Vitamin D3 Confers Protection from Autoimmune Encephalomyelitis Only in Female Mice

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Vitamin D₃ Confers Protection from Autoimmune Encephalomyelitis Only in Female Mice

Karen M. Spach* and Colleen E. Hayes²†

The prevalence of multiple sclerosis (MS) is a chronic, inflammatory, neurodegenerative disease marked by autoreactive T lymphocyte and mononuclear cell infiltration into the CNS, demyelination, oligodendrocyte loss, and axonal degeneration (1). MS often shows a relapsing-remitting course, later developing into chronic progressive disease. For unknown reasons, MS afflicts women twice as often as men. The etiology of MS is uncertain, but available immunologic, genetic, and epidemiologic data suggest that MS is an autoimmune disease that develops in genetically susceptible individuals who are exposed to as-yet undefined environmental risk factors (2, 3). Identifying these environmental factors and elucidating how they increase autoimmune disease risk could guide new strategies to prevent MS.

There is a very strong inverse correlation between MS disease prevalence and UVB exposure (4–9), implying that UVB exposure is strongly protective with respect to MS. Because UVB exposure is required for vitamin D₃ synthesis and vitamin D₃ has important immunoregulatory functions, we proposed that vitamin D₃ may mediate the protective effects of UVB in MS (10–12). Consistent with this hypothesis, MS risk and severity were lowest among individuals who ingested large amounts of fish oil, a rich vitamin D₃ source (13–15), or took vitamin D supplements (16). Also, the periodicity of MS relapses and remissions correlated with seasonal changes in vitamin D₃ supplies from UVB exposure (17). Furthermore, the transcriptional regulatory functions of the hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) are mediated by the nuclear vitamin D receptor (VDR) (18), and genetic epidemiology studies showed that the VDR b allele correlated with MS risk in the Japanese (19, 20). Finally, studies showing that 1,25-(OH)₂D₃ markedly inhibited induction of the MS model disease, experimental autoimmune encephalomyelitis (EAE) (21–28), and reversed established EAE disease (25, 29) have provided strong evidence in favor of the hypothesis that vitamin D₃ may perform anti-inflammatory and neuroprotective functions in MS. Thus, a robust and diverse body of evidence supports the hypothesis that vitamin D₃ insufficiency may increase the MS risk and, conversely, that adequate vitamin D₃ may decrease this risk.

Although it is well established that 1,25-(OH)₂D₃ inhibits EAE, no experiments have examined whether the hormone precursor, vitamin D₃ (30), can inhibit EAE. We addressed this question in the present research. B10.PL mice were fed diets with or without vitamin D₃, immunized with myelin basic protein (MBP) and evaluated for EAE disease. Surprisingly, we found that vitamin D₃ significantly inhibited EAE in female but not male mice, and ovariectomy (OVX) abrogated this protective effect. Gender-based differences in vitamin D₃ metabolism in the CNS correlated with reduced EAE susceptibility in intact, vitamin D₃-fed female mice. Thus, there was synergy between ovarian tissue and vitamin D₃ with respect to EAE inhibition, with the ovarian tissue controlling vitamin D₃ metabolism and anti-inflammatory functions in the CNS. We discuss a possible mechanism for ovarian control of vitamin D₃ metabolism in the inflamed CNS, the implication that sunlight deprivation may contribute to the female predominance of MS, and the possible combined use of female hormones and vitamin D₃ to reduce the MS risk and/or severity.

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2 Address correspondence to Dr. Colleen E. Hayes, Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, 433 Babcock Drive, Madison, WI 53706. E-mail address: hayes@biochem.wisc.edu

3 Abbreviations used in this paper: MS, multiple sclerosis; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24-OHase, 25-dihydroxyvitamin D₃-24-hydroxylase; 25-(OH)₂D₃, 25-hydroxyvitamin D₃; -D, mice fed no vitamin D₃; +D, mice fed 1 Ìg/day of vitamin D₃; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; OVX, ovariectomized; SHAM, sham-operated; VDR, vitamin D receptor.
Materials and Methods

Mice

The B10.PL(73NS)Sn mice were obtained originally from The Jackson Laboratory and bred in the pathogen-free mouse colony of the Department of Biochemistry. Mice were housed at 25°C with a 12-h light-dark cycle and 40–60% humidity. The drinking water was provided ad libitum. Before experiments, the mice were fed commercial mouse chow containing 0.33 g/day vitamin D₃ and 1% calcium (lab diet no. 5008; PMI Nutrition International). All animal experimentation was conducted in accord with accepted standards of humane animal care, as outlined in the ethical guidelines. The Institutional Animal Care and Use Committee approved the experimental protocols.

