# ROLE OF VITAMIN K IN INTERACTION BETWEEN 1,25-DIHYDROXYVITAMIN $D_3$ RECEPTORS AND DNA

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In the modern view of the endocrine system of vitamin D, the hormonally active metabolites of this vitamin synthesized in the kidneys, 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$], exerts its functions and, in particular, in intestinal calcium absorption, by interaction with a specific intracellular receptor [7].

The structure of this receptor and regulation of its activity have received little study. It has been found that 1,25$(OH)_2D_3$ and other vitamin $D_3$ metabolites [6], and also glucocorticoids and retinoids [5, 9] are able to regulate the concentration of 1,25$(OH)_2D_3$ receptors. Pyridoxal-5'-phosphate reduces interaction of 1,25-$(OH)_2D_3$ receptors with nuclear binding sites [8], and an alimentary phosphorus deficiency reduces their affinity for the hormone [4]. For interaction of the 1,25-$(OH)_2D_3$ — receptor complex with chromatin, it must undergo modification by phosphorylation [10]. The mechanisms of inactivation of the 1,25-$(OH)_2D_3$ receptor, so far as we are aware, have not been described. In this paper we give data indicating a role of vitamin K in this process.

## EXPERIMENTAL METHOD

Male Wistar rats weighing initially about 50 g were used. In the first experiment the animals were given a diet for 1 month containing different quantities of vitamins D and K, and 0.62% Ca and 0.53% P. The groups of animals are described in Table 1. The vitamin K (vikasol) content in the diet was 200 μg/kg, and vitamin $D_3$ was given perorally in a dose of 1 μg (40 IU) per animal every other day. In the second experiment, rats on a diet adequate in vitamin D received the vitamin K antagonist 3-[α-(4'-nitrophenyl)-β-acetylethyl]-4-hydroxycoumarin in a dose of 30 mg/kg body weight for 5 days. In the third experiment the animals were kept on a low-calcium diet (0.03% Ca, 0.7% P), adequate in vitamin D, for 17 days, and then for 5 days on a high-calcium diet (2.4% Ca, 0.7% P). Half of these rats received the vitamin K antagonist in a dose of 15 mg/kg during the last 12 days of the experiment.

Separate quantitative determination of vacant and endogenously (in vivo) occupied 1,25-$(OH)_2D_3$ receptors in the chromatin fraction of the small intestinal mucosa was carried out by the method described previously [1, 2]. During determination of the kinetic binding parameters, 1,25-$(OH)_2D_3$ receptors were extracted with hypertonic TED-KCl buffer (10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol — DTT, 0.3 mM phenylmethylsulfonyl fluoride — PMSF, 0.3 M KCl, pH 7.4).

Aliquots of the high-speed cytosol (100 μl, 0.2 mg protein) were incubated for 17 h at 0-4°C with increasing (0.055-1.8 nM) concentrations of $^3$H-1,25-$(OH)_2D_3$. The free and bound ligands were separated by adsorption of the latter on hydroxyapatite [1]. Radioactivity was extracted from the residue with a mixture of chloroform and methanol (1:2).

DNA-cellulose (5-6 mg DNA/g) was obtained by the method in [3], using DNA from sturgeon sperm. A suspension of DNA-cellulose in TED-buffer (50%, wt./v) was added to the cytosol $^3$H-1,25-

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TABLE 1. Concentration of Vacant and in Vivo Occupied Nuclear 1,25-$(OH)_2D_3$ Receptors, Kinetic Parameters, and DNA-Cellulose Binding of Cytoplasmic 1,25-$(OH)_2D_3$ Receptors in Small Intestinal Mucosa of Rats Differing in Their Vitamin D and K Intake

| Group of animals | Concentration of nuclear receptors, fmoles/mg protein | | | Cytoplasmic receptors | | Percent of binding with DNA-cellulose |
|---|---|---|---|---|---|---|
| | vacant | occupied | percent of occupied receptors | $B_{max}$, fmoles/mg protein | $K_d$, pM | |
| 1- (+D, +K) | 63,0±19,3 | 7,69±1,8 | 10,9 | 320 | 186 | 30,3 |
| 2- (+D, —K) | 41,6±13,7 | 9,64±3,5 | 18,8 | 299 | 200 | 90,4 |
| 3- (—D, +K) | 69,4±19,7 | <1,0 | <2,0 | 253 | 210 | 37,9 |
| 4- (—D, —K) | 53,6±14,0 | <1,0 | <2,0 | 220 | 175 | 85,0 |
| 5- (—D, +K, +D,1 day) | 65,4±22,7 | 10,2±3,4 | 13,5 | 258 | 238 | 43,7 |
| 6- (—D, —K, +D,1 day) | 44,8±8,5 | 7,80±2,1 | 14,8 | 243 | 160 | 59,1 |
| 7- (—D, +K, +D,5 days) | 60,8±12,9 | 8,30±1,2 | 12,0 | 295 | 217 | 31,5 |
| 8- (—D, —K, +D,5 days) | 38,6±3,2 | 8,33±0,1 | 17,4 | 225 | 190 | 65,- |

Legend. +D and —D) Animals replete with and deprived of vitamin D; +K and —K) animals replete with and deprived of vitamin K. 1 Day and 5 days) Time of administration of vitamin $D_3$. Here and in Table 2, data presented as arithmetic mean of 3 to 5 determinations ± standard error, or as mean of two determinations of a pooled preparation from two or three animals.

