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The Effect of Excess Dietary Vitamin A on Vitamin K-dependent Carboxylation in Rat Liver Microsomes

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ABSTRACT

The rate of vitamin K-dependent carboxylation of endogenous liver microsomal proteins and an exogenous peptide substrate for carboxylase were measured to test the effects of excess vitamin A on vitamin K function in rats. *In vitro* vitamin A incubation in normal rat microsomes of vitamin K-sufficient rats did not influence the carboxylation rates of either endogenous prothrombin precursors or a peptide substrate added. Similarly, vitamin A incubation in microsomes from control and excess vitamin A-fed rats that were on vitamin K-free diet did not change the rate significantly within the respective groups ; however, the rates of endogenous protein carboxylation from excess vitamin A-fed rats tended to be increased by the *in vitro* vitamin A addition compared to that of control rats. Excess vitamin A-fed rats had 2- to 3-fold higher carboxylase activities of endogenous protein carboxylation either with or without the *in vitro* vitamin A incubation than did control rats. In an *in vivo* study, carboxylase activities with an added exogenous peptide substrate were not influenced by excess intake of vitamin A. However, the endogenous protein carboxylation rate was about 2- to 3- fold higher for excess vitamin A-fed rats than for control rats. Carboxylase activities tended to be increased in both control and excess vitamin A-fed rats as the study progressed . The effect of excess amounts of vitamin A on endogenous protein carboxylation appeared as early as one week post-initiation of the diet. The result of this study indicate that excess vitamin A produces toxic effect rapidly, and that excess dietary vitamin A increase the rate of carboxylation of endogenous protein, mainly prothrombin precursors, which is an indication of vitamin K deficiency.

KEY WORDS : Vitamin A · Vitamin K · Vitamin K-dependent carboxylase activity.

Introduction

Hypervitaminosis A, as a result of an intake of excess vitamin A over a period of time, leads to hemorrhages and hypoprothrombinemia[1]. However, supplementation with additional vitamin K can counteract these symptoms[2]. Wostmann and Knight[3] demonstrated an antagonistic effect between vitamin A and K in the germfree rat. An excess

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dietary supplementation of vitamin A produced a hemorrhagic syndrome which is a typical vitamin K deficiency symptom. Matschiner et al[4)5)]. observed that excess vitamin A induced vitamin K deficiency as measured by prothrombin concentration in the blood. A preliminary study conducted in our laboratory revealed that vitamin K was capable of alleviating *in vitro* vitamin A-induced hemolysis. Other investigators[6)7)] also found that hypervitaminosis A and E caused prolonged prothrombin time. March et al.[7)] observed that hypoprothrombinemia induced by vitamin E was rapidly reversed by vitamin K injection in chicks, indicating an increased requirement of vitamin K in the presence of high amounts of vitamin E.

Recently, above normal levels of vitamin A therapy for prevention and/or treatment of cancer has been tested and proven effective, and megadoses of vitamin A are being used for treatment of dermatological conditions. Additionally, anticoagulant therapy for coronary diseases is commonly used which causes an increase in abnormal prothrombin (des−γ−carboxy prothrombin) levels as in vitamin K deficiency states[8)9)]. Therefore , megadoses of vitamin A and anticoagulant therapy may influence vitamin K status and further its requirement.

Vitamin A has been suggested to have an effect on membranes[10)]. Megadoses of vitamin A cause damage in plasma membranes as well as other membranes of intercellular organelles[11)12)]. This could be inhibited by a number of isoprenoid compounds such as vitamin E and K[10)] which suggests that there may be some interactions among fat-soluble vitamins.

This report describes experiments testing the effects of excess vitamin A on the vitamin K-dependent incorporation of $H^{14}CO_3$ into the endogenous microsomal prothrombin precursor and into an exogenous peptide substrate. Since substantial amounts of vitamin K-dependent carboxylase are located in rough endoplasmic reticulum of liver[13)] we used rat liver microsomes in our study to determine if excess dietary vitamin A influences solubilization of vitamin K-dependent carboxylase by measuring carboxylation of the exogenous substrate (the pentapeptide, Phe−Leu−Glu−Glu−Leu). Also carboxylase activity of endogenous protein precursors was measured to determine the effects of hypervitaminosis A on prothrombin precursor levels.

