlsbad, CA) according to the instruction manual, which 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 (5-30µg/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 Na2HPO4, 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 in 1%BSA and 0.05% Tween 20 in TBS. After three additional washes, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) in the antibody incubation buffer described above 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

Effect of 24,25(OH)₂D₃ on catalase redistribution in isolated chick enterocytes

In isolated chick enterocytes, 130 pM 1,25(OH)₂D₃ has been shown to stimulate phosphate uptake after 5 minutes [3] and 24,25(OH)₂D₃ was reported to abolish that effect [19]. In this study, time course experiments on catalase localization in isolated cells were compared among ethanol control, 1,25(OH)₂D₃ and 24,25(OH)₂D₃ treatments. In initial experiments, catalase localization by confocal microscopy was determined by adding hormones before primary antibody Ab365 to allow potential redistribution of the receptor. Chick intestinal cells were cultured in petri dishes, treated with 0.01% ethanol, 6.5nM 24R,25(OH)₂D₃, 200pM 24S,25(OH)₂D₃, or 360pM 1,25(OH)₂D₃, concentrations that have been shown to be equivalent to circulating levels[20], for selected times (15 sec, 30 sec, 7 min, 10 min, 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 2.1A shows shorter hormone/ethanol incubation time points and Figure 2.1B shows longer hormone/ethanol incubation 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. These results are shown quantitatively in Figure 2.1C, and indicate no significant differences with time or a slight increase in redistribution was seen with time. Figure 2.2 depicts results from experiments in which cells were treated with 24R,25(OH)₂D₃ for shorter (Figure 2.2A) and longer (Figure 2.2B) time points, in which there were obvious increases in nuclear staining (indicated by yellow arrows) as well as surface staining relative to controls. Quantification of nuclear staining is depicted in Figure 2.2C. Significant differences were observed at 15 seconds after hormone treatment. Figure 2.3 depicts results from experiments in which cells were treated with 24S, 25(OH)₂D₃ for shorter (Figure 2.3A) and longer (Figure 2.3B) time points, in which there were slight increases in nuclear staining relative to controls, but to a much lesser extent compared to 24R,25(OH)₂D₃ treatment. Figure 2.3C provides quantification of the data, and illustrates that 24S,25(OH)₂D₃ treatment caused 100%-111% change in nuclear staining, relative to cells treated with 24R,25(OH)₂D₃. Figure 2.4 depicts results from experiments in which cells were treated with 1, 25(OH)₂D₃ for shorter (Figure 2.4A) and longer (Figure 2.4B) time points, in which there were no increases in nuclear staining nor surface staining relative to controls, since 1,25(OH)₂D₃ does not compete with 24R,25(OH)₂D₃ for binding to catalase. These results are shown quantitatively in Fig. 2.4C. In a previous study, it was found that 24R,25(OH)₂D₃ 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} [21]. One possible gene regulatory mechanism could be catalase binding to STAT3 [22]. 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 Ab 365 was first added to cells for 30 min, and subsequently incubated with ethanol vehicle, 1,25(OH)_{2}D_{3}, 24S,25(OH)_{2}D_{3}, or 24R,25(OH)_{2}D_{3}. Figure 2.5 depicts control cells treated with 0.01% ethanol for shorter (Figure 2.5A) and longer (Figure 2.5B) time points revealed obvious surface staining, but little inside the nucleus. As shown in Figure 2.6A and 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 primary antibody. Similarly, treatment of cells with addition of primary antibody before hormone treatment inhibited the effects of 24S,25(OH)_{2}D_{3} (Figure 2.7A, and 2.7B), and as expected, no effect of 1,25(OH)_{2}D_{3} treatment on catalase localization was found (Figure 2.8A and 2.8B).

To further elucidate the observations above, catalase-staining intensity inside the nucleus was quantified with ImageJ and Adobe Photoshop CS5 software. Figure 2.9 depicts comparisons among ‘hormones first treatments’—in which cells were treated with hormones/ethanol vehicle prior to primary antibody Ab 365. There was a significant
increase (P<0.01) in the intensity within the 24R,25(OH)_2D_3 treated group, a smaller increase (P<0.05) in the intensity within the 24S,25(OH)_2D_3 treated group was also observed, while the other groups stayed the same. Figure 2.10 depicts a comparison among cells treated with primary antibody Ab 365 prior to hormones/ethanol, or ‘antibody first treatments’. While blocking the cell surface catalase, there was no obvious increase in the nuclear staining intensity among the four groups, indicating that the nuclear redistribution mediated by 24R,25(OH)_2D_3 is indeed from cell surface catalase.

