Vitamin D₃ Action on RNA Synthesis in Rat Intestinal Mucosa

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Vitamin D₃ action on uridine-5-³H incorporation into rat intestinal mucosal RNA was studied. The uridine was administered 1 hour after 2,000 IU vitamin D₃ treatment. Specific activity of RNA in nuclear and supernatant fractions was increased by vitamin D₃ at 15 minutes after uridine-5-³H administration. Two hours after uridine-5-³H administration, specific activity of RNA in nuclei, mitochondria and supernatant were also increased, but it was decreased in microsome.

Mucosal RNA was further analyzed by methylated albumin-kieselguhr column. It was found that uridine-5-³H incorporation into sRNA was increased and that into rRNA was decreased with vitamin D₃ administration.

DNA dependent RNA polymerase activities were studied in isolated nuclei obtained from rat intestinal mucosal cell. Mg²⁺-activated DNA dependent RNA polymerase was not influenced at 1 hour after vitamin D₃ administration, however, decreased at 3 hours and 16 hours after vitamin D₃ treatment. These decreases of activity would be corresponded with decreases of uridine-5-³H incorporation into rRNA. Mn²⁺ and (NH₄)₂SO₄-activated DNA dependent RNA polymerase was not influenced by vitamin D₃.

Many workers have already shown the essential role of vitamin D (calciferol) in mediating the absorption of calcium from the small intestine. It has required some lag period to appear the physiological response of the enhanced calcium absorption after the administration of vitamin D₃ (1). The hypothesis was proposed that vitamin D₃-mediated enhancement of intestinal calcium transport is achieved indirectly through RNA and protein synthesis. This hypothesis was primarily based on the finding that actinomycin D inhibits the intestinal response to vitamin D (2, 3).

In our laboratory, Moriuchi *et al.* have

demonstrated the evidences that ³H-vitamin D₃ was found inside nuclei in rat intestinal mucosa with the electronmicroscopic radioautography (4). Calcium-binding protein was purified by Wasserman and Taylor (5) in chick intestinal mucosa and also in the rat by Moriuchi (6) and Ooizumi (7). This protein is hardly found in rachitic rat, and the presence of two kinds of the protein in vitamin D₃-treated rats suggests that it may have been induced by vitamin D₃ (7).

In this paper, we studied on the RNA metabolism in rat intestinal mucosa cell to elucidate the primary action of vitamin D₃.

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Some evidences were presented in the uridine- $5\text{-}^3\text{H}$ incorporation into the intestinal cellular fraction and also into the RNA fractions separated by the methylated albumin-kieselguhr column. The DNA dependent RNA polymerase activities were also observed.

EXPERIMENTAL PROCEDURE

1. Preparation of Rats

Young female rats weighing 50–60 g were fed Rachitogenic diet No. 2, U.S.P. (N.B.Co.) for 4 weeks. Crystalline vitamin D_3 ² (Tokyo Chem. Ind. Co.) was dissolved in ethanol and propylenglycol mixture (1:9), and 2,000 I.U. was administered orally into the vitamin D deficient rats.

Animals were given 50 or 200 μCi of uridine- $5\text{-}^3\text{H}$ (specific activity 5.2 Ci/mmol, purchased from Daiichi Chem. Co.) per 50 g body weight 2 hours prior to sacrifice.

After decapitation, small intestinal mucosa was immediately prepared as described in the previous reports (6,7). Three to four grams of the combined mucosa obtained from 3–4 rats were homogenized in 0.25 M sucrose in 0.05 M tris-HCl buffer (pH 7.5) containing 0.025 M KCl, 0.005 M MgCl_2 (0.25 M sucrose-TKM) (8), with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Five strokes of homogenation were carried out in an ice bath, and the homogenate was filtered with nylon cloth to remove connective tissues.

2. Cellular Fractionation

Cellular fractionation was carried out essentially to the procedure of Haussler *et al.* (9). The homogenate was centrifuged for 10 minutes at $800\times g$, and the resulting pellet containing crude nuclei was washed with 0.25 M sucrose-TKM at $800\times g$ for 10 minutes and 0.1 % Triton X-100 in 0.25 M sucrose-TKM at $1,000\times g$ for 10 minutes. Pellet was gently homogenized with 2.2 M sucrose-TKM, adjusted to 1.75 M sucrose concentration by diluting with 0.25 M sucrose TKM and centrifuged at $65,000\times g$ for 1 hour in a Hitachi 55P ultracentrifuge with an RP40A rotor.

Mitochondria were obtained by centrifuging at $8,000\times g$ supernatant by centrifugation at $105,000\times g$ for 1 hour. Pellets were maintained at -20° until used.