Materials and Methods

Chemicals : $NaH^{14}CO_3$ (57mCi/mmol) and NCS tissue solubilizer were purchased from Amersham (Arlington Heights, IL). Vitamin K_1 was purchased from Sigma(St. Louis, MO) and reduced to vitamin K_1 hydroquinone as described by Sadowski et al.[14)]. The pentapeptide, Phe−Leu−Glu−Glu−Leu was obtained from Vega-Fox Biochemicals(Tucson, AZ) and dithiothreitol from Calbiochem(San Diego, CA). All other chemicals were of analytical reagent grade.

Experimental diets : Control diet, prepared as designated by the American Institute of Nutrition (AIN−76A , Teklad, Madison, WI) contained 4000 IU vitamin A/kg diet in the form of retinyl palmitate. For excess vitamin A diet, 100 times more retinyl palmitate was added to the control diet. Vitamin K-free diets were prepared by excluding vitamin K from the respective diet.

Treatment of animals : Male Sprague-Dawley rats obtained from Harlin Industries, Inc.(Indianapolis, IN) were housed individually in open mesh wire bottom cages that prevented coprophagy in a room thermostatically maintained at 21℃ and 40% relative humidity with alternating 12 hour periods of light and dark.

Experimental design : For the *in vitro* study, rats were fed regular rat chow and fasted 18 hours prior to sacrifice. Rat liver microsomal suspension were incubated with or without retinol and the carboxylase activities were measured. And also , rats fed either control or excess vitamin A containing diets for 4-8 weeks were provided vitamin K-free diet for 8 days , and treated the same manner as above.

For the *in vivo* study, rats were fed either control or excess vitamin A containing diet for a designated period until 8 days (except period I) before the respective assay at which time they were fed the vitamin K-free diet. Then the rats were fasted 18-21 hours prior to the assay. Experimental periods were designed as follows : period I, 1-7 days post-initiation on experimental diet : period II, 8-21 days : period III, 22-35 days : period IV, 36-49 days : period V, 50-63 days : period VI, 64-77 days (Table 1).

Sample preparation : Livers of fasted rats were homogenized in two parts (w/v) of cold buffer containing 0.25 M sucrose, 0.025 M imidazole, pH 7.2 (buffer A) and centrifuged at 10,000×g for 10 minutes to obtain the postmitochondrial supernatant. Microsomal pellets were prepared from centrifugation of postmitochondrial supernatant at 105,000×g for 60 minutes in an ultracentrifuge (Model L5-50, SW 41 rotor, Beckman Instruments, Palo Alto, CA). The pellets were surface washed and resuspended to an equivalent volume of postmitochondrial supernatant with 0.25 M sucrose, 0.025 M imidazole, 0.5 M KCl, 1.0 mM dithiothreitol(buffer B) by a loose fitting Dounce homogenizer.

Vitamin A incubation : Retinol(final concentration 0.05μM) was added to the microsomal suspension and incubated at room temperature for 20 minutes prior to initiation of the carboxylase assay for determining the *in vitro* effect of vitamin A. In this study, 0.05μM of retinol was used since a concentration curve showed it to be the optimum concentration for carboxylation of both endogenous proteins and the peptide substrates.