TABLE 2. Concentrations of Vacant and in Vivo Occupied 1,25-$(OH)_2D_3$ Receptors in Kidneys and Intestinal Mucosa and Binding of in Vitro Occupied 1,25-$(OH)_2D_3$ Receptors with DNA-Cellulose in Rats Receiving the Vitamin K Antagonist

| Group of animals | Condition of preincubation | Concentration of receptors, fmoles/mg protein | | | Percent of binding with DNA-cellulose |
|---|---|---|---|---|---|
| | | vacant | occupied | percent of occupied receptors | |
| **Kidneys** | | | | | |
| Control | Complete system | 77,8±14,5 | 8,79±0,10 | 10,2 | 41,1 |
| | +$K_1$ | 106±5,3 | 9,56±1,47 | 8,27 | 37,2 |
| | —$K_1$ | 84,8±12,0 | 7,74±0,75 | 8,36 | 36,3 |
| Animals receiving vitamin K antagonist | Complete system | 111±7,8 | 13,9±0,18* | 11,1 | 32,5 |
| | +$K_1$ | 83,0±16,8 | 16,4±0,35*,** | 16,5 | 94,2 |
| | —$K_1$ | 79,2±3,9** | 17,9±0,37*,** | 18,4 | 86,9 |
| **Mucosa** | | | | | |
| Control | Complete system | 273±18,8 | 19,1±0,35 | 6,54 | 27,4 |
| | +$K_1$ | 242±10,0 | 17,2±0,54 | 6,63 | 42,6 |
| | —$K_1$ | 292±15,7 | 16,6±2,28 | 5,38 | 28,8 |
| Animals receiving vitamin K antagonist | Complete system | 267±40,1 | 20,0±4,31 | 6,97 | 31,3 |
| | +$K_1$ | 212±13,2 | 21,1±2,29 | 9,05 | 75,0 |
| | —$K_1$ | 264±19,3 | 28,3±1,23* | 10,7 | 67,8 |

Legend. Complete carboxylating system included vitamin $K_1$, NADH, and $NaHCO_3$ (see: Experimental Method); +$K_1$) vitamin $K_1$ in ethanol and buffer; —$K_1$) buffer with NADH, $NaHCO_3$, and ethanol. *) Significant difference ($p<0.05$) from corresponding value in control group, **) significant difference from corresponding value for complete system in the same group.

$(OH)_2D_3$—receptor complex up to a KCl concentration of 0.15 M and incubated for 45 min at 0-4°C. The DNA-cellulose residue was washed twice with TED buffer and 0.5% Triton X-100.

In rats receiving the vitamin K antagonist the possible effect of vitamin K-dependent γ-carboxylation on the properties of the 1,25-$(OH)_2D_3$ receptors was estimated. For this purpose a homogenate of mucosa and kidneys in TD-KCl buffer (10 mM Tris-HCl, 10 mM DTT, 0.3 mM PMSF, 0.3 M KCl, pH 7.4) was centrifuged for 20 min at 15,000g and at 4°C. Triton X-100 was added to the supernatant in a concentration of up to 2% to solubilize the microsomes, and the product was centrifuged for 1 h at 105,000g, 4°C. Aliquots of cytosol (1 ml, 10 mg protein) were incubated for 3 h at 0-4°C, either in the presence of a complete carboxylating system (0.1 mM vitamin $K_1$, 2 mM NADH, 1 mM $NaHCO_3$) [11], or with the addition of vitamin $K_1$ only or of the buffer only. The cytosol was diluted with TED-KCl buffer to a protein concentration of 1 mg/ml. A concentration of vacant and in vivo occupied 1,25-$(OH)_2D_3$ receptors and binding of in vitro occupied receptors with DNA-cellulose were determined as described above, with the exception that, because of the unusually high nonspecific binding of $^3H$-1,25-$(OH)_2D_3$ by vitamin