Experimental diets

The synthetic diet was formulated to contain all essential nutrients, except vitamin D₃ (31). The vitamin D₃ (cholecalciferol; Acros Organics) was dissolved in absolute ethanol (1 mg/ml) and stored in the cold. Each 1 kg of synthetic diet was suspended in 1.5 liters of boiling 1.0% agar (Difco) water and cooled. Before the mixture cooled completely, an amount of vitamin D₃ was added to the diet to provide 0 (−D diet), 1 (+D diet), or 5 μg/day vitamin D₃, calculated based on a daily measured consumption of 4.0 g dry weight of diet/mouse. An oil-soluble vitamin mix without vitamin D₃ was then added to the cooled diet. Fresh synthetic diet was prepared weekly, stored at 4°C, and provided to the mice three times per week.

EAE induction and evaluation

Age- and sex-matched groups of mice were continuously fed the +D or −D diet beginning at age 8 wk. EAE was induced at age 8 wk by injecting MBP isolated from guinea pig spinal cords and pertussis toxin (List Biological Laboratories) as described previously (25). EAE disease severity was evaluated daily as follows: 0, normal; 1, limp tail; 2, paraparesis with a clumsy gait; 3, hind limb paralysis; 4, hind- and fore-limb paralysis; and 5, moribund. At several times during the study, mice were weighed, and blood samples were obtained from the tail vein. At the conclusion of the study, each mouse was euthanized. An oil-soluble vitamin mix without vitamin D₃ was added to the spinal cords, which were then flash frozen with liquid nitrogen and stored at −70°C before RNA or 1,25-(OH)₂D₃ extraction. Alternatively, the spinal cords were divided into six equal sections, aligned vertically, snap frozen in OCT compound (Sakura Finetek) and stored at −70°C for histopathology. The blood was clotted and centrifuged, and the decanted serum was frozen at −70°C before analysis.

Histopathology

The frozen spinal cords, divided into six equal sections to ensure a representative view of the spinal cord, were cryosectioned transversely at 10 μm, fixed in 37% paraformaldehyde, stained with Gill’s No. 3 H&E (Sigma-Aldrich), and examined using a Zeiss Axioskop microscope equipped with a Plan-Neofluar ×20/0.5 objective. Bright field images were acquired with AxioVision 3.0 software controlling an Axiocam digital camera. Each spinal cord section was divided into quadrants; 20 quadrants/slide and 2 slides/mouse were scored. The meninges, gray matter, and white matter of the 40 quadrants were scored in a blinded fashion as 0, 1, 2 or 3, based on the presence or the absence of infiltrating cells in each of the regions on the spinal cord. The histopathology score was recorded as the percentage of spinal cord quadrants that showed a readily identifiable inflammatory cell infiltrate.

25-(OH)D₃ and 1,25-(OH)₂D₃ analysis

The serum was extracted, and the 25-hydroxyvitamin D₃ (25-(OH)D₃) (DiaSorin) and 1,25-(OH)₂D₃ (Nichols Institute) concentrations were determined in duplicate with radioimmunoassay kits, according to the manufacturer’s protocols. The spinal cords were first extracted with a chloroform-methanol-4% KCl in water (1:2:0.8 v/v) mixture to recover the vitamin D metabolites (32). The spinal cord extracts were then assayed in duplicate for 1,25-(OH)₂D₃. A spike-recovery control was performed with each 1,25-(OH)₂D₃ extraction by adding 100 pg of 1,25-(OH)₂D₃ to a crushed spinal cord from a vitamin D₃-depleted mouse (fed −D diet for >28 days). The extraction was then completed, the 1,25-(OH)₂D₃ was assayed in duplicate, the percentage recovery was calculated, and a recovery correction factor was applied to the experimental data. The hormone recovery averaged 70 ± 10%.