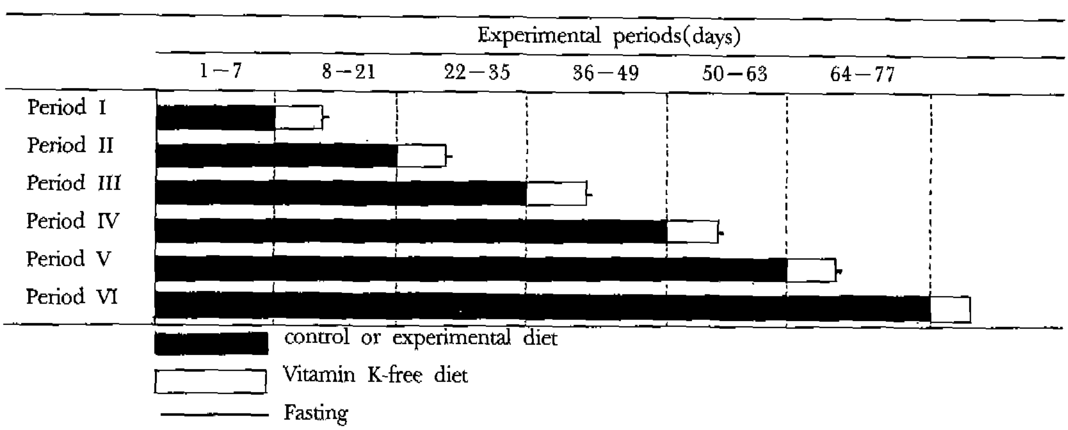
Carboxylase assays : The microsomal preparation (0.4 ml) was incubated with 50μl of 5 mM Phe−Leu−Glu−Glu−Leu , 5μl of 1 mCi/ml $NaH^{14}CO_3$, and 10μl of vitamin K_1 hydroquinone in ethanol at 17℃ for 30 minutes[13]. Then 1.0 ml of 10%

Table 1. Experimental design

In vitro study

Diet	Incubation
Regular rat chow	−vitamin A +vitamin A
Control diet	
Excess vitamin A	−vitamin A +vitamin A

In vivo study



ice-cold trichloroacetic acid(TCA) was added to 0.2 ml of the incubation mixture, and then centrifuged to obtain the TCA-precipitated proteins. The TCA-precipitated pellets were washed and dissolved in 0.7 ml of NCS and gassed with CO_2 for 6 minutes. The supernatants obtained from the TCA-precipitation were saved and also gassed as above. Then 0.4ml of the gassed sample was transferred to scintillation vials containing 12ml of scintillation fluid (ACS). Radioactivity of both TCA-treated supernatants and pellets were determined in a liquid scintillation counter(Model LS-1800, Beckman Instruments, Palo Alto, CA).

Protein assay : Protein content in the microsome was determined by the procedures of Lowry , et al.[15].

Statistical Analysis : The one way analysis of variance test was used to determine significant differences among group mean for each parameter investigated. The group mean were further tested by a t-test to determine differences($p<0.05$) between individual means.

Results

In vitro carboxylase activities of endogenous protein precursors and exogenous pentapeptide substrates are presented in Table 2. Carboxylation of precursor protein which are mainly prothrombin precursors in vitamin A-incubated liver microsomes tended to be higher than the ones without vitamin A, but the difference was not statistically significant. Exogenous carboxylation also showed a similar trend with no statistical significance.

However , marked differences ($P<0.05$) in endogenous carboxylation were observed between control and excess vitamin A-fed rats when both groups were incubated with vitamin A *in vitro* (Table 3). The values for rats fed excess vitamin A were almost triple those of the control rats. Although vitamin A incubation did not influence the carboxylation rates significantly within respective groups, vitamin A excess rats appeared to be more susceptible to vitamin A incubation than did the control rats. Exogenous carboxylation was not influenced by vitamin A incubation.

Results of an *in vivo* study of vitamin K-dependent carboxylase activity are shown in Table 4 and Fig. 1. Carboxylase activities of the both control and excess vitamin A rats were not different during period I(1 to 7 days post-initiation of the experimental diet). However, there were significant differences between the activities of the two groups from period II to VI($P<0.05$). Carboxylation rates increased as the study progressed in the both groups as can be seen in Fig. 1 ; however, this was more obvious in the excess vitamin A rats than in the control rats. Carboxylase activities within each group were increased between period I and II and

Table 2. *In vitro* effects of vitamin K-dependent carboxylase activity of endogenous proteins and a peptide substrate in microsomes from rats fed vitamin K-containing diets

Substrate	Carboxylase Activity		P
	−Vitamin A**	+Vitamin A***	
	(d pm/30 min/mg protein)		
Endogenous proteins	485± 96*	706± 141	N.S.
Peptide substrate	2426± 171	2967± 372	N.S.