An independent approach was taken to verify these findings. In these experiments, cells were treated with either ethanol vehicle, 24S,25(OH)_2D_3, or 24R,25(OH)_2D_3 for 10 and 25 min, collected by centrifugation, and resuspended in homogenization buffer for subcellular fractionation. Aliquots of P_1 (nuclei, brush borders, and unbroken cells), P_2 (peroxisomes, lysosomes, mitochondria, Golgi, and basal lateral membranes, and S_2 (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)_2D_3 treatment compared to 24S,25(OH)_2D_3 (Figure 2.11). One possible explanation might be there was a higher concentration of 24R,25(OH)_2D_3 in the incubations than 24S,25(OH)_2D_3. Catalase bands intensity were then quantified using ImageJ software. Figure 2.12 indicates a significant increase (P<0.01) in catalase band intensity among 24R,25(OH)_2D_3 treatments. However, the fact that redistribution from cell surface and organelles to the nucleus following 24S,25(OH)_2D_3 was detected by western analysis indicates that confocal microscopy is the less sensitive method. The results shown in Figure 2.11 also indicate that the commercially available antibody is less specific than Ab 365 [17].
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.
Figure 2.1 Time course study of catalase localization in isolated intestinal cells: ethanol control treatment. Figure 2.1A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.1B indicates longer incubation time points (25 min, 30 min, 40 min, 50 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. Cells were allowed to adhere overnight in the absence of serum at 37°C with 5% CO₂/95% air. The following day, media were replaced with GBSS-0.1% BSA. Ethanol was then added for the indicated times, after which cells were fixed and stained with anti-catalase antibody and Alexa Fluor 594 and fluorescein conjugated Phalloidin (not shown). Cell images were observed and taken using Zeiss Confocal Microscope LSM 710, 60 x oil objective. Images were edited with ImageJ and Adobe Photoshop CS5.
Figure 2. 2 Time course study of catalase localization in isolated intestinal cells: 6.5 nM 24R,25(OH)2D3 hormone treatment. Cells were isolated, cultured, and stained as described in the legend to Figure 2.1. Catalase punctate staining inside nucleus is indicated using a yellow arrow. Figure 2.2A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.2B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Figure 2.2C presents a bar graph of intensity of nuclear staining vs. time. Values represent means ± S.E.M for n =7 independent experiments. Significant differences (**P<0.01) were determined using the student t-test, are relative to corresponding controls.
Figure 2.3 Time course study of catalase localization in isolated intestinal cells: 200 pM 24S,25(OH)2D₃ hormone treatment. Figure 2.3A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.3B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Figure 2.3C presents a bar graph of intensity of nuclear staining vs. time. Values represent means ± S.E.M for n =7 independent experiments. Significant differences (**P<0.01) (*P<0.05) were determined using the student t-test, are relative to corresponding controls, are relative to corresponding controls.
Figure 2.4 Time course study of catalase localization in isolated intestinal cells: 360 pM 1,25(OH)$_2$D$_3$ hormone treatment. Figure 2.4A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.4B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Figure 2.4C indicates catalase nuclear punctate staining intensity between ethanol control treatment and 1,25(OH)$_2$D$_3$ hormone treatment. Values represent means ± S.E.M for n = 7 independent experiments.
Figure 2.5 Time course study of catalase localization in isolated intestinal cells: 0.01% ethanol (final concentration) after prior incubation with antibody. Figure 2.5A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.5B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Cells were isolated, cultured, and stained as describe in the legend to Figure 2.1.
Figure 2.6 Time course study of catalase localization in isolated intestinal cells: 6.5 nM 24R,25(OH)₂D₃ hormone treatment after prior incubation with antibody. Figure 2.6A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.6B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Cells were isolated and cultured as described in Fig. 2.1 The following day, media were replaced with primary anti-catalase antibody for 30 min prior to addition of steroid and the indicated incubation periods. Cells were fixed and stained as described in Figure 2.1. Figure 2.6C indicates catalase nuclear punctate staining intensity between ethanol control treatment and 24R,25(OH)₂D₃ hormone treatment. Values represent means ± S.E.M for n = 7 independent experiments.
Figure 2.7 Time course study of catalase localization in isolated intestinal cells: 200 pM 24S,25(OH)₂D₃ hormone treatment after prior treatment with antibody. Figure 2.7A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.7B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Figure 2.7C presents a bar graph with quantification of the observed nuclear fluorescence. The following day, media were replaced with primary anti-catalase antibody for 30 min prior to addition of steroid for the indicated times. Cells were processed for microscopy as describe in the legend to Figure 2.1. Figure 2.7C indicates catalase nuclear punctate staining intensity between ethanol control treatment and 24S,25(OH)₂D₃ hormone treatment. Values represent means ± S.E.M for n=7 independent experiments.
Figure 2.8 Time course study of catalase localization in isolated intestinal cells: 360 pM 1,25(OH)_{2}D_{3} hormone treatment after prior treatment with antibody. Figure 2.8A indicates shorter incubation time points (15 sec, 30 sec, 7 min, 10 min, 15 min); Figure 2.8B indicates longer incubation time points (25 min, 30 min, 40 min, 50 min, 60 min). Figure 2.8C presents a bar graph depicting quantification of nuclear staining intensity for the various time points. Cells were isolated and cultured as described in Figure 2.1. The following day, media were replaced with primary anti-catalase antibody for 30 min. Cells were fixed and stained with Alexa Fluor 594 and Phalloidin. 1,25(OH)_{2}D_{3} hormone was then added for the indicated times. Figure 2.8C indicates catalase nuclear punctate staining intensity between ethanol control treatment and 1,25(OH)_{2}D_{3} hormone treatment. Values represent means ± S.E.M for n = 7 independent experiments.
Figure 2. 9 Western analyses on effect of vitamin D and time on catalase expression in the nucleus. Cells were isolated and nuclei extracted using nuclear extract kit. Anti-catalase was used as the primary antibody. 10µg of protein was loaded for each lane.
Figure 2.10 Western blot of nuclear extract catalase band intensity quantified by ImageJ software. Values represent means ± S.E.M for n = 3 independent experiments. Significant differences (*P<0.05; ** P<0.01) were determined using Student’s t-test, are relative to corresponding ethanol controls, and are indicated by an asterisk.
Discussion