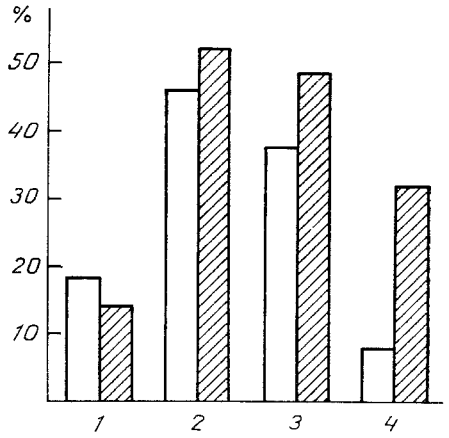


Fig. 1. Change in percentage of 1,25-$(OH)_2D_3$ receptors occupied in vivo in kidney cytosol of rats receiving vitamin K antagonist against the background of a low calorie diet. 1) Background data; 2) animals kept for 10 days on low calcium diet; 3) animals receiving vitamin K antagonist for 7 days against the background of a low-calcium diet; 4) animals continuing to receive vitamin K antagonist for 5 days against the background of high calcium diet. Shaded columns represent group of rats receiving vitamin K antagonist, unshaded columns — control group. Ordinate, percentage of occupied receptors. Data shown as mean of two determinations of pooled preparation from two or three animals (see caption to Table 1 also).

D-binding protein, 25-$OHD_3$ was added to the kidney cytosol for 1 h before the end of incubation, in a concentration of 50 nM.

## EXPERIMENTAL RESULTS

The vitamin K deficiency did not affect the level of occupied 1,25-$(OH)_2D_3$ receptors but it caused a moderate (not statistically significant because of the wide scatter) decrease in the concentration of vacant nuclear receptors, as a result of which the percentage of occupied receptors increased. A similar type of changes in the concentration of vacant and percentage of occupied 1,25-$(OH)_2D_3$ receptors was observed in combined vitamin D and K deficiency, and also when vitamin $D_3$ was given to animals deprived of this vitamin (Table 1). The maximal capacity and affinity of 1,25-$(OH)_2D_3$ receptors in vitamin K deficiency were unchanged, but in vitamin D deficiency their capacity was reduced without any change in their affinity for the ligand.

The sharp (by 2-2.5 times) increase in binding of the 1,25-$(OH)_2D_3$—receptor complex (holoreceptor) with DNA-cellulose was an unexpected effect of vitamin K deficiency (Table 1). This effect was also observed in combined vitamin D and K deficiency and also when such animals were given vitamin $D_3$.

Such a significant effect of vitamin K deficiency on binding of the holoreceptor with DNA raised the question of the mechanism of this effect. Since the principal biochemical function of vitamin K is its role in post-translation γ-carboxylation of some Ca-binding proteins [11], we attempted to evaluate the possible connection of this process with changes discovered in the properties of the 1,25-$(OH)_2D_3$ receptor.

For this purpose 1,25-$(OH)_2D_3$ receptors extractable from the intestinal mucosa and kidneys of rats receiving the vitamin K antagonist by buffer with high ionic strength in the presence of the detergent Triton X-100 were used. This cytosol, which also contains dissolved glutamyl

carboxylase, was incubated in vitro with the carboxylating system under near-optimal conditions both for γ-carboxylation and for subsequent binding with the 1,25-$(OH)_2D_3$ receptor.

In this experiment binding of the cytosol 1,25-$(OH)_2D_3$–receptor complex from small intestinal mucosa and kidneys of rats receiving the vitamin K antagonist with DNA-cellulose, just as in the first experiment, was twice or three times higher than in the control animals (Table 2). The number of occupied receptors in the kidneys of the rats receiving the vitamin K antagonist was almost twice as many as in intact animals. These data suggest that the effect of more intensive binding of the holoreceptor with DNA in vitamin K deficiency, which we observed, is realized not only in the model system in vitro, but also under physiological conditions in vivo.

Incubation of cytosol of the small intestinal mucosa and kidneys of intact rats with the complete system essential for vitamin K-dependent carboxylation of endogenous substrates (vitamin $K_1$ + NADH + $HCO_3^-$ + DTT) did not affect the parameters of binding of the holoreceptor with DNA-cellulose. Incubation of the cytosol of the mucosa and kidneys of rats receiving the vitamin K antagonist, with the same complete system led to a decrease in binding of the holoreceptor with DNA-cellulose to the level characteristic of intact animals. Incubation with vitamin $K_1$ alone, without NADH and $HCO_3^-$, had no such action. At the same time, carboxylation of endogenous substrates of kidney cytosol of rats receiving the vitamin K antagonist led to a decrease in the concentration and percentage in vivo occupancy of the hormone receptors. This effect was not observed in the cytosol of the intestinal mucosa.

