9-Cis Retinoic Acid Reduces 1α,25-Dihydroxycholecalciferol-Induced Renal Calcification by Altering Vitamin K-Dependent γ-Carboxylation of Matrix γ-Carboxyglutamic Acid Protein in A/J Male Mice1,2

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Abstract

Matrix γ-carboxyglutamic acid protein (MGP), a vitamin K-dependent protein, is involved in regulation of tissue calcification. We previously reported that 9-cis retinoic acid (RA) mitigates 1α,25-dihydroxycholecalciferol [1,25(OH)2D3]-induced renal calcification in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung cancer A/J male mouse model. This raised the question if the mechanism(s) underlying this calcification involves vitamin K. We assessed expression and vitamin K-dependent γ-carboxylation of MGP and vitamin K concentrations [phylloquinone (PK), as well as its conversion product, menaquinone-4 (MK-4)] in tissues obtained from NNK-injected A/J male mice fed 1,25(OH)2D3 (2.5 μg/kg diet; D group) ± RA (15 mg/kg diet) for 20 wk. Renal calcification was only observed in the D group (2/10; 20% of the group). Renal MGP mRNA and uncarboxylated MGP (ucMGP) increased in response to D (P < 0.05) but not in response to RA or RA + D. In contrast, γ-carboxylated MGP increased to 2.2-fold of the control in response to D + RA (P < 0.05) but not in response to RA or D alone. Although all diets contained equal amounts of PK, the kidney MK-4 concentration was higher in the D group (P < 0.05) and lower in the RA group (P < 0.05) compared with the RA + D or control groups. Renal PK concentrations were lower in the RA and RA + D groups than in the control and D groups (P < 0.05). These data suggest that 9-cis RA mitigated 1,25(OH)2D3-induced renal calcification by modifying the 1,25(OH)2D3-induced increase in ucMGP. The mechanisms by which 9-cis RA and 1,25(OH)2D3 alter vitamin K concentrations warrant further investigation. J. Nutr. 138: 2337–2341, 2008.

Introduction

The vitamin K-dependent protein, matrix γ-carboxyglutamic acid protein (MGP),3 plays a role in the regulation of calcification in cartilage and soft tissue (1–3). The function of MGP depends on a post-translational modification of specific glutamate residues that are converted into γ-carboxyglutamic acid (Gla) residues by a vitamin K-dependent carboxylase (4). MGP may be an inhibitor of vascular calcification. Evidence for this stems from its calcium-binding Gla motif and resulting inhibition of cartilage calcification (5,6). MGP in γ-carboxylated form (cMGP) is present in normal arteries, whereas plaques with calcification have uncarboxylated forms of MGP (ucMGP) (2).

Both vitamins A and D influence expression and/or synthesis of MGP (7,8). There is some suggestion that vitamin D has a direct effect on the vitamin K-dependent γ-carboxylase system (7,9). 9-cis Retinoic acid (RA) is a ligand for RA receptors and retinoid X receptors (RXR) and can induce vitamin K-dependent MGP mRNA in human cells, including osteoblasts, articular cartilage chondrocytes, and fibroblasts (8). In contrast, RA functions as an inhibitor of MGP gene expression in rat renal and human breast cancer cell lines (10), as well as in nonmammalian species (11).

Previously, we demonstrated that both 1α,25-dihydroxycholecalciferol [1,25(OH)2D3] and 9-cis RA inhibited lung carcinogenesis in tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-initiated A/J mice (12). Unexpectedly, the addition of 9-cis RA mitigated 1,25(OH)2D3-induced renal calcification (12). However, the mechanisms involved were unclear. Although it is biologically plausible that 9-cis RA and 1,25(OH)2D3 have roles in the expression and vitamin...
K-dependent γ-carboxylation of MGP, which would explain the differences in renal calcification among the different treatment groups, it is not known if these were the mechanisms involved in this rodent model of carcinogenesis. In the present study using archived samples from our earlier work, we hypothesized that the previously observed 1,25(OH)2D3-induced abnormal renal calcification in NNK-initiated A/J mice would be associated with elevated total MGP and ucMGP. Further, we hypothesized that the 9-cis RA would inhibit the 1,25(OH)2D3-elevated MGP, thereby preventing the renal calcification. Because the MGP requires vitamin K for γ-carboxylation, we examined the vitamin K concentrations [phylloquinone (PK) as well as its conversion product, menaquinone-4 (MK-4)] in the renal and hepatic tissues.