In this study, localization of catalase inside chick intestinal cells was determined using confocal microscopy. Figure 2.1 depicts no redistribution of catalase in vehicle ethanol control treatments after 15 sec to 60 min incubation. Figure 2.2 indicates an obvious catalase redistribution to nucleus at every indicated incubation time point in 24R,25(OH)₂D₃ treatments. Figure 2.3 depicts no obvious redistribution of catalase in 24S,25(OH)₂D₃ treatments, the same observation can be found in Figure 2.4, where no redistribution of catalase was found after 1,25(OH)₂D₃ treatment. In order to test whether the observed redistribution of catalase to the nucleus after 24R,25(OH)₂D₃ treatments are from the cell surface, another set of experiments were undertaken. On the day of the experiments, primary antibody was added to the cells before treatment with vehicle or hormones to block internalization of cell surface catalase. Figure 2.5 depicts no redistribution of catalase in vehicle control treatments after 15 sec to 60 min of incubation. In Figure 2.6, no catalase redistribution is observed at any indicated incubation time point in 24R,25(OH)₂D₃ treatment, which indicates that adding primary antibody prior to 24R,25(OH)₂D₃ treatment completely abolishes the effect. In Figure 2.7 and 2.8, no redistribution of catalase was observed either after 24S,25(OH)₂D₃ or 1,25(OH)₂D₃ treatment. In order to quantify the catalase punctate staining intensity, each image was edited and quantified using Adobe Photoshop CS5. As indicated in with each figure, when vehicle control or hormones were added prior to primary antibody, a significant increase (200%, P<0.01) in the nuclear punctate staining intensity is observed in 24R,25(OH)₂D₃ treatments, compared to vehicle control treatments as well as 1,25(OH)₂D₃ treatment [190%, increase of 24R,25(OH)₂D₃ relative to 1,25(OH)₂D₃]. An
increase in the punctate staining intensity is also observed in 24S,25(OH)\textsubscript{2}D\textsubscript{3} treatments, but to a lesser extent (130% relative to ethanol control; 110% relative to 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment; P<0.05). When vehicle control or hormones are added after primary antibody addition, as indicated in Figure 2.10, all four treatments (vehicle control, 24R,25(OH)\textsubscript{2}D\textsubscript{3}, 24S,25(OH)\textsubscript{2}D\textsubscript{3}, and 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment) share the same pattern. No significant difference is observed among the four treatment groups, indicating cell surface catalase is required as the receptor for redistribution. Western analyses were undertaken to further prove the hypothesis tested above. In Figure 2.11, a denser band was observed in the nuclear extract sample in both 24R,25(OH)\textsubscript{2}D\textsubscript{3} and 24S,25(OH)\textsubscript{2}D\textsubscript{3} treatments, relative to vehicle controls. As indicated in Figure 2.12, catalase band intensity was quantified by ImageJ software. There is a significant increase in the band intensity after 24R,25(OH)\textsubscript{2}D\textsubscript{3} treatment (300%, P<0.01) and a slight increase in the band intensity after 24S,25(OH)\textsubscript{2}D\textsubscript{3} treatment (140%, P<0.05), which is in agreement with the results from experiments above.

The combined data indicate that 24,25(OH)\textsubscript{2}D\textsubscript{3} treatment of intestinal cells results in the migration of cell surface catalase to the nucleus. This in turn strongly suggests that 24R,25(OH)\textsubscript{2}D\textsubscript{3} has effects on the genome in addition to the reported rapid effects on phosphate transport. Microarray analyses may in the future identify particular genes or groups of genes that are modulated by 24,25(OH)\textsubscript{2}D\textsubscript{3}.

Taken together, these results suggest that the changes in catalase localization that occur during the time points tested with 24R,25(OH)\textsubscript{2}D\textsubscript{3} treatment may have potential clinical significance in phosphate homeostasis and in the development of the development of pharmacological therapies.
References


CHAPTER 3

EFFECTS OF VITAMIN D ON CATALASE EXPRESSION AND PHOSPHATE UPTAKE IN CHICKEN INTESTINAL CELLS

Abstract

Many researchers now recognize that the steroid 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] has biological activity. In previous work from our lab, it has been reported that 24,25(OH)₂D₃ decreases catalase protein levels in cultured intestinal cells over a period of several hours. An effect on catalase gene expression combined with rapid signaling would conclusively demonstrate that 24,25(OH)₂D₃ is not a ‘scrap’ metabolite. In the current work, we report that 24R,25(OH)₂D₃ is the effective metabolite for decreasing ³²P uptake and catalase gene expression, but not for protein regulation. We have used male chick intestinal cells treated with either vehicle, 24,25(OH)₂D₃ or 1,25(OH)₂D₃ for each condition. ³²P uptake assays showed a decrease in uptake after 24R,25(OH)₂D₃ treatment in both suspension cells and cultured cells. RT-PCR further indicated a down-regulation of catalase gene expression after treatment with 24R,25(OH)₂D₃ compared to vehicle control and 1,25(OH)₂D₃. Protein expression was then checked using western blotting, with a result of no change in protein expression levels. By understanding molecular and cellular actions of 24,25(OH)₂D₃ in chick intestine, progress will be made in enhancing phosphate and calcium absorption in animals to supply the minerals for adequate bone growth, and cellular metabolism.

Introduction

1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the most studied vitamin D metabolite, exerts
its effect through both genomic and non-genomic (more accurately termed pre-genomic) mechanisms. Non-genomic actions are rapid, regulated by membrane-associated receptors [1] and signal transduction pathways, while genomic actions take longer, and are exerted largely through transcription family factors to control gene expression [2]. Another vitamin D metabolite, 24,25(OH)\textsubscript{2}D\textsubscript{3}, is produced mainly when an animal is calcium, phosphate, and 1,25(OH)\textsubscript{2}D\textsubscript{3} replete. The inhibitory action of 24,25(OH)\textsubscript{2}D\textsubscript{3} on phosphate absorption is observed across species. It has been reported that 24,25(OH)\textsubscript{2}D\textsubscript{3} inhibits the rapid 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated stimulation of \textsuperscript{32}P transport in the perfused chick duodenal loop [3, 4], as well as \textsuperscript{45}Ca transport [5]. Catalase, which has been identified as an endogenous 24,25(OH)\textsubscript{2}D\textsubscript{3} binding protein [6], is in part bound to the cell surface. It has been reported that 24R,25(OH)\textsubscript{2}D\textsubscript{3}, not 24S,25(OH)\textsubscript{2}D\textsubscript{3}, is the effective metabolite for catalase redistribution [7]. In earlier work we also found that 24,25(OH)\textsubscript{2}D\textsubscript{3} decreases catalase protein levels in cultured intestinal cells over a period of several hours [8].