Spinal cord transcript analysis

The GAPDH, IFN-γ, VDR, CYP27B1, and CYP24A1 transcript abundance was measured in the spinal cord by real-time PCR. Total cellular RNA was extracted from frozen spinal cord samples using TRI Reagent (Molecular Research Center). The GAPDH transcripts were amplified with the reverse transcription system (Promega). To assess whether the RNA was intact, the GAPDH transcripts were PCR amplified. Only RNA samples that yielded a GAPDH amplicon were used for further study.

Real-time PCR was performed as described with minor modifications (33). The PCR (25 μl) contained 0.5–1 μg of cDNA, 50 nM of each primer, 12.5 μl of 2× SYBRGreen PCR Master Mix (Applied Biosystems), and 2.25 μl of H₂O. The amplification was accomplished with the GeneAmp 5700 Sequence Detection Systems instrument (Applied Biosystems) programmed for incubations of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. Published primers for the GAPDH, IFN-γ, and CYP27B1 were used (34, 35). The VDR primers were designed using Primer Express Software (Applied Biosystems); they were forward, 5′-GCCACAGCACTTACCGCAT-3′, and reverse, 5′-TAGGTGCGCA GAATTGGAAGG-3′. The CYP24A1 primers, designed by Dr. J. W. Pike (Department of Biochemistry, University of Wisconsin, Madison, WI) were forward, 5′-ACCCCAAGGTCCGATGACATC-3′, and reverse, 5′-CCAGTTGTTGCGTCCAGGAGG-3′. Primers were purchased from Integrated DNA TECHNOLOGIES or Invitrogen Life TECHNOLOGIES. To generate a standard curve, cDNA representing each specific amplification was gel purified and quantified by absorbance at 260 nm. Each real-time PCR included reactions with serially-diluted standard cDNA. The standard cDNA copy number, calculated from the absorbance and the dilution, was plotted vs the threshold cycle, Ct. The transcript copy number in each unknown sample was determined from Ct by reference to the appropriate standard curve. The data were calculated as transcript abundance relative to GAPDH as the internal standard.

Data analysis

Individual mice were analyzed, and the mean and SD or SEM were calculated for each group of mice. The group sizes are given in the table and figure legends. The significance of differences between the group means was determined using the Mann-Whitney rank sum test, Student’s t test, Block analysis, or χ² test as indicated (36). A value of p < 0.05 was considered significant.

Results

The experiments reported here used the EAE model to investigate how vitamin D₃ nutrition might influence MS risk. Mice were fed synthetic diets that provided 0 (−D diet) or 1 μg/day (+D diet) of vitamin D₃ continuously beginning at age 4 wk. The +D diet had three times as much vitamin D₃ as the commercial laboratory mouse diet. At age 8 wk, the mice were primed with MBP and subsequently evaluated for EAE disease. The +D female mice had a significantly lower EAE incidence, peak severity, and cumulative disease index than the −D females or the +D or −D males; none of which differed significantly for any EAE disease parameter (Fig. 1A; Table I). Spinal cord IFN-γ transcript analysis and histopathology corroborated the clinical data. The +D female spinal cord had few IFN-γ transcripts (1 ± 1 copies/10³ GAPDH copies; p < 0.02) 10 days postpriming, when IFN-γ synthesis peaks, whereas the −D female and +D and −D male spinal cord had many IFN-γ transcripts (5–23 copies/10³ GAPDH copies). Furthermore, the +D female mice showed very little pathology on day 22, the first plateau of acute clinical disease, whereas the −D females and +D and −D males had lesions with inflammatory cell infiltration (Fig. 1B). Taken together, the clinical, histological, and immunological data show that vitamin D₃ decreased EAE disease in female but not male mice.