*Mean± SE of 4 rats per group

**Incubated with 10μl of ethanol at room temperature for 20min

***Incubated with 10μl of 2.5μM retinol(final conc. 0.05 μM) at room temperature for 20min

Table 3. In vitro effects of vitamin A incubation on vitamin K-dependent carboxylase activity in microsomes from control and excess vitamin A-fed rats
(Respective diets were vitamin K-free for 7 days prior to the assay)

Group	Endogenous protein		P	peptide substrate		P
	−Vitamin A	+Vitamin A**		−Vitamin A	+Vitamin A**	
	(dpm/30 min/mg protein)					
Control	403± 60*	367± 48	N.S.	1490± 132	1497± 157	N.S.
Excess vitamin A	778± 149	1000± 169	N.S.	1406± 108	1630± 288	N.S.
P	0.05	0.05		N.S.	N.S.	

*Mean± SE of 4 rats per group
**Incubated with 10μl of 2.5 μM retinol(final conc. 0.05 μM) at room temperature for 20min

Table 4. The *in vivo* study on vitamin K-dependent carboxylation of endogenous proteins and peptide substrate in microsomes from control and excess vitamin A-fed rats

Experimental period*	Endogenous protein		P	Peptide substrate		P
	Control	Excess Vitamin A		Control	Excess Vitamin A	
	(dpm/30 min/mg protein)					
I	183± 17**	307± 80	N.S.	1178± 55	1321± 66	N.S.
II	234± 14	741± 39	0.05	1280± 94	1280± 118	N.S.
III	217± 39	713± 44	0.05	1263± 154	1214± 105	N.S.
IV	263± 24	736± 77	0.05	1126± 41	1127± 72	N.S.
V	388± 57	920± 57	0.05	1364± 41	1127± 72	N.S.
VI	464± 63	1052± 57	0.05	1311± 29	1323± 106	N.S.
Average	297± 26**	774± 53	0.05	1250± 35	1279± 40	N.S.

*Period I, 1~7 days post-initiation on experimental diet ; Period II, 8~21 days ; Period III, 22~35 days ; Period IV, 36~49 days ; Period V, 50~63 days ; Period VI, 64~77 days
**Mean± SE
N.S., Not significant

thereafter stabilized until period IV and again increased between period V and VI. Vitamin A excess rats had approximately 2− to 3− fold higher carboxylase activity than did the control rats. Total values of all periods were about 2.5 times greater in excess vitamin A rats . Exogenous carboxylation, using a peptide substrate, did not show significant differences in any of the experimental periods.

Discussion

Membrane effects of vitamin A have been suggested by many investigators for decades . Membrane microviscosity was reduced by half at a concentration of 10^{-5} M or lower of retionic acid which was even more potent than Triton X-100, sodium dodecyl sulfate or lysophosphatidylcholine[10]. White [16] suggested that chronic oral ingestion of vitamin A may cause normochromic macrocytic anemia in humans, probably due to its direct effect on the lipoprotein membrane of the mature erythrocytes. A former study in our laboratory[17] demonstrated that incubation with 0.2μM retinol stimulated rat liver microsomal NADH-cytochrome c reductase, an intrinsic membrane enzyme with a non-luminal orientation. Since the active site of vitamin K-dependent carboxylase is thought to be accessible only from the microsomal lumen[13], vitamin A may influence the activity by penetrating the lipid bilayer of the microsomal membrane as detergents would.