Lowering the Ca content in the diet of the rats from 0.6 to 0.03% led to a sharp rise in the level of occupied 1,25-$(OH)_2D_3$ receptors in the kidneys (Fig. 1). Administration of the vitamin K antagonist had hardly any effect on the concentration of occupied 1,25-$(OH)_2D_3$ receptors in rats receiving a low calcium diet, but largely prevented the decrease in the percentage of occupied hormone receptors with an increase in the calcium content of the diet up to 2.4%.

These results definitely indicate the importance of vitamin K-dependent γ-carboxylation of endogenous substrates for functioning of 1,25-$(OH)_2D_3$ receptors.

The explanation of the possible role of γ-carboxylation in modification of 1,25-$(OH)_2D_3$ receptors may be derived from the suggestion that glutamic acid residues are present in the molecule of the receptor of this hormone. Since the action of 1,25-$(OH)_2D_3$ is accompanied by an increase in the flow of $Ca^{++}$ into the target cell, the possibility cannot be ruled out that this cation may bring about inactivation of the receptor through a feedback mechanism. The mechanism of this inactivation may consist of interaction of $Ca^{++}$ with γ-carboxylglutamic acid residues in or near the DNA-binding domain of the receptor, leading to reduction of its ability to bind with DNA, or it may act as the signal for elimination of this domain, but does not affect binding of 1,25-$(OH)_2D_3$ with the receptor.

The effect of vitamin K on 1,25-$(OH)_2D_3$ receptors may also be mediated by some modifying factor dependent on this vitamin. By analogy with the known blood clotting factors, it may be a serine proteinase. However, the presence of PMSF, an inhibitor of this activity, in the buffer used to extract the receptors makes it exceedingly likely that this possibility can be ruled out.

Further investigations with highly purified 1,25-$(OH)_2D_3$ receptors are essential for a fuller explanation of the functional significance of the vitamin K- and $Ca^{++}$-dependent post-translation modification of this protein.

The results thus demonstrate for the first time the existence of a vitamin K-dependent, Ca-sensitive mechanism regulating the properties of preformed 1,25-$(OH)_2D_3$ receptors.

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# STIMULATION OF INTERLEUKIN-2 MEDIATED ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY LIPOPOLYSACCHARIDE AND MURAMYL DIPEPTIDE IN VITRO

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Lipopolysaccharide (LPS) and muramyl dipeptide (MDP) are glycoconjugates of bacterial origin which possess immunostimulating properties [11]. The writers showed previously that these substances have a synergic action on production of tumor necrosis factor (TNF) in vitro and in vivo and on regression of syngeneic tumors [2, 5].

It has been suggested that immune rejection of tumors during treatment with LPS and/or TNF takes place in two successive stages: a) necrosis of the tumors, b) the development of antitumor immunity [4, 13]. For the second stage to take place, a sufficient quantity of growth factors must be present and, in particular, of interleukin-2 (IL-2), which induces generation or reactivation of specific and nonspecific antitumor killer cells [8, 16].

IL-2 is widely used in tumor immunotherapy [12]. It is an interesting question whether combined immunotherapy with LPS, MDP, and IL-2 is more effective than treatment with glycoconjugates and lymphokine separately. The aim of the present investigation was to discover how LPS and/or MDP affect the ability of IL-2 to activate the the proliferative and killer functions of human peripheral mononuclear cells in vitro.

## EXPERIMENTAL METHOD

Peripheral blood mononuclear cells (PBMS) were obtained from nine healthy male and female blood donors by fractionation of whole blood on a Ficoll—Verografin density gradient [3]. The following reagents were used: *E. coli* 055:B5 LPS were obtained from Difco, USA; MDP was generously provided by N. V. Bovin, of the Institute of Biotechnology, Ministry of the Medical and Biological Industry of the USSR; recombinant human IL-2 was from the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga.

To study modulation of the proliferative response of PBMS the cells were first incubated for 24 h in a concentration of $2.5 \cdot 10^6$/ml in 2 ml of medium RPMI-1640 (Flow Laboratories, England), enriched with 10% fetal calf serum (Flow Laboratories), 2 mM L-glutamine, 10 mM HEPES buffer, and 50 µl/ml gentamicin, in 24-well panels (Linbro, Flow Laboratories, England) at 37°C in the presence of 5% $CO_2$ without immunomodulators, and in the presence of LPS (10 ng/ml), MDP (10 µg/ml), or a combination of both.

The cells were washed 3 times, counted, and dropped into 96-well panels (Costar, USA) in a dose of $2 \cdot 10^5$ per well. Meanwhile IL-2 was added to the wells in different concentrations. Each version of the experiment was repeated 3 times. The PBMS were incubated for 48 h at 37°C in the presence of 5% $CO_2$. $^3$H-Thymidine (specific activity 25 Ci/mmole) was added to each well 4 h before the end of culture. The cells were transferred to filters (Flow Laboratories) with

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