Materials and Methods

Animals. The results reported in the present study are based on analysis of archived samples obtained from a study designed to test for additive, synergistic, or antagonistic effects of 1,25(OH)2D3 in combination with 9-cis RA on lung tumor development, as previously described (12). This protocol was approved by the Human Nutrition Research Center on Aging Institutional Animal Care and User Committee. Briefly NNK, a tobacco-specific carcinogen, was used to induce tumors in A/J mice. Male A/J mice (6 wk old) were assigned to 4 experimental groups: the control group, the RA group, which was fed 15 mg 9-cis RA/kg diet, the D group, which was fed 2.5 μg 1,25(OH)2D3/kg diet (LKT Laboratories), and the RA + D group, which was fed 2.5 μg 1,25(OH)2D3/kg diet and 15 mg 9-cis RA/kg diet. All 4 groups received an intraperitoneal injection of 100 mg NNK/kg body weight, and were fed an AIN-93M powdered semipurified diet (Dyets) (13) for 20 wk. Mice were food-deprived overnight prior to terminal exsanguination under deep anesthesia. The liver and kidney were snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis. Archived samples were obtained from a previous study (12), so there were limited samples available for use in the present study. Specifically, there were only sufficient samples from 10 animals per group for measurements of mRNA in kidney and vitamin K concentrations in liver and kidney, and 3 animals per group for measurement of MGP in kidney.

Kidney calcification. Three independent researchers who were unaware of the treatment groups examined both kidneys for discoloration or irregular surface indicative of calcium deposits in the kidneys, and results are reported as both incidence and percentage of animals in each group with evidence of gross kidney calcification. Selected kidneys from each group were processed histologically and stained using hematoxylin-eosin and von Kossa techniques to confirm the presence or absence of kidney calcium deposits, as previously described (12).

MGP mRNA. Total RNA was isolated from mouse kidney tissue (80 mg) using TriPure Isolation Reagent (Roche Diagnostics) according to the manufacturer’s protocol. CDNA was prepared from the RNA samples using Moloney murine leukemia virus RT (Invitrogen) and an automated thermal cycler (MJ Research PTC-200, Bio-Rad Laboratories) and quantified using a fluorescence-based real-time detection method (ABI PRISM 7000 Sequence Detection system, Perkin-Elmer Applied Biosystems) using SYBR Green reagents (Invitrogen). Melting curves were analyzed following the cycling program to confirm the specificity of the reaction for the amplicon. For each sample and each gene, PCR was carried out in duplicate. Sequences for primers (Sigma-Genosys) are as follows: MGP forward primer, 5′-CGAATCTCAGAAGACATGGA; MGP reverse primer, 5′-ATGCCAGCCCTCTGTTGAG; β-actin forward primer, 5′-TACCTGAGCCAGAGATGCC; β-actin reverse primer, 5′-CCACAGGATTCGACAGAA.

MGP protein. Mouse kidney tissue (50 mg wet weight) was homogenized in whole cell extract buffer. The protein concentration of the cell lysate was determined with a Coomassie Plus protein assay (Bio-Rad). SDS-PAGE and immunoblotting were performed using 15% SDS-PAGE and polyvinylidene difluoride membrane. The membranes were blocked with 5% bovine serum albumin and successively incubated in buffers containing the affinity-purified rabbit uMGP antibody, cMGP antibody (14), and β-actin antibody (Sigma) overnight. The membranes were then incubated for 1 h at room temperature using horseradish peroxidase conjugated anti-rabbit secondary antibodies. The blots were visualized by enhanced chemiluminescence development using a western blotting detection system. Semi-quantitative analysis of MGP protein and normalization to β-actin were measured by densitometry analysis of enhanced chemiluminescence films scanned using a Bio-Rad GS-710 scanner. For each sample and each antibody, western blotting was conducted in duplicate.

Vitamin K analysis. Mouse liver and kidney (100 mg wet weight) from each animal were homogenized using a PowerGen homogenizer (Fisher Scientific) in PBS. The internal standard, K1(25), was used for kidney homogenate analysis. Liver contained interfering substances that coelute with K1(25), (15), so 2′,3′-dihydrovitamin K was used as an internal standard for liver homogenate analysis. Concentrations of 2 forms of vitamin K, PK and MK-4, were measured in homogenates by reversed-phase HPLC, as described elsewhere (15).

Statistical analysis. Data are reported as means ± SD. The main effects of RA and D intake, as well as the interaction between RA and D, on renal cMGP and ucMGP expression, were analyzed by 2-way ANOVA. Because there was an interaction between RA and D with respect to both renal cMGP and ucMGP expression (P < 0.03), we subsequently used a 1-way ANOVA with Tukey’s honestly significant difference for multiple comparisons to determine the effect of nutrient intake on the renal MGP mRNA, ucMGP, and cMGP expression and on the ratio of ucMGP:cMGP separately. For the analyses of tissue concentrations of vitamin K, a natural log transformation was applied prior to formal analyses to improve normality of skewed distributions. However, for the purposes of presentation, the data in Table 1 are untransformed. The main effect of nutrient intake on the tissue concentrations of total vitamin K, as well as for the individual forms of vitamin K (PK and MK-4), was also analyzed using a 1-way ANOVA with Tukey’s honestly significant difference for multiple comparisons. All analyses were carried out using SPSS v14.0. Significance was set at P < 0.05.