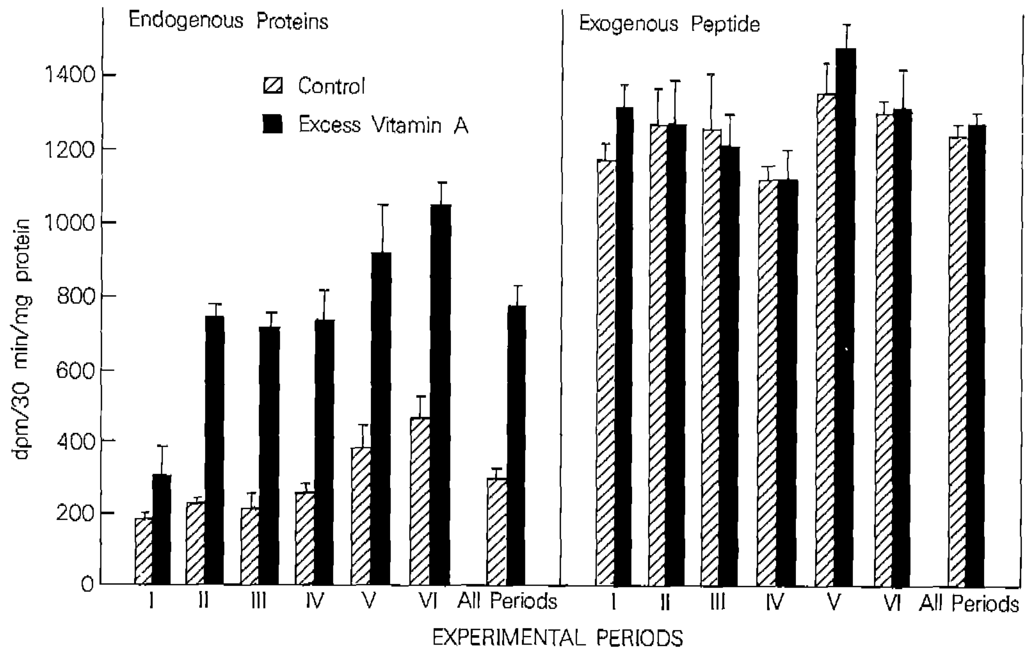


Fig. 1. Carboxylation rates of endogenous proteins and an exogenous peptide substrate in vitamin K-deficient rats.

However, in this study, exogenous carboxylation of the peptide substrate with vitamin A incubation was slighly higher but the differences between the two groups were not significant probably due to large variations in individual rats.

Similar effects on peptide carboxylation were observed *in vivo* as well as when microsomes of either control or vitamin A excess rats were incubated *in vitro* with excess amounts of vitamin A . Although not significant, the magnitude of the differences were much smaller in the *in vivo* study than those in the *in vitro* study. These results could be explained by a competitive effect between peptide substrate and endogenous protein substrate for vitamin K-dependent carboxylase . Kappel and Olson[18)19)] already reported that the presence of endogenous microsomal proteins caused an initial lag in synthetic pentapeptide carboxylation , and preincubation of microsomes prior to peptide addition showed no such lag phase since protein substrates were already carboxylated during the preincubation period. The result in our study may be due also to a higher carboxylation rate of endogenous proteins in excess vitamin A-fed rats. This may indicate that excess vitamin A-fed rats had higher content of endogenous protein substrates which were carboxylated in preference to peptide substrates added since those microsomes were not preincubated in the present study. Therefore, the carboxylation rates of peptide substrates for excess vitamin A groups were not significantly higher than the ones for control groups as expected.

Much greater carboxylation of endogenous proteins were observed in excess vitamin A-fed rats with or without vitamin A incubation. Vitamin A incubation did not increase the carboxylation rates within the respective groups. Although it was not significant, it appeared that excess vitamin A-fed rats were more susceptible to the vitamin addition. Higher carboxylation rates were also observed in the *in vivo* study during all periods except period I. This indicated that vitamin A may be toxic as early as one week in young postweanling rats.

Earlier studies reported that normal rat liver was lower in prothrombin precursors than that of rats fed vitamin K-deficient diet for 1 week[20]. Moreover, anti-vitamin K drugs (i.e., sodium warfarin) caused an accumlation of endogenous substrates for carboxylase[8)9)]. Other studies[8)21)22)] reported that vitamin K-dependent carboxylase activity in the liver was inhibited in vitamin K dificiency or by administration of vitamin K antagonists, and abnormal prothrombin which is a noncarboxylated protein was released into the blood. This abnormal prothrombin is not a normal component of blood since it is undetectable in healthy subjects who are not deficient in vitamin K. Therefore, results of the present study may be explained by the possibility that excess vitamin A ingestion produces more endogenous protein substrates for carboxylase, an indication of vitamin K deficiency. This observation may be due to inhibition of vitamin K absorption by excess intake of vitamin A as described by Matschiner et al.[5]. In their study, vitamin K requirement was increased to that of germfree rats and fecal vitamin K was almost doubled when rats were fed 50 IU of retinoic acid/g of diet.