A second study tested a higher vitamin D₃ dose in male mice to determine whether males simply required more vitamin D₃ than females for vitamin D₃-mediated protection from EAE. Feeding 5 μg/day vitamin D₃ elevated the serum 25-(OH)D₃ to 133 ± 43 nmol/L, increased the serum calcium, and decreased the body...
Vitamin D₃ inhibited EAE in female but not male mice. A. Clinical EAE disease in +D and −D mice. Adult mice were fed synthetic diets formulated to provide 0 (○) or 1 µg/day (●) of vitamin D₃ for 30 days. EAE was induced, synthetic diet feeding was continued, and disease severity was scored daily. Shown is the composite mean from 25 to 28 mice/group evaluated in four separate experiments. The female groups were significantly different on days 11–21 and day 26 onward, whereas the +D female and male groups were significantly different on days 10–14, 16–23, and 25–38 (p < 0.05; Student’s t-test). B. Histopathological EAE disease in +D and −D mice (representative images from three mice per group). Spinal cords were collected 22 days post-MBP immunization, snap frozen, cryosectioned, fixed, and stained. The arrows highlight inflammatory lesions.

Table I. Dietary vitamin D₃ delayed the onset and reduced the severity of EAE in female but not male micea

<table>
<thead>
<tr>
<th>sex</th>
<th>Dietary Vitamin D₃ (µg/day)</th>
<th>Incidence (%)</th>
<th>Onset (day)</th>
<th>Mortality (%)</th>
<th>Peak Disease Severity</th>
<th>Cumulative Disease Index</th>
<th>Sections with Lesions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>0</td>
<td>100</td>
<td>16 ± 5</td>
<td>18</td>
<td>2.7 ± 1.2</td>
<td>45 ± 35</td>
<td>63 ± 15</td>
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<tr>
<td>female</td>
<td>1</td>
<td>68*</td>
<td>19 ± 7</td>
<td>0*</td>
<td>1.3 ± 0.7*</td>
<td>18 ± 15*</td>
<td>25 ± 17*</td>
</tr>
<tr>
<td>male</td>
<td>0</td>
<td>100</td>
<td>16 ± 6</td>
<td>4</td>
<td>2.3 ± 1.0</td>
<td>33 ± 21</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>male</td>
<td>1</td>
<td>100</td>
<td>19 ± 10</td>
<td>0</td>
<td>2.0 ± 0.8</td>
<td>32 ± 19</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>female SHAM</td>
<td>1</td>
<td>73††</td>
<td>16 ± 5††</td>
<td>31</td>
<td>1.7 ± 1.3</td>
<td>9 ± 9†</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>female O VX</td>
<td>1</td>
<td>100</td>
<td>12 ± 4</td>
<td>11</td>
<td>2.3 ± 1.0</td>
<td>16 ± 9</td>
<td>65 ± 3</td>
</tr>
</tbody>
</table>

a The experimental design is presented in the Fig. 1 legend. The clinical data shown are the composite mean ± SD from four separate experiments (15–28 mice/group). The histopathology data are from three mice per group. The statistical tests performed on the data were the χ² test (incidence and mortality), the Student t test (disease onset, severity, and cumulative disease index), and the Mann-Whitney rank sum test (histopathology data). For within-sex comparisons, the * indicates p < 0.05 and ** indicates p < 0.01. For between-sex comparisons, the # indicates p < 0.05 and ## indicates p < 0.01. For SHAM and OVX group comparisons, the † indicates p < 0.05 and ‡ indicates p < 0.01.

b Mice with a clinical score ≥ 1 for 2 consecutive days were considered to have EAE.

c The cumulative disease index is the sum of the clinical scores for the 42 days postimmunization for the original experiments and 22 days postimmunization for the surgery experiments.

After 30 days of the synthetic diet feeding, the female mice were SHAM or OVX, allowed to recover from surgery for 10 days, and primed with MBP while diet feeding continued throughout the study.
CYP27B1 and CYP24A1 transcripts were measured. These transcripts were analyzed on day 10 post-MBP priming, when clinical signs attributable to activated macrophages in the CNS were first evident. We reasoned that differences in synthesis or inactivation early in the disease process would have the greatest impact on disease outcome. The kidney CYP27B1 and CYP24A1 transcripts were also quantified. The data showed that the kidney and spinal cord CYP27B1 transcripts (Table II) and the kidney CYP24A1 transcripts (Fig. 3A) did not vary significantly with gender, diet, or MBP priming. In sharp contrast, the spinal cord CYP24A1 transcripts (Fig. 3B) did not vary significantly with gender, diet, or MBP priming. The decreased spinal cord CYP24A1 mRNA in the MBP-primed +D females might be due to decreased VDR expression because the multiple vitamin D-responsive elements in the CYP24A1 promoter couple gene expression to VDR activity (38). Normal mice had one to three VDR copies per 10^3 GAPDH copies in the spinal cord, regardless of gender or diet (Table II). These transcripts increased 10 days post-MBP priming with the increase in inflammatory cell infiltration into the CNS but still did not vary significantly with gender or diet. The +D males had a substantially higher CYP24A1-VDR transcript ratio than the +D females.