In the current study we report that in intestinal cells 24R,25(OH)\textsubscript{2}D\textsubscript{3} decreased \textsuperscript{32}P uptake and catalase gene expression after 7 min treatment at the mRNA level. As mRNA levels cannot be used as surrogates for corresponding protein levels without verification [9], we performed western blotting to confirm the relation, with a result of no change in protein expression.

**Experimental**

**Animals**

All surgical procedures were approved by the Institutional Animal Use and Care Committee at Utah State University (Logan, UT). White leghorn cockerels (Privett
Hatchery; Portales, NM) were obtained on the day of hatch and raised for 3-7 weeks on a commercially available vitamin D-replete diet (Nutrena Feeds; Murray, UT). 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.

Cell Isolation and Cell Culture

The chick enterocytes were isolated with citrate chelation media (96 mM NaCl, 27 mM sodium citrate, 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, 10-12]). 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$$\cdot$6H$_2$O, 0.28 mM MgSO$_4$$\cdot$7H$_2$O, 0.9 mM CaCl$_2$, pH 7.3). Aliquots of the cell suspension were pipetted into 35 mm plastic Petri dishes (Falcon, Scientific Products, Franklin Lakes, NJ) containing 2 ml RPMI-1640 (Hyclone; Logan, UT) and antibiotics (100 units/ml penicillin, 100mg/ml streptomycin; Sigma-Aldrich Chemical Company, St. Louis, MO). The cells were incubated overnight or longer at 37ºC with 5% CO$_2$/95% air in the absence of serum to promote cell adhesion.

$^{32}$P uptake in suspension and cultured intestinal cells

Chick intestinal cells were isolated by citrate chelation as described above and centrifuged at low speed (500 x g, 5 min, 4ºC). Cell pellets were resuspended in room
temperature Gey’s Balanced Salt Solution. 2.2 mL of cell suspensions were pipetted into a polypropylene tube along with 1 µCi/ml (1 Ci = 37 GBq) H$_3^{32}$PO$_4$, final concentration (Perkin Elmer, Boston, MA). 100 µl of cell suspensions were removed at T = -5 and -1 min to establish basal uptake. At T = 0 min, cells were treated with either vehicle (0.01% ethanol, final concentration), 300 pM 1,25(OH)$_2$D$_3$, 6.5 nM 24R,25(OH)$_2$D$_3$ or 200 pM 24S,25(OH)$_2$D$_3$, and incubated at room temperature. At T = 1, 3, 5, 7, 10 min, 100 µl of cell suspension aliquots were transferred to 900 µl ice-cold GBSS in 1.5 ml microcentrifuge tubes to stop phosphate uptake. Samples were put on ice until centrifuged at 1000 x g for 5 min, 4ºC. Supernatant was decanted and any residual liquid was carefully swabbed with Kimwipes while still in the inverted position. Pellets were resuspended in 500 µl double distilled water, aliquots were used for protein determination using a UV spectrophotometer (Beckman Coulter) and radionuclide measurement using a multi-purpose scintillation counter (Beckman) [8]. Values obtained for the treated phase were normalized to average basal phase, and represent means ± S.E.M for n independent experiments. Time course data indicated that optimal responsiveness to 24,25(OH)$_2$D$_3$ occurred at 7 min of steroid treatment.

Chicken intestinal cells were isolated by the citrate chelation protocol as previously described. Under sterile conditions, 200 µl aliquots of cell suspension were pipetted into 35 mm plastic Petri dishes (Falcon, Scientific Products) containing 3 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 with 5% CO$_2$/95% air at 37ºC. No serum was added to promote better cell adherence.

The next day, media were aspirated and cells were pre-incubated at room
temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS-0.1%BSA containing 4 μCi of H$_3^{32}$PO$_4$ with either 300 pM 1,25(OH)$_2$D$_3$, 200 pM 24S,25(OH)$_2$D$_3$ or 6.5 nM 24R,25(OH)$_2$D$_3$ were added and incubated for an additional 7 min. Adherent cells were washed three times with 4 ml ice-cold GBSS and lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. Finally, cells were collected by scrapping and aspirating. Aliquots were used for protein determination using a UV spectrophotometer (Beckman Coulter, City State) and radionuclide measurement using a multi-purpose scintillation counter (Beckman) [8]. Values represent means ± S.E.M for N independent experiments.

siRNA transfection in cultured intestinal cells

Chicken intestinal cells were isolated by the citrate chelation protocol as previously described. Under sterile conditions, 200 µl aliquots of cell suspension were pipetted into 35 mm plastic Petri dishes (Falcon Scientific Products; Franklin Lakes, NJ) containing 2 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 with 5% CO$_2$/95% air at 37ºC prior to transfection. No serum was added to promote better cell adherence.

The next day, cells were transfected with 100 nM scrambled siRNA (Santa Cruz Biotechnology; Santa Cruz, CA), or 80 pmols siRNA against catalase, under RNase-free, sterile conditions. Cells were then incubated 5-7 hours at 37ºC in a CO$_2$ incubator. 1 ml of normal growth medium containing 2 times the normal serum and antibiotic concentration (2x normal growth medium) were added without removing the transfection mixture. After another additional 18-24 hours incubation, media were aspirated and replaced with RPMI-1640 with 10% FBS. The following procedures were conducted
after 24 hours of addition of fresh medium.