Another explanation for the vitamin A-induced changes observed in our study may be that lysosomal enzymes are released under excess vitamin A administration. This may influence the change in the properties of membranes that could stimulate protein synthesis[23)24)]. Stimulation of intercellular synthesis of fibronectin by retinoic acid has been demonstrated already by Bolmer and Wolf[25)26)]. In this respect, the increased endogenous protein carboxylation observed in excess vitamin A-fed rats in the present study may be due partly to the toxic effect of vitamin A on membrane and protein synthesis.

An interesting observation made in our study was that carboxylase activity was increased as the experiment progressed, not only in the excess vitamin A rats but also in the control rats. Mean values for period VI of both control and excess vitamin A groups were about 2~3 times greater than those for period I.

This could be explained by the observations made by Olson et al.[27] that vitamin A concentration in the liver had a tendency to be increased with age in American children who died of various causes. Its concentration was low until 3 months, increased rapidly up to 4 years, and then remained constant throughout adolescence . If this is true in rats also, vitamin A accumluation in the liver of control diet-fed rats is likely which, in turn, is likely to influence vitamin K-dependent carboxylation.

The present study showed that excess vitamin A, indeed, did affect vitamin K-dependent carboxylation. However, since it is unclear what influences excess vitamin A has on the vitamin K-dependent carboxylase system this mechanism requires further study. The following questions may be asked. Are the changes observed with excess vitamin A due to inhibition of vitamin K absorption or is the effect at the membrane level or might it be both ? Since megadoses of vitamin A are being used therapeutically in the treatment of some dermatological lesions, and anticoagulant therapy for coronary heart diseases is rather common, more attention should be given to the vitamin K nutritional status when it is stressed by the use of high amounts of vitamin A .

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비타민 A 과량 섭취가 흰쥐의 간 Microsome의 비타민 K-dependent Carboxylation에 미치는 영향

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본 연구에서는 비타민 A 과량 섭취가 비타민 K 기능에 미치는 영향을 알아보기 위하여 carboxylase의 기질인 쥐의 간내 microsome 단백질과 첨가된 펩티드의 vitamin K-dependent carboxylation rate를 측정 하였다. *In vitro* 실험에서는 정상 vit.A 섭취군의 간 microsome을 vit.A 로 incubation했을 때 간 내 prothrombin 선구 물질이나 첨가된 peptide기질의 carboxylation rate는 영향을 받지 않았다. 이와 비슷한 양상으로서 무비타민 K 식이와 함께 비타민 A를 정상수준 혹은 과잉 수준으로 섭취한 쥐를 비교한 경우에는 동일군 간에는 carboxylation rate에 유의한 차이를 나타내지 않았다.그러나 비타민 A 과잉군의 간내 단백질의 carboxylation rate 는 대조군에 비하여 증가하는 경향이었다. 비타민 A 과잉군은 비타민 A로 incubate한 경우나 하지 않은 경우 모두 대조군에 비하여 약 2~3 배 의 carboxylase 활성을 보였다. *In vivo* study 에서는 첨가된 peptide에 대한 carboxylase활성은 비타민 A 과잉 섭취에 의하여 영향을 받지 않았다. 그러나 간 내 단백질의 carboxylation rate는 비타민 A 과잉군이 대조군에 비하여 2~3 배나 더 높았다. Carboxylase 활성은 대조군이나 비타민 A 과잉군 모두 연구기간이 진행될수록 더 증가하였다. 그리고 간 내 단백질의 carboxylation에 대한 비타민 A 과잉 효과는 실험 식이를 시작한 후 일주일 정도에서 나타나기 시작하였다. 그러므로 이 연구 결과는 비타민 A 과잉 시에는 과잉증이 빠른 시일내에 일어나며, 비타민 A 과잉은 비타민 K 결핍의 지표인 prothrombin 의 선구물질을 증가시킨다는 것을 시사한다.