Thus, EAE susceptibility correlated directly with a sex-based disparity in spinal cord CYP24A1 transcripts and inversely with the spinal cord 1,25-(OH)_{2}D_{3}. One interpretation of these data is that +D females and males produced spinal cord 1,25-(OH)_{2}D_{3} at similar rates, but the +D females inactivated it more slowly, allowing it to accumulate and inhibit EAE. The interpretation that males inactivated 1,25-(OH)_{2}D_{3} more rapidly could explain why substantially more 1,25-(OH)_{2}D_{3} was needed to prevent EAE in males than in females (24).

The experimental design is presented in Fig. 1 legend. The samples were collected on the indicated days; CNS designates spinal cord samples. The RNA was extracted, and a real-time PCR analysis was performed in triplicate. The values shown are the composite mean ± SEM for three separate experiments. The day 0 and day 22 data are from 5–7 samples/group. The day 10 data are from 8–14 samples/group. For between-gender comparisons, # indicates p < 0.01 (Mann-Whitney rank sum test).

### Table II. The spinal cord CYP24A1 transcripts decreased with MBP priming to a significantly lower level in the female mice than in the male mice

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sample</th>
<th>Day Relative to MBP Priming</th>
<th>Transcript Copies/10^3GAPDH Copies by Sex and Dietary Vitamin D_{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−D</td>
<td>+D</td>
</tr>
<tr>
<td>VDR</td>
<td>CNS</td>
<td>0</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>10</td>
<td>25.7 ± 7.0</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>Kidney</td>
<td>0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>Kidney</td>
<td>22</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>CNS</td>
<td>0</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>CNS</td>
<td>10</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>Kidney</td>
<td>0</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>Kidney</td>
<td>22</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>CNS</td>
<td>0</td>
<td>2.7 ± 1.4</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>CNS</td>
<td>10</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

*The experimental design is presented in Fig. 1 legend. The samples were collected on the indicated days; CNS designates spinal cord samples. The RNA was extracted, and a real-time PCR analysis was performed in triplicate. The values shown are the composite mean ± SEM for three separate experiments. The day 0 and day 22 data are from 5–7 samples/group. The day 10 data are from 8–14 samples/group. For between-gender comparisons, # indicates p < 0.01 (Mann-Whitney rank sum test).
ever, vitamin D3 did not protect the OVX, MBP-primed sexes of mice had equivalent serum 1,25-(OH)2D3 concentrations, indicating that vitamin D3 protected intact female mice (Fig. 4). How-

d onset and less severe EAE than the SHAM inter-

vention (data not shown). The SHAM based differences in vitamin D3 metabolism in the CNS. The two

based disparity in EAE susceptibility apparently derives from sex-

D3 inhibited severe EAE only in female mice. This striking sex-

We have provided evidence that nontoxic levels of dietary vitamin

Discussion

We have provided evidence that nontoxic levels of dietary vitamin D3 inhibited severe EAE only in female mice. This striking sex-

accumulation in the spinal cord, rather, female hormones directly

exactly how 1,25-(OH)2D3 inhibits EAE.

at least two mechanisms might explain why the MBP-primed

D females prevented CYP24A1 gene induction more completely than the +D males. Female hormones might directly or indirectly repress this gene or male hormones might stimulate gene expression. To distinguish these possibilities, mice were continuously fed a +D or −D diet beginning 30 days before OVX, castration, or sham surgery (SHAM). Ten days postsurgery, they were primed with MBP, diet feeding was continued, and EAE disease was analyzed. The castrated, MBP-primed +D and −D males did not differ significantly for any EAE disease parameter, indicating that male hormones did not impede vitamin D3-mediated disease prevention (data not shown). The SHAM +D females had a delayed onset and less severe EAE than the SHAM −D females, confirming that vitamin D3 protected intact female mice (Fig. 4A). However, vitamin D3 did not protect the OVX, MBP-primed +D females (Fig. 4A), and these females did not accumulate 1,25-(OH)2D3 in the spinal cord (Fig. 4B). These results support the interpretation that male hormones did not suppress 1,25-(OH)2D3 accumulation in the spinal cord, rather, female hormones directly or indirectly facilitated this accumulation.