Western Blots

The isolated intestinal cells described above were collected by centrifugation at 500 x g, 5 min (4°C), and resuspended in 30 ml of GBSS. Cells were then cultured overnight, then transfected the next day according to the siRNA transfection protocol (Santa Cruz). On the day of use, cells were treated with 0.01% ethanol, 6.5 nM 24R,25(OH)₂D₃ or 300 pM 1,25(OH)₂D₃ for 7 min at room temperature. Media was removed and cells were lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. 10µg of total protein were loaded in each lane.

SDS-PAGE and western blot analyses were used to determine immunoreactive levels of catalase in controls and cells treated with vitamin D metabolites. Protein was determined with the Bradford reagent (Bio-Rad, Hercules, CA) and then samples (5-30µg/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 37°C in blocking solution (0.1% wt/vol BSA) in Tris-buffered saline (PBS; 0.9% NaCl in 20 mM Tris-HCl, pH 7.4), and incubation with primary antibody (Abcam Inc., Cambridge, MA) in 1% BSA and 0.05% Tween 20 in PBS overnight at 4°C. After three additional washes, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) in the same incubation buffer 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 ImageJ software.

RNA isolation and RT-PCR using SYBR green

On the day of use, total RNA was isolated with TRIzol reagent (Sigma) using glycogen as a carrier because of low cell numbers (<10^4). For RT-PCR, 100 ng of total RNA was used for RT reactions to make cDNA. The primer for the 259-bp catalase product in the forward direction was a 23-mer (5’-CTG GCA ACC CAA TAG GAG ATA AG-3’), and for the reverse direction, (5’-GCA ACA GTG GAG AAC CGT ATA G-3’), a 22-mer was used. The primer for the 545-bp GAPDH product in the forward direction was a 20-mer (5’-AGT CGG AGT CAA CGG ATT TG-3’), and for the reverse direction, (5’-TCT TCT GTG TGG CTG TGA TG-3’), a 20-mer was used. The primers were validated using end point PCR, with subsequent experiments using real time PCR. Power SYBR Green PCR Master Mix (Applied Biosystems, UK) was used for RT-PCR. The RT-PCR detection procedure was the MiniOpticon System (Bio-Rad) and results were analyzed with the company’s software.

**Determination of Protein Concentration**

The Bradford assay was performed to determine sample protein concentrations. After homogenization of cells, 10 µl of sample was added to 790 µl double distilled water followed by 200 µl of Bradford reagent (Bio-Rad). The contents were mixed, set at room temperature for 10 min for color development, and analyzed by spectrophotometry at 595 nm. Bovine gamma globulin (Sigma) was used as the standard.
Statistical Analysis

Values are expressed as the means ± S.E.M for the number of independent experiments indicated in the figure legends. The data were analyzed for significance using Student’s t-test and significant and differences were determined with a 95% confidence level (P<0.05).

Results

Time course study of $^{32}$P uptake after hormone treatments in intestinal cells suspensions

Uptake studies in suspension were first undertaken to determine an optimal time point of hormone effect prior to studies using cultured adherent cells, which are technically more difficult. Chick intestinal cells were isolated with citrate chelation media described above and resuspended in 40 mL GBSS, aliquoted, and then treated with 0.01% ethanol, 130 pM 1,25(OH)$_2$D$_3$, or 6.5 nM 24,25(OH)$_2$D$_3$ with 1 µCi $^{32}$H$_3$PO$_4$/ml of cell suspension at room temperature. The selected steroid concentrations have been shown to be equivalent to circulating levels [13]. Incubations were for selected times (1min, 3min, 5min, 7min, 10min), and uptake stopped by transferring aliquots of cell suspension into ice-cold GBSS at the indicated times. Cells were pelleted by low speed centrifugation (1000 x g, 5 min, 4°C), supernatants decanted, and any residual GBSS was carefully swabbed with Kimwipes while the tubes were still in an inverted position. Cells were resuspended in 0.5 ml reagent grade water, and aliquots were taken for determination of protein and measurement of radionuclide. Figure 3.1 illustrates $^{32}$P uptake levels with different hormone treatment conditions. As shown, 1,25(OH)$_2$D$_3$ had an overall stimulatory effect on $^{32}$P uptake, 24R,25(OH)$_2$D$_3$ had an inhibitory effect on $^{32}$P uptake,
while 24S,25(OH)₂D₃ had no change relative to the ethanol control treatment. As illustrated in Figure 3.1, both 1,25(OH)₂D₃ and 24R,25(OH)₂D₃ hormone treatments led to significant changes: 1,25(OH)₂D₃ at T = 7 min with a 140% increase (P < 0.01) and for 24R,25(OH)₂D₃ at T = 7 min with an 136 percent decrease, each relative to vehicle controls (P < 0.01). The 7 min time point was then chosen for the rest of the study.
Figure 3.1 - Time course of 1,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$ or 24S,25(OH)$_2$D$_3$ - mediated $^{32}$P uptake in chicken intestinal cell suspensions, relative to vehicle controls. Cells were isolated with citrate chelation media, and collected by low speed centrifugation (500 x g, 5 min, 4°C), cell pellets were resuspended in room temperature Gey's Balanced Salt Solution (GBSS). Cells were treated with 360 pM 1,25(OH)$_2$D$_3$, 6.5 nM 24,25(OH)$_2$D$_3$ or vehicle (0.01% ethanol, final concentration) with 1 µCi $^{32}$H$_3$PO$_4$/1 ml of cell suspension at room temperature and stopped by transferring aliquots of cell suspension into ice-cold GBSS at the indicated times. Cells were pelleted by low speed centrifugation (1000 x g, 5 min, 4°C), supernatants decanted, and any residual GBSS was carefully swabbed with Kimwipes while the tubes were still inverted. Cells were resuspended in 0.5 ml reagent grade water, and aliquots were taken for determination of protein and measurement of radionuclide. Data were calculated as cpm/µg protein and then related to corresponding basal levels. Values represent mean ± S.E.M for n=5 independent experiments. Significant differences (*P<0.05, **P<0.01) were determined using Student’s t-test, are relative to corresponding controls.
Chick intestinal cells were isolated with citrate chelation media and resuspended in 40 mL GBSS and cultured overnight as described above. The next morning, cells were pre-incubated at room temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS-0.1%BSA containing 4 µCi of H$_3^{32}$PO$_4$ with one of three hormones was added and the cells incubated for an additional 7 min. Adherent cells were washed three times with 4 ml ice-cold GBSS and lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. Finally, cells were collected by scrapping and aspirating, and aliquots were taken for determination of protein and measurement of radionuclide. Figure 3.2 shows $^{32}$P uptake after treatments with different hormones or vehicle. According to Figure 3.2, a similar pattern is observed as in Figure 3.1: 1,25(OH)$_2$D$_3$ had a stimulatory effect on $^{32}$P uptake to 220% of vehicle controls (P<0.05), 24R,25(OH)$_2$D$_3$ had an inhibitory effect to 140% of vehicle controls (P<0.05), while 24S,25(OH)$_2$D$_3$ had no effect under these conditions. Thus treatment of adherent cells in culture gave results equivalent to those in suspension, allowing further studies with siRNA.
Figure 3.2 Effect of vehicle, 1,25(OH)₂D₃ or 24,25(OH)₂D₃ on ³²P uptake in cultured chicken intestinal cells. On the days of experiments, cells cultured overnight were pre-incubated at room temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS-0.1%BSA containing 4 µCi of H₃²PO₄ with vehicle, 1,25(OH)₂D₃, 24S,25(OH)₂D₃ or 24R,25(OH)₂D₃ were added and incubated for an additional 7 min. Adherent cells were washed three times with 4 ml ice-cold GBSS and lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. Finally, cells were collected by scraping and aspirating, and aliquots were taken for determination of protein and measurement of radionuclide. Data were calculated as cpm/µg protein and then related to corresponding vehicle controls. Values represent means ± S.E.M for n = 8 independent experiments. Significant differences were determined using the student t-test, and are indicated by an asterisk (*P<0.05, relative to vehicle control; ** P<0.01, relative to vehicle control).