3. Extraction of RNA

RNA extraction with the hot phenol method of Scherrer and Darnell (10) was further modified as follows. Cellular particles were suspended in 0.01 M acetate buffer (pH 5.1), and sodium dodecyl sulphate was added to final concentration of 0.5 % and bentonite to approximately 20 mg per g tissue.

Each fraction was emulsified with an equal volume of 80 % phenol and the mixture was shaken for 15 minutes at 60° . The mixture was rapidly chilled and the emulsion was broken by centrifugation at $5,000\times g$ for 5 minutes at 4° . The aqueous phase was extracted twice with hot phenol. After the extraction, RNA was precipitated from the clear aqueous solution by the addition of 0.1 volume of 20 % sodium acetate and two volumes of 95 % ethanol. After the storage for 12–18 hours at -20° , RNA was precipitated by centrifugation, and then dissolved in 0.01 M acetate buffer (pH 5.1).

4. RNA Fractionation by Methylated Albumin-Kieselguhr Column

RNA was fractionated on the methylated albumin-kieselguhr column with a linear gradient elution of NaCl from 0.4 to 1.6 M in 0.05 M phosphate buffer (pH 6.5) (11). Optical density (260 m μ) of each effluent was recorded by the Uvicon (Toyo Kagaku Co.). Radioactivity was measured in the observed peak fractions. The effluents were combined and precipitated with addition of 1/5 volume of 50 % trichloroacetic acid, and collected on 25 mm Millipore filter membranes of 0.45 μ pore size. After washing with 5 % trichloroacetic acid, the membranes were dried at 50° and placed in scintillation vials with 10 ml of toluene based scintillator (PPO 1 g in 1 liter of toluene).

5. Assay of DNA dependent RNA Polymerase

Purified nuclei were used for enzyme sources. Incubation mixtures for the Mg^{2+}

² Biological activities of vitamin D_3 was kindly checked by Dr. Katsui G. (Eisai Co.).

activated RNA polymerase activity consisted of 0.1 ml of nuclear suspension containing about 0.1 mg of DNA, 50 μ moles of tris-HCl buffer pH 8.5, 2.5 μ moles of MgCl₂, 10 μ moles of cysteine, 2 μ moles of NaF, 5.0 μ moles of phosphoenolpyruvate, 10.0 μ g of pyruvate kinase, 0.2 μ moles each of GTP, CTP, and ATP, and 0.01 μ moles (50 μ Ci/ μ mole) of ³H-UTP (final volume 0.5 ml) (12). Incubation was carried out for 15, 30, 45 minutes at 37°.

Incubation mixture for the Mn²⁺-(NH₄)₂SO₄ activated RNA polymerase activity consisted of 0.1 ml of nuclear suspension, 50 μ moles of tris-HCl buffer pH 7.5, 2.0 μ moles of MnCl₂, 0.05 ml of saturated ammonium sulphate (pH 7.5), 5.0 μ moles of phosphoenolpyruvate, 10 μ g of pyruvate kinase, and 0.2 μ moles each of GTP, CTP, and ATP (final volume 0.5 ml). After incubation for 15 minutes, 0.01 μ moles of ³H-UTP (50 μ Ci/ μ mole) (12) was added, and the incubation was continued further for 45 minutes.

The reaction was stopped by addition of 1 ml of ice cold 10 % trichloroacetic acid. RNA was extracted by the modified method of Schneider (13) as follows. The reaction mixture was centrifuged at 2,000 $\times$ g for 10 minutes and the precipitate was washed with 1 ml of ice cold 10 % trichloroacetic acid, then washed twice with 1 ml of 95 % ethanol. The lipid free tissue residue was suspended in 1 ml of 5 % trichloroacetic acid, and heated for 15 minutes at 90° with occasional stirring. Insoluble residue was precipitated by centrifugation and washed with 1 ml of 5 % trichloroacetic acid. The combined extracts were used for RNA fraction.

6. Determination

Radioactivity in the extracted RNA fraction was determined with the addition of dioxane based scintillator (PPO 5 g, naphthalene 100 g in 1 liter of dioxane) by Beckman Liquid Scintillation Counter L.S. 100 in the external standard method.

RNA was assayed by the orcinol reaction (14) with standard of yeast RNA (Sigma). DNA was assayed by the diphenylamine reaction (15) with standard of calf thymus DNA (Sigma).