At least two mechanisms might explain why the MBP-primed +D females prevented CYP24A1 gene induction more completely than the +D males. Female hormones might directly or indirectly repress this gene or male hormones might stimulate gene expression. To distinguish these possibilities, mice were continuously fed a +D or −D diet beginning 30 days before OVX, castration, or sham surgery (SHAM). Ten days postsurgery, they were primed with MBP, diet feeding was continued, and EAE disease was analyzed. The castrated, MBP-primed +D and −D males did not differ significantly for any EAE disease parameter, indicating that male hormones did not impede vitamin D3-mediated disease prevention (data not shown). The SHAM +D females had a delayed onset and less severe EAE than the SHAM −D females, confirming that vitamin D3 protected intact female mice (Fig. 4A). However, vitamin D3 did not protect the OVX, MBP-primed +D females (Fig. 4A), and these females did not accumulate 1,25-(OH)2D3 in the spinal cord (Fig. 4B). These results support the interpretation that male hormones did not suppress 1,25-(OH)2D3 accumulation in the spinal cord, rather, female hormones directly or indirectly facilitated this accumulation.

Discussion

We have provided evidence that nontoxic levels of dietary vitamin D3 inhibited severe EAE only in female mice. This striking sex-

Based differences in vitamin D3 metabolism in the CNS. The two

sexes of mice had equivalent serum 1,25-(OH)2D3 concentrations, but in the CNS, the relatively EAE disease-free +D females had significantly less CYP24A1 mRNA encoding the inactivating 24-

OGase enzyme and therefore higher 1,25-(OH)2D3 levels than the EAE-diseased +D males. The higher 1,25-(OH)2D3 level provided very potent anti-inflammatory and neuroprotective activities in the intact +D females. OVX eliminated this protective mechanism. The OVX female mice did not accumulate 1,25-(OH)2D3 in the CNS. Lacking the 1,25-(OH)2D3-mediated anti-inflammatory and neuroprotective functions, the OVX females sustained severe inflammation-induced CNS damage and progressive motor function loss. Our data provide the first direct evidence that the ovary regulates vitamin D3 metabolism and 1,25-(OH)2D3-mediated anti-inflammatory and neuroprotective functions in the CNS.