The lack of effect by 24S,25(OH)₂D₃ suggested that no further testing with this metabolite need be performed.
Effect of vitamin D on cultured chicken enterocytes on catalase gene expression

Chick enterocytes were isolated, cultured, and transfected as described above. Assays were performed 24 hours after the addition of fresh medium. Total RNA was prepared with TRIzol reagent, 100 ng was used for cDNA synthesis and 100 ng was used for RT-PCR with primers specific for catalase or GAPDH as described above. Figure 3.3 provides data as a fold ratio normalized to GAPDH expression. Cells were treated with test substance for 7 min. siRNA transfection had no effect on gene transcription levels in cells treated with ethanol or 1,25(OH)$_2$D$_3$ compared to those treated with scramble RNA (Figure 3.4). This finding suggests that catalase siRNA treatment was not effective in reducing catalase expression. However, comparisons between the scrambled siRNA treatments reveal that 1,25(OH)$_2$D$_3$ increased catalase gene expression relative to ethanol treatment controls (P<0.01). Conversely, 24R,25(OH)$_2$D$_3$ decreased catalase gene expression (P<0.01) compared to the ethanol control. When treated with scrambled control siRNA, the 1,25(OH)$_2$D$_3$ treatment group showed an increase in catalase mRNA expression relative to ethanol controls, while the 24R,25(OH)$_2$D$_3$ treatment group had a decrease in catalase mRNA expression, relative to ethanol controls (Figure 3.4).
Figure 3.3 Effect of vitamin D on gene expression for catalase in cultures of chicken intestinal cells. Cells were transfected with scrambled siRNA or catalase siRNA according to siRNA transfection protocol (Santa Cruz, CA). After transfection, cells were incubated with ethanol control or test hormones at room temperature for 7 min. Total RNA was prepared with the TRizol reagent, and 100 ng was used for RT-PCR with primers specific for catalase or GAPDH. Values represent means ± S.E.M for n=6 independent experiments. Significant differences were determined using Student's t-test (**P<0.01).
Effect of $^{32}$P uptake after different hormone treatments at 7 min in cultured transfected enterocytes

Chick enterocytes were isolated with citrate chelation media and resuspended in 40 mL GBSS cultured overnight at 37°C with 5% CO$_2$/95% air to promote cell adhesion. The next day, cells were transfected with either scrambled control siRNA or siRNA directed against catalase according to the siRNA transfection protocol, and were incubated for an additional 20 hours at 37°C with 5% CO$_2$/95% air. Assays were performed 24 hours after the addition of fresh medium. Cells were pre-incubated at room temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS-0.1%BSA containing 4 µCi of H$_3$PO$_4$ with vehicle or hormones were added and incubated for an additional 7 min. Adherent cells were washed three times with 4 ml ice-cold GBSS and lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. Finally, cells were collected by scraping and aspirating, and aliquots were taken for determination of protein and measurement of radionuclide. Although as demonstrated in Figure 3.4, catalase siRNA was ineffective in reducing catalase expression; comparisons between the scramble treated cells revealed that treatment with 1,25(OH)$_2$D$_3$ increased $^{32}$P uptake (P<0.01) and 24R,25(OH)$_2$D$_3$ reduced $^{32}$P uptake (P<0.01) relative to ethanol controls.
Figure 3.4 Effect of vehicle, 1,25(OH)₂D₃ or 24R,25(OH)₂D₃ on ³²P uptake in transfected cultured chicken enterocytes. Cells were transfected with scrambled control siRNA or catalase siRNA according to the siRNA transfection protocol (Santa Cruz Biotechnology, CA). On the day of the experiments, transfected cells were pre-incubated at room temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS-0.1%BSA containing 4 µCi of H₃²PO₄ and either vehicle or 1,25(OH)₂D₃, 24S,25(OH)₂D₃ or 24R,25(OH)₂D₃ were added and incubated for an additional 7 min. Adherent cells were washed three times with 4 ml ice-cold GBSS and lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED. Finally, cells were collected by scraping and aspirating, and aliquots were taken for determination of protein and measurement of radionuclide. Data were calculated as cpm/µg protein and then related to corresponding basal levels. Open bars represent data from scrambled siRNA treatments; Filled bars represent data from siRNA treatments. Values represent means ± S.E.M for n =4 independent experiments. Significant differences (**P<0.01) were determined using Student’s t-test, are relative to corresponding treatments also transfected with scramble RNA.
Effect of vitamin D on protein expression of catalase in cultured chicken enterocytes