Results

Renal calcification. In the D group, 2 of the 10 mice had renal calcification (20%), whereas no mice in the control, RA, and RA + D groups had calcification. As previously reported (12), body weights did not differ among the groups at baseline and final body weight was not affected by the addition of RA, although it was lower in the D group (18.80 ± 2.76 g) than in the control (23.36 ± 1.98 g), RA (21.24 ± 2.64 g), and D + RA (22.14 ± 2.55 g) groups (P < 0.05).

<table>
<thead>
<tr>
<th>TABLE 1 Vitamin K concentrations in NNK-injected A/J male mice kidney and liver after supplementation of RA, D, or RA + D for 20 wk 1, 2</th>
<th>Kidney</th>
<th>Control</th>
<th>RA</th>
<th>D</th>
<th>RA + D</th>
<th>pmol/g wet tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK</td>
<td>17.2 ± 4.9</td>
<td>10.1 ± 3.9</td>
<td>37.8 ± 21.2</td>
<td>19.9 ± 6.7</td>
<td>31.6 ± 16.0</td>
<td>16.0 ± 8.3</td>
</tr>
<tr>
<td>PK</td>
<td>52.7 ± 32.2</td>
<td>8.7 ± 3.6</td>
<td>52.0 ± 31.6</td>
<td>16.0 ± 8.3</td>
<td>16.0 ± 8.3</td>
<td>16.0 ± 8.3</td>
</tr>
<tr>
<td>Total</td>
<td>70.0 ± 36.1</td>
<td>18.7 ± 5.1</td>
<td>89.8 ± 33.6</td>
<td>35.6 ± 14.6</td>
<td>35.6 ± 14.6</td>
<td>35.6 ± 14.6</td>
</tr>
<tr>
<td>Liver</td>
<td>MK</td>
<td>1.8 ± 3.1</td>
<td>ND 3</td>
<td>2.1 ± 3.7</td>
<td>0.7 ± 1.4</td>
<td>34.0 ± 7.1</td>
</tr>
<tr>
<td>PK</td>
<td>46.5 ± 10.2</td>
<td>13.7 ± 1.6</td>
<td>34.0 ± 7.1</td>
<td>16.1 ± 4.1</td>
<td>16.1 ± 4.1</td>
<td>16.1 ± 4.1</td>
</tr>
<tr>
<td>Total</td>
<td>48.3 ± 9.8</td>
<td>13.7 ± 1.6</td>
<td>36.1 ± 7.5</td>
<td>16.8 ± 4.1</td>
<td>16.8 ± 4.1</td>
<td>16.8 ± 4.1</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 10. Means in a row with superscripts without a common letter differ, P < 0.05.
2 ND, Values were below the minimum detectable concentration of 0.05 pmol/g wet tissue.

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MGP mRNA. There was an increase in the expression of MGP mRNA in the D group compared with the control \((P < 0.01)\) (Fig. 1). In contrast, there were no changes in MGP mRNA in the RA or the RA+D groups compared with the control.

UcMGP and cMGP. The ucMGP was higher \((P < 0.05)\) in the D group compared with the control, RA, and RA+D groups (Fig. 2). In contrast, the cMGP was significantly higher in the RA+D group compared with the other groups \((P < 0.05)\). The ucMGP/cMGP ratio was higher in the D group than in all other groups and was lower in the RA+D group than in all other groups other than RA \((P < 0.05)\).

Tissue concentrations of PK and MK-4. The AIN-93M powdered semipurified rodent diet contained 840 ± 15 μg PK/kg diet, but no menaquinones, as confirmed by direct analysis. No menadione was added to the diet. In kidney, MK-4 concentrations were higher in response to D and lower in response to RA compared with either the control group or the RA+D group \((P < 0.05)\) (Table 1). Liver MK-4 concentrations did not differ among the groups. The PK concentrations in liver and kidney were lower in the RA and RA+D groups than in the control and D groups \((P < 0.05)\).

Discussion

High doses of vitamin D cause hypercalcemia, hypercalciuria, and ectopic soft tissue calcification (16,17), although the mechanisms are unclear. In agreement with earlier studies (18,19), we previously reported that high doses of 1,25(OH)2D3 induced extensive renal calcification in NNK-injected A/J mice (1). In this study, we demonstrated that the 1,25(OH)2D3-induced renal calcification in the NNK-injected A/J mice was associated with elevated total and ucMGP in the kidney. MGP knockout mice have extensive arterial calcification, demonstrating that MGP acts as an inhibitor of calcification (1). The vitamin K-dependent Gla region in MGP is directly involved in a bone morphogenetic protein-2/MGP interaction, which inhibits vascular calcification (2,20,21). Therefore, we conclude that soft tissue calcification induced by 1,25(OH)2D3 increases ucMGP. Because the ucMGP cannot inhibit the progression of renal calcification, there is an increased incidence of renal calcification.