Precisely how 1,25-(OH)2D3 inhibits EAE is still not entirely clear (12). Many in vitro experiments have shown that 1,25-(OH)2D3 prevented immature dendritic cells from maturing and producing the costimulatory molecules and cytokines needed for priming T lymphocytes (39), but we found no evidence for this mechanism in vivo in the EAE model system (27). Other in vitro experiments have suggested that 1,25-(OH)2D3 may facilitate Th type 2 responses rather than Th type 1 responses, but we and others (12, 26, 27) found no evidence for this type of activity in vivo in the EAE model system. We found a requirement for Rag-1-dependent cells other than the encephalitogenic Th type 1 cells in the mechanism (27), whereas others found that CD8+ T cells were not essential (28). We interpreted these data as suggesting that the 1,25-(OH)2D3 may be supporting the function of regulatory T cells that maintain peripheral tolerance to self (27). Other data from our lab showed that 1,25-(OH)2D3 reduced the number of inflammatory cells in the CNS by sensitizing these cells to apoptotic signals (25, 29). Additional experiments are underway to define more precisely how 1,25-(OH)2D3 inhibits EAE.
The nuclear VDR is expressed in the CNS (40, 41), and the 1,25-(OH)_{2}D_{3} performs a variety of neuroprotective functions that reduce CNS damage and motor function loss. The VDR was detected in the hippocampus (neurons and astrocytes), where it appeared to support hippocampal cell survival (42), and in brain regions involved in memory and cognition, implicating 1,25-(OH)_{2}D_{3} in memory processing (43). The VDR was also present in hypothalamic neurons, implicating 1,25-(OH)_{2}D_{3} in the control of hypothalamic peptidergic systems (44), and in oligodendrocytes (45) and astrocytes (46), suggesting possible roles for 1,25-(OH)_{2}D_{3} in the control of myelination and blood-brain barrier maintenance. We found that 1,25-(OH)_{2}D_{3} regulated the cadherin-related neuronal receptor I, glial fibrillary acidic protein, and other genes associated with neuroprotection (29). The 1,25-(OH)_{2}D_{3} stimulated the synthesis of neurotrophins, including nerve growth factor (46, 47), a neurotrophin that performs differentiative, protective, and repair functions with respect to sensory and sympathetic neurons (48). It also stimulated the synthesis of neurotrophic tyrosine kinase receptor type 2 (29) and other neurotrophin receptors (49). Furthermore, the 1,25-(OH)_{2}D_{3} enhanced the expression of Ca^{2+} binding proteins (29, 50) and decreased the mRNAs encoding the α(1C) and α(1D) pore-forming subunits of the L-type Ca^{2+} channels (51). These 1,25-(OH)_{2}D_{3}-mediated changes would be expected to reduce Ca^{2+} and decrease the vulnerability of neurons to excitotoxic damage, because Ca^{2+} channel increases enhanced neuronal sensitivity to excitotoxic insults and correlated with brain aging and motor impairments.

Sunlight exposure provides >90% of the human vitamin D_{3} requirement (30). In our experiments, we varied the dietary vitamin D_{3} levels in the +D and −D mice to model humans with high and low sunlight exposure, respectively. In humans, the serum 25-(OH)D_{3} varies with sunlight exposure and dietary vitamin D_{3} (52), although the serum 1,25-(OH)_{2}D_{3} does not (53). People with plentiful sunlight exposure, e.g., individuals living at low latitudes and working outdoors (lifeguards or farmers), had ∼100–200 nmol/L serum 25-(OH)D_{3} (52). Conversely, sunlight-deprived individuals had ∼10–40 nmol/L serum 25-(OH)D_{3} (54); examples are people living at latitudes >34°N or >3°S, where vitamin D_{3} synthesis does not occur year-round (55) or living and working mainly indoors (submarine sailors, office workers, the homebound elderly, children in school and daycare). By comparison, the +D mice had 80–120 nmol/L serum 25-(OH)D_{3}, whereas the −D mice had 5–20 nmol/L, similar to humans with high or low sunlight exposure, respectively. To sustain >70 nmol/L serum 25-(OH)D_{3} during the winter months, healthy men required an estimated 3000–4000 IU of vitamin D_{3} daily (52, 54, 56, 57).

To explain why vitamin D_{3} did not protect OVX female mice, we hypothesize that estrogen derived from ovarian tissue was essential to prevent CYP24A1 gene induction and allow the 1,25-(OH)_{2}D_{3} to accumulate and mediate anti-inflammatory and neuroprotective functions. This hypothesis is based on data showing that estrogen is protective in MS and EAE. In MS, relapse rates declined during pregnancy and escalated postpartum, concurrently with pregnancy-induced changes in estrogen levels (58, 59). Moreover, estrogen therapy reduced the MS severity in nonpregnant, female MS patients (60, 61). A small study found that most MS patients experienced more severe symptoms with declining estrogen levels after menopause (62). With respect to EAE, female mice were more susceptible to EAE than males (63), and OVX females had accelerated EAE disease (64). Pregnancy protected female mice from EAE induction and progression (64–67). Moreover, estrogen treatment of normal or OVX females at the time of EAE induction suppressed EAE disease (64, 68, 69), whereas progesterone treatment had no effect (70). It is possible that the protective functions of estrogen in MS and EAE may reflect in part a role for estrogen in regulating vitamin D_{3} metabolism in the CNS. Ongoing studies are testing this hypothesis by evaluating vitamin D_{3}-mediated inhibition of EAE in OVX mice with and without hormone replacement.