Chick enterocytes were isolated, cultured, and transfected as described above. Assays were performed 24 hours after the addition of fresh medium. Cells were pre-incubated at room temperature for 5 min in 1 ml GBSS-0.1%BSA. Then 1 ml of GBSS containing vehicle or hormones were added and incubated for additional 7 min. Adherent cells were washed, lysed, and collected as described. 10 µg of protein for each treatment was used for SDS-PAGE and western blotting. Protein band intensity was determined using ImageJ software. Figure 3.5 shows no change in protein expression among control treatments. In contrast, 1,25(OH)₂D₃ increased catalase protein levels, relative to ethanol treated controls transfected with scramble siRNA (P<0.01), while 24R,25(OH)₂D₃, decreased catalase expression compared to 1,25(OH)₂D₃ (P<0.05) and ethanol controls. This result indicated that protein levels are not proportional to mRNA in cultured chicken enterocytes after 7 min treatment with vitamin D.
Figure 3.5 Effect of vitamin D on protein levels of catalase in cultured chicken enterocytes. Cells were transfected with scrambled siRNA or catalase siRNA according to siRNA transfection protocol (Santa Cruz, CA). Treatment with vehicle or hormones was for 7 min. Cells were lysed with 0.5 ml 0.1% (v/v) Triton X-100 in TED, 10 µg of proteins were used for SDS-PAGE and western blotting. Figure 3.5A shows a western blot; Figure 3.5B depicts densitometric analyses of the bands shown. Values represent means ± S.E.M for n=3 independent experiments. Significant differences were determined using the student t-test, are relative to corresponding controls, and are indicated by asterisks (**P<0.01) (*P<0.05).
Discussion

Previous experiments with primary cultures of chick intestinal cells exposed to 24,25(OH)₂D₃ for 5 h have demonstrated that 24R,25(OH)₂D₃ is capable of decreasing both catalase specific activity and catalase protein levels [8]. In this study with suspended chick enterocytes treated with 24R,25(OH)₂D₃, a decrease in phosphate uptake occurs as early as 1 min and is significantly lower than ethanol controls at 7 min. Although the treated/average basal values increase in both 24S,25(OH)₂D₃ and 24R,25(OH)₂D₃ with time, 24S,25(OH)₂D₃ remained more closely to basal values compared to 24R,25(OH)₂D₃. In contrast, the decrease in ³²P uptake is sustained at an average of 150% below controls from 1 to 10 min in 24R,25(OH)₂D₃ treatments. A similar result is observed with primary cultured chick enterocytes. After a 7 min incubation with hormones or the vehicle ethanol (a time point determined by previous study in cell suspension), a significant decrease in phosphate uptake is observed in 24R,25(OH)₂D₃ treatments (at an average of 140% below ethanol controls).

With equivalent results observed in both suspension and cultured chick enterocytes, further siRNA study was undertaken to determine whether this manipulation affects 24R,25(OH)₂D₃ mediated changes in catalase gene expression and/or protein expression. Unfortunately, catalase siRNA treatment was not effective in reducing catalase gene expression. Nonetheless, several important observations were made. A decrease in 1,25(OH)₂D₃-mediated phosphate uptake is observed with a 7 min after 24R,25(OH)₂D₃ treatment combined with scrambled siRNA (P<0.01). An increase in 1,25(OH)₂D₃-mediated phosphate uptake is observed with 7 min after 1,25(OH)₂D₃ treatment combined with scrambled siRNA (P<0.01). Catalase gene expression was
determined using RT-PCR, and a significant 281% decrease is observed in 24R,25(OH)₂D₃ treated cells with scramble siRNA relative to the corresponding ethanol control (P<0.01). The inhibitory effect was again diminished with catalase siRNA.

Protein levels of catalase were determined by western blots after 7 min of steroid treatment. With catalase antibody directed against chick catalase as primary antibody, fainter bands were observed in cultured cells treated with 24R,25(OH)₂D₃, while 1,25(OH)₂D₃ treatment produced denser bands (Figure 3.5A). Densitometric analysis (Figure 3.5B) indicated a significantly lower level of immunoreactive protein in cultured intestinal cells treated for 7 min with 24R,25(OH)₂D₃, relative to either corresponding control or 1,25(OH)₂D₃ treatments (P<0.01). This observation is in agreement with similar effect observed in 24 h samples for chick intestinal cells and kidney cells [8].

These combined data indicated that 24R,25(OH)₂D₃ has significant physiological consequences in maintaining phosphate homeostasis even though the siRNA did not work in manipulating catalase gene expression in cultured chick enterocytes.
References


Summary

1,25-dihydroxyvitamin D₃ has proven to be the up-regulating steroid hormone for phosphate homeostasis. This secosteroid hormone exerts its effect through two different mechanisms, either via a nuclear vitamin D receptor (nVDR) to express a slow action to generate genomic responses or through the more rapid action of 1,25D₃-MARRS receptor to generate pre-genomic actions of ion transport and signal transduction inside cells [1-6]. From previous studies, it was demonstrated in chick enterocytes that the signal transduction mechanism of 1,25(OH)₂D₃-mediated rapid uptake of phosphate was mediated by PKC [7, 8].