RESULTS

1. Vitamin D₃ Effect on Uridine-5-³H Incorporation into Rat Intestinal Mucosal RNA

Vitamin D₃ effect on uridine-5-³H incorporation into rat intestinal mucosal RNA was observed. 2,000 I.U. vitamin D₃ was administered orally to vitamin D deficient rats at 1, 3, and 14 hours before uridine-5-³H administration. Twenty microcuries of uridine-5-³H was given intraperitoneally 2 hours before decapitation (16). The incorporation was slightly increased at 1 hour after vitamin D₃ treatment (Fig. 1). The time course of uridine-5-³H incorporation into intestinal mucosal RNA was observed with vitamin D deficient and vitamin D₃ fed rats. In this experiment 2,000 I.U. of vitamin D₃ was administered orally 1 hour before uridine-5-³H administration (Fig. 2). The specific activity of the intestinal mucosal RNA exhibited rapid increase, which reached maximum at 1 hour after the uridine administration and kept its level at least for 1 hour. The specific activity of intestinal mucosal

FIG. 1 Time course of vitamin D₃ effect on uridine-5-³H incorporation into rat intestinal mucosal RNA

2,000 I.U. vitamin D₃ was administered orally to vitamin D deficient rats at 1, 3 and 14 hours before uridine-5-³H administration. 20 μ Ci of uridine-5-³H was given intraperitoneally 2 hours before decapitation. Data were the mean of 5 determinations.

FIG. 2 Time course of uridine-5-³H incorporation into intestinal mucosal RNA

Rats were received intraperitoneally 50 μ Ci uridine-5-³H per 50 g rat and sacrificed at 15, 60 and 120 minutes after uridine administration. 2,000 I.U. vitamin D₃ was administered orally 1 hour before uridine treatment.

—●—, vitamin D₃ fed rat; ---●---, vitamin D deficient rat. Data were the mean of 6 determinations.

RNA obtained from vitamin D₃ fed rats showed a little higher value than that of vitamin D deficient rats for about 2 hour period observed after uridine-5-³H administration.

2. Vitamin D₃ Effect on Uridine-5-³H Incorporation into Subcellular RNA Fractions of Rat Intestinal Mucosa

Uridine-5-³H incorporation into intestinal mucosal RNA was slightly increased at 1 hour after vitamin D₃ treatment. This result would be ascribed to the observation on whole intestinal mucosal RNA. Therefore, vitamin D₃ effect was further investigated on the uridine-5-³H incorporation into subcellular RNA fractions (Table 1). Rats were received uridine-5-³H (50 μ Ci/50g) at 1 hour after vitamin D₃ administration, and then sacrificed at 15 minutes (early stage of incorporation) or 2 hours (steady stage of incorporation) after uridine administration.

In the early stage of uridine-5-³H incorporation into RNA, nuclear RNA was labeled primarily, but mitochondrial, microsomal and supernatant RNA were not so much labeled

TABLE 1
Vitamin D₃ effect on uridine-5-³H incorporation into subcellular RNA of rat intestine

fraction	rat		D deficient		D ₃ fed	
	15 minutes		2 hours		15 minutes	
Homogenate	235 ± 26	289 ± 23	259 ± 22	311 ± 18	163 ± 12	525 ± 34
Mitochondria	140 ± 8	452 ± 36	79 ± 10	235 ± 10	401 ± 33	155 ± 12
Microsome	64 ± 8	323 ± 30	97 ± 9	356 ± 31	140 ± 12	455 ± 23
Nuclei	278 ± 4	97 ± 9	401 ± 33	155 ± 12	140 ± 12	455 ± 23
Supernatant	77 ± 3	356 ± 31	140 ± 12	455 ± 23	140 ± 12	455 ± 23

cpm/mg RNA ± S.E.M. (6 determinations)

2,000 I.U. vitamin D₃ was administered orally 1 hour before uridine injection, and 50 μ Ci uridine-5-³H intraperitoneally per 50 g body weight 15 minutes or 2 hours before sacrifice.

as nuclear RNA. The specific activity of nuclear and supernatant RNA was enhanced significantly by vitamin D₃ treatment, but that of mitochondrial and microsomal RNA was not influenced.

On the other hand, in the steady stage, uridine-5-³H incorporation into nuclear RNA was decreased, and that of mitochondrial, microsomal and supernatant RNA was increased. These results would be coincided with the evidence for RNA transport from nucleus to the cytoplasm in mammalian cells (17-19). The specific activity of nuclear, mitochondrial and supernatant RNA was enhanced by vitamin D₃ administration, but that of microsomal RNA was rather depressed.