The 9-cis RA alone did not affect MGP gene expression or vitamin K-dependent γ-carboxylation of MGP. This is consistent with a previous study in human osteosarcoma cells, in which 9-cis RA did not modify the γ-carboxylation of a vitamin K-dependent protein (9). Our observation that 9-cis RA attenuated 1,25(OH)2D3-induced MGP expression in the kidney could be due to the interaction between the vitamin D receptor and RXR, because both are required for binding to vitamin D response element (VDRE) in the promoter region of target genes. Whereas 1,25(OH)2D3 dramatically enhances vitamin D receptor-RXR heterodimer interaction with the VDRE, 9-cis RA inhibits this association (22). Our data suggest that the possible antagonistic effect of 9-cis RA on the promoter of the MGP gene through the VDRE needs further study using in vitro approaches.

Another unexpected finding of this study was the influence of both 9-cis RA and 1,25(OH)2D3 on vitamin K tissue concentrations, suggestive of an interaction among fat-soluble vitamins A, D, and K. Because the diets were identical in PK concentrations across all treatment groups, we hypothesized that there would be equivalent PK and MK-4 concentrations in kidney and liver across all treatment groups. Furthermore, we projected that the increased MGP expression in response to 1,25(OH)2D3 suppleme-
mentation would result in more ucMGP, because there was not a concomitant increase in vitamin K intake and MGP requires vitamin K for γ-carboxylation. However, 1,25(OH)2D3 increased the conversion of PK to MK-4 as suggested by the decrease in PK in the liver and an increase in renal MK-4. The PK in the kidney remained unchanged. It is unclear how PK is converted to MK-4. One possible route is through the desaturation of the phytyl side chain to produce the geranygeranyl group of MK-4. Another possible route is the removal of the phytyl side chain to release menadione, which is subsequently prenylated (23–25). Although we are not aware of previous in vivo studies that report a 1,25(OH)2D3-induced, tissue-specific conversion of one form of vitamin K to another, renal vitamin K-dependent γ-carboxylyase activity is stimulated by 1,25(OH)2D3 in vitro and in vivo (26,27). One of the products of this γ-carboxylyase activity, vitamin K epoxide, has been identified as critical in this conversion process (24,25). Alternatively, it is plausible that 1,25(OH)2D3 increases synthesis of currently unidentified enzymes involved in this conversion. Although the mechanism by which PK is converted to MK-4 is unknown, MK-4 has been reported to have unique roles based on in vitro and in vivo studies. For example, MK-4 is more effective than PK in preventing calcification induced by treatment with vitamin K antagonists (2). Moreover, the anticancer and antioxidant activity of MK-4 is more effective than PK in hepato-cellular carcinoma, leukemia, lung, colon, and gastric cancer cell lines (28,29). It is not known if 1,25(OH)2D3 would have an impact on these functions should it increase the final concentrations of MK-4.

In this study, there were consistently lower PK and MK-4 concentrations in response to the addition of 9-cis RA. It has been previously reported that rats given an excess of vitamin A have decreased blood clotting, which may be prevented by administration of vitamin K. In contrast, no adverse effects of excessive amounts of vitamin A on vitamin K-dependent blood coagulation were reported in an avian species (30). Our study design precludes speculation on the actual mechanism of antagonism, although similar antagonistic effects on vitamin K concentrations have been reported in response to vitamin E supplementation (31).

One major limitation of this study was the use of a carcinogen-injected mouse to study the interrelationships between 1,25(OH)2D3, 9-cis RA and vitamin K in the regulation of renal calcification. The original observation that 9-cis RA mitigates 1,25(OH)2D3-induced renal calcification was made in a carcinogen-injected mouse (12) for which the present study was a continuation. It will be necessary to confirm these findings in mice that are not exposed to a carcinogen prior to treatment with either 1,25(OH)2D3 or 9-cis RA. Another limitation was the lower final body weight in the D group only.

In conclusion, 1,25(OH)2D3-induced renal calcification may be due to high ucMG. The 9-cis RA appears to mitigate this 1,25(OH)2D3-induced renal calcification by decreasing the ucMGP:ucMGP ratio. The mechanism(s) underlying the influence of 1,25(OH)2D3 and 9-cis RA on γ-carboxylation of MGP, vitamin K concentrations, and the conversion of PK to MK-4 warrants further investigation.

Literature Cited