Catalase has been proven to be the cellular binding protein for 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] [9]. Both 24,25(OH)₂D₃ and the production of H₂O₂ have been demonstrated to inhibit catalase activity [10]. Somjen et al. [11] reported that 24R,25(OH)₂D₃, not 24S,25(OH)₂D₃ was the active metabolite in bone. However, a previous study found that the two isomers of 24,25(OH)₂D₃ had different effects on phosphate transport in vivo [12]. The inhibitory effect is mainly performed by 24R,25(OH)₂D₃ since it was capable of decreasing phosphate absorption after a 1h injection in vivo, while 24S,25(OH)₂D₃ was found to be increasing phosphate transport at 5h in chicks in vivo but the latter effect was diminished after 10h. This suggests another receptor may be involved, or that the steroid may stimulate other pathways to mediate phosphate absorption in vivo [12]. Previous work done in the perfused duodenal loop
system has proved that the antagonism of the stimulatory effect of \(1,25(OH)_2D_3\) by \(24,25(OH)_2D_3\) was seen 10 min after the onset of perfusion [13]. In these studies, the main focus was on how \(24,25(OH)_2D_3\) effects phosphate uptake in chick enterocytes in vitro.

The first series of experiments were performed to determine the effects of the two isomers of \(24,25(OH)_2D_3\) on localization of catalase in chick enterocytes. The present work demonstrated that \(24R,25(OH)_2D_3\) is the effective metabolite for catalase redistribution in vitro, not \(24S,25(OH)_2D_3\). Male chick intestinal cells were treated with either vehicle, \(24R,25(OH)_2D_3\) or \(1,25(OH)_2D_3\), and punctate staining was observed on the cell surface and in the cytoplasm for all time points tested. Figure 2.2 depicts results from \(24R,25(OH)_2D_3\) treatments, in which there were obvious increases in nuclear staining as well as surface staining relative to controls (Figure 2.1). Figure 2.3 shows results from \(24S,25(OH)_2D_3\) treatments, in which there was an obvious decrease in punctuate nuclear staining compared to \(24R,25(OH)_2D_3\) treatments. Additional western analyses were performed to confirm the punctate staining inside the nucleus was from the redistribution of cell surface catalase.

Further experiments were undertaken to examine the origin of the nuclear staining by confocal microscopy experiments. Primary antibody was first added to cells for 30 min in order to block surface staining, and subsequently incubated with vehicle, \(1,25(OH)_2D_3\), \(24R,25(OH)_2D_3\) or \(24S,25(OH)_2D_3\). This work demonstrated that ligand binding to cell surface catalase induced redistribution to the nucleus, but was blocked by the antibody. Figure 2.6 indicates nuclear redistribution after \(24R,25(OH)_2D_3\) treatment did not occur, indicating that the cell surface catalase induced redistribution was blocked
by the antibody. A similar effect was not found in either 24S,25(OH)_2D_3 nor control treatments. Catalase punctate staining intensity was also quantified by densitometry.

The second series of experiments were performed to determine the relationship between vitamin D metabolites, catalase expression and phosphate uptake in chicken intestinal cells. It has been reported that 24,25(OH)_2D_3 decreases catalase protein levels in cultured intestinal cells over a period of several hours [14], an effect on catalase gene expression combined with rapid signaling would conclusively demonstrate that 24,25(OH)_2D_3 is not a ‘scrap’ metabolite. The present work demonstrated that 24R,25(OH)_2D_3 is the effective metabolite for decreasing phosphate uptake and catalase gene expression, not 24S,25(OH)_2D_3. Figure 3.1 and Figure 3.2 illustrates ³²P uptake levels with different hormone treatment conditions in normal enterocytes, in which 24R,25(OH)_2D_3 has a inhibitory effect on phosphate uptake, while 24S,25(OH)_2D_3 does not. Figure 3.3 demonstrates the effect of different hormone treatments on ³²P uptake in transfected cultured enterocytes. A decrease in ³²P was observed only in 24R,25(OH)_2D_3 treatment (P<0.05), indicating an elimination of the steroid-catalase effect on decreasing phosphate uptake. As shown in Figure 3.4, only 24R,25(OH)_2D_3 treatment led to a decrease in catalase mRNA expression. It was also determined that 24R,25(OH)_2D_3 had no effect on catalase protein expression.

Conclusions

From these recent results, we can then conclude that:

1. 24R,25(OH)_2D_3, not 24S,25(OH)_2D_3, is the effective metabolite for catalase redistribution in chicken enterocytes in vitro.
2. Western analysis and additional confocal microscopy experiments confirmed that the punctate staining inside the nucleus arose from the redistribution of cell surface catalase.

3. 24R,25(OH)₂D₃ is the effective metabolite for decreasing phosphate uptake as well as catalase gene expression determined by RT-PCR, but did not have an effect on protein expression as determined by western analysis.

**Future Study**

1. Determination of the possible mechanism involved in catalase binding to STAT3 [15];
2. Evaluation of whether 24R,25(OH)₂D₃ mediates the production of separate hormones that regulate phosphate transport.

**Benefit**

This study provides some basic framework of cellular and molecular mechanism of 24,25(OH)₂D₃ regulated phosphate homeostasis, and increases our understanding of the effects of 24,25(OH)₂D₃ on phosphate transport in vitro. The results from this research may open new ways for drugs or gene therapies to regulate phosphate homeostasis. Furthermore, this research also has implications in agriculture. Since the production of manure high in phosphate in poultry farms is a serious problem, phosphate in manure of production animals could be diminished.
References