3. Vitamin D₃ Effect on Uridine-5-³H Incorporation into the RNA Fractionated by Methylated Albumin-Kieselguhr Column

Vitamin D₃ effect on the subcellular RNA fractions was rather complicated in the steady stage of uridine-5-³H incorporation than in the early stage. The intestinal mucosal RNA was further fractionated by methylated albumin-kieselguhr column to identify subcellular RNA and to know the precise mechanism of vitamin D₃.

The elution pattern of intestinal mucosal RNA showed three peaks which were designated as peak I, II and III according to the elution orders (Fig. 3). Peak I and III were identified as sRNA and rRNA respectively using authentic sRNA and rRNA, but peak II

FIG. 3 *Methyalted albumin-kieselguhr column chromatography of intestinal RNA obtained from vitamin D deficient (A) and vitamin D₃ fed rats (B)*

2,000 I.U. vitamin D₃ was administered orally 3 hours before sacrifice. About 5 mg RNA was applied to the column (20×155 mm), and eluted with a linear concentration gradient of NaCl ranging from 0.4 M (400 ml) to 1.6 M (400ml). Optical density (260 mμ) was recorded by the Uvicon.

could not be identified. In nuclear RNA, three peaks were also observed. Peak I was found in supernatant RNA and peak III was in microsomal RNA.

The elution pattern of intestinal RNA obtained from the methyalted albumin-kieselguhr

column was not influenced by the vitamin D₃ treatment. However, uridine-5-³H incorporation into the RNA fractionated by methyalted albumin-kieselguhr column was influenced by vitamin D₃ administration. The specific activity of peak I and II were increased and that of peak III was decreased by vitamin D₃ (Table 2). The increase of specific activity in the intestinal supernatant of vitamin D₃ fed rat would be resulted from sRNA and the decrease in microsome of vitamin D₃ fed group would be resulted from rRNA.

4. Vitamin D₃ Effect on the DNA dependent RNA Polymerase

The most remarkable effect of vitamin D₃ was observed in nuclear RNA at 15 minutes and 2 hours after uridine-5-³H administration. Therefore, the vitamin D₃ effect on nuclear RNA synthesis was studied with isolated nuclei

TABLE 2
Uridine-5-³H incorporation into the RNA fractionated by methyalted albumin-kieselguhr column

RNA fractions	D deficient	D ₃ fed
I	856 ± 52	1,194 ± 102
II	1,439 ± 101	2,349 ± 160
III	863 ± 88	532 ± 46

cpm/mg RNA ± S.E.M. (6 determinations)

2,000 I.U. vitamin D₃ was administered orally 3 hours before sacrifice. 200 μCi uridine-5-³H was administered intraperitoneally per 50 g of rat 2 hours before being killed.

FIG. 4 Vitamin D₃ effect on the DNA dependent RNA polymerase activities

2,000 I.U. vitamin D₃ was administered orally 1 hour before decapitation. The activity was expressed as p moles of ³H-UTP (50 μCi/μmoles) incorporated into RNA per mg of DNA.

(A), Mg²⁺-activated RNA polymerase; (B), Mn²⁺ and (NH₄)₂SO₄-activated RNA polymerase.

TABLE 3

Vitamin D₃ effect on DNA dependent RNA polymerase activities in isolated rat intestinal nuclei

polymerase	rat D deficient	D ₃ fed		
		1 hour	3 hours	16 hours
Mg ²⁺ -activated RNA polymerase	483 ± 32	518 ± 41	399 ± 21	241 ± 18
Mn ²⁺ and (NH ₄) ₂ - SO ₄ -activated RNA polymerase	553 ± 37	606 ± 45	630 ± 42	704 ± 33

p moles/mg DNA ± S.E.M. (6 determinations)

2,000 I.U. vitamin D₃ was administered orally 1, 3 and 16 hours before decapitation. The activity was expressed as p moles of ³H-UTP (50 μCi/μ moles) incorporated into RNA per mg of DNA. Incubation was at 37° for 45 minutes.

to obtain further informations.

DNA dependent RNA polymerase activity in isolated nuclei was observed in the presence of either Mg²⁺ or Mn²⁺ and (NH₄)₂SO₄ (Fig. 4). Mg²⁺-activated DNA dependent RNA polymerase activity was not influenced at 1 hour after vitamin D₃ administration. However, it was depressed at 3 hours after vitamin D₃ administration. This action of vitamin D₃ on the Mg²⁺-activated DNA dependent RNA polymerase was further emphasized at 16 hours after vitamin D₃ treatment (Table 3). On the other hand, Mn²⁺ and (NH₄)₂SO₄-activated DNA dependent RNA polymerase was not influenced by vitamin D₃.