There is substantial published evidence that estrogen stimulates 1,25-(OH)_{2}D_{3} accumulation in women as we found in female mice. Firstly, the serum 1,25-(OH)_{2}D_{3} increased 2-fold from the early follicular phase of the menstrual cycle, when estrogen levels are low, to the time of ovulation, when estrogen levels are high (71, 72). Secondly, the serum 1,25-(OH)_{2}D_{3} rose 2–3-fold during the high estrogen period of pregnancy (73). Finally, the serum 1,25-(OH)_{2}D_{3} increased >50% in young women (74) and postmenopausal women (75–78) receiving estrogen therapy. These data establish a cause-effect relationship between increased estrogen and higher 1,25-(OH)_{2}D_{3} levels in the serum. Moreover, the data are consistent with the hypothesis that estrogen may strongly influence vitamin D_{3} metabolism and the anti-inflammatory and neuroprotective functions of the vitamin D_{3} endocrine system in the CNS. In this way estrogen, sunlight, and vitamin D_{3} may function synergistically to reduce MS risk and/or severity in women.

It is not known why female and male mice had equivalent CYP24A1 gene expression in the CNS before MBP priming, but the intact, female mice had significantly lower CYP24A1 gene expression than males after MBP priming. In the kidney, there is a feedback inhibition loop wherein the 1,25-(OH)_{2}D_{3} and the vitamin D_{3} strongly induce the CYP24A1 gene encoding the hormone-inactivating 24-OHase through dual vitamin D-responsive elements in the CYP24A1 gene promoter (38). The 24-OHase then converts 1,25-(OH)_{2}D_{3} into an inactive metabolite, thereby maintaining the plasma 1,25-(OH)_{2}D_{3} concentration within very narrow limits (37). Under noninflammatory conditions in the CNS, the 1,25-(OH)_{2}D_{3} concentration may also be maintained within narrow limits because glial cells express the VDR and reportedly induce the CYP24A1 gene in response to 1,25-(OH)_{2}D_{3} (79). However, the feedback inhibition loop does not operate in activated macrophages, where IFN-γ signaling prevents CYP24A1 gene induction and allows the 1,25-(OH)_{2}D_{3} to accumulate (80).

We theorize that female mice had lower CYP24A1 gene expression in the CNS than males after MBP priming because of estrogen-related differences in IFN-γ synthesis and signaling in activated macrophages. Estrogen reportedly stimulated IFN-γ production via an estrogen-responsive element in the IFN-γ promoter (81). Moreover, IFN-γ signaling activated Stat1, and activated Stat1 prevented the VDR from transactivating the CYP24A1 promoter (82). Thus, the intact females, through estrogen action, may have produced more IFN-γ in the CNS soon after MBP priming, and the IFN-γ may have rapidly and completely terminated CYP24A1 gene expression in the activated macrophages, allowing the 1,25-(OH)_{2}D_{3} to accumulate in the spinal cord. By day 10, when clinical disease signs were first evident in the males and the −D females, the +D mice may have partially resolved the inflammation and decreased the IFN-γ production, explaining why the IFN-γ transcripts were not abundant in this group at this time point. This interpretation is consistent with an unexplained protective role of IFN-γ in EAE (83, 84). Current experiments are testing this hypothesis through studies of vitamin D_{3}-mediated inhibition of EAE in intact and OVX female mice with a targeted disruption of the IFN-γ gene.

The research reported here has significant implications for MS. If humans have a similar gender difference in vitamin D_{3} metabolism in the CNS, then sunlight deprivation would increase the MS risk more significantly in women than in men. In this manner, a
gender bias with regard to benefits from a protective environmental factor could introduce a gender bias in disease incidence, relating to the unexplained female bias in MS incidence (85). If men do not completely prevent CYP24A1 gene induction in activated macrophages, then they may not benefit from sunlight’s protective effects or from vitamin D₃-based therapeutic strategies to inhibit MS. A second important implication is that the protective effects of sunlight and vitamin D₃ may decline significantly in menopausal women, when there are decreasing supplies of estrogen to prevent UVB exposure or high dietary vitamin D₃ to slow MS progression or reduce MS severity in peri- and postmenopausal women. It is our hope that these insights will guide new strategies to reduce the prevalence and impact of MS and possibly other autoimmune diseases.

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Disclosures

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References