Observation on RNA polymerase at 3 hours after vitamin D₃ administration will correspond with the experiment of uridine-5-³H incorporation at 2 hours following 1 hour after vitamin D₃ administration (steady stage of incorporation). It is reported that Mg²⁺-activated DNA dependent RNA polymerase is located in nucleoli and produces rRNA, Mn²⁺ and (NH₄)₂SO₄-activated DNA dependent RNA polymerase is in ex-nucleoli and produces DNA like RNA (20). From these results, decrease in rRNA synthesis by vitamin D₃ would be due to the decreased activity of Mg²⁺-activated DNA dependent RNA polymerase.

DISCUSSION

The intestinal calcium absorption stimulated by vitamin D₃ was reduced with the administration of actinomycin D (2,3). Vitamin D₃ has been reported to stimulate the synthesis of calcium binding protein (5-7). Thus vitamin D₃ would be expected to control the RNA synthesis.

In the observation for vitamin D₃ effect on uridine-5-³H incorporation into mucosal RNA it was slightly increased at 1 hour after the vitamin D₃ administration. On the other hand, uridine-5-³H incorporation into mucosal RNA showed rapid increase, which reached maximum at 1 hour and kept its level at least for 1 hour.

From these results, vitamin D₃ effect on

uridine incorporation into subcellular RNA was observed in the early stage and steady stage of uridine-5-³H incorporation at 1 hour after vitamin D₃ administration. In the early stage, vitamin D₃ stimulated the uridine-5-³H incorporation into nuclear RNA. The same result was reported by Lawson (21) on the incorporation of orotic acid-5-³H into chick intestinal nuclear RNA at 20 minutes after vitamin D₃ injection. In this experiment, orotic acid-5-³H was injected 10 minutes before sacrifice. Stimulation of nuclear RNA synthesis will induce calcium binding protein and the incorporation of uridine-5-³H into supernatant RNA would be also increased. In our experiments supernatant RNA was identified as sRNA by methylated albumin-kieselguhr column. Remarkable effect of vitamin D₃ was not observed on the uridine-5-³H incorporation into microsomal and mitochondrial RNA.

In the steady stage, vitamin D₃ stimulated RNA synthesis in nuclei, mitochondria and cytoplasm. However, RNA synthesis in microsome was depressed by vitamin D₃ and this RNA was identified as rRNA by methylated albumin-kieselguhr column. Similar preferential inhibition of the synthesis of rRNA has been reported in rat thymus cell treated with prednisolone (22). Physiological significance of this decreased RNA synthesis will be investigated in following papers.

On the other hand, DNA dependent RNA polymerase activities were observed in the isolated nuclei to know the vitamin D₃ effect on nuclear RNA synthesis. The observation on RNA polymerase activities at 1 or 3 hours after vitamin D₃ administration corresponds with the observation of early stage or steady stage of uridine-5-³H incorporation. Mg²⁺-activated DNA dependent RNA polymerase activity was decreased at 3 hours after vitamin D₃ administration, and it was manifest at 16 hours. Mg²⁺-activated RNA polymerase is known to synthesize the rRNA (20). Therefore, the decreases of Mg²⁺-activated DNA dependent RNA polymerase activity will influence the uridine-5-³H incorporation into microsomal RNA in the steady stage. On the other hand, no change was found on the

activity of Mn²⁺ and (NH₄)₂SO₄-activated DNA dependent RNA polymerase. Lawson *et al.* (21) have reported that the activities of both enzymes were slightly decreased by vitamin D₃ administration. However, they observed the enzyme activities at 5 hours after 2.5 μg vitamin D₃ or 1 hour after 125 μg vitamin D₃ administration. The enzyme activities were observed in the experimental conditions of long period after small doses of vitamin D₃ administration and short period after large doses of vitamin D₃ administration.

In the present time, it will be considered that nuclear RNA synthesis was stimulated by vitamin D₃ in the early stage. This would be primary action of vitamin D₃ and then induce the calcium binding protein. The secondary action of vitamin D₃ will be observed in the steady stage. Decreases of uridine-5-³H incorporation into microsomal RNA and Mg²⁺-activated DNA dependent RNA polymerase activity will be derived from the secondary action of vitamin D₃. Induction of calcium binding protein by vitamin D₃ administration will increase calcium content in nuclei, and calcium ion will compete with magnesium ion in a site of the template. From these results, Mg²⁺-activated DNA dependent RNA polymerase was decreased with vitamin D₃ in the steady stage. Several regulatory steps will be involved in the process of RNA synthesis to cause the synthesis of calcium binding protein. Vitamin D₃ action on the subcellular membrane should be also considered.

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