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Age- and brain-region-specific effects of dietary vitamin K on myelin sulfatides

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Abstract

Dysregulation of myelin sulfatides is a risk factor for cognitive decline with age. Vitamin K is present in high concentrations in the brain and has been implicated in the regulation of sulfatide metabolism. Our objective was to investigate the age-related interrelation between dietary vitamin K and sulfatides in myelin fractions isolated from the brain regions of Fischer 344 male rats fed one of two dietary forms of vitamin K: phyloquinone or its hydrogenated form, dihydrophyloquinone for 28 days. Both dietary forms of vitamin K were converted to menaquinone-4 in the brain. The efficiency of dietary dihydrophyloquinone conversion to menaquinone-4 compared to dietary phyloquinone was lower in the striatum and cortex, and was similar to those in the hippocampus. There were significant positive correlations between sulfatides and menaquinone-4 in the hippocampus (phyloquinone-supplemented diet -12mo and 24mo; dihydrophyloquinone-supplemented diet - 12mo) and cortex (phyloquinone-supplemented diet -12mo and 24 mo). No significant correlations were observed in the striatum. Furthermore, sulfatides in the hippocampus were significantly positively correlated with MK-4 in serum. This is the first attempt to establish and characterize a novel animal model that exploits the inability of dietary dihydrophyloquinone to convert to brain menaquinone-4 to study the dietary effects of vitamin K on brain sulfatide in brain regions controlling motor and cognitive functions. Our findings suggest that this animal model may be useful for investigation of the effect of the dietary vitamin K on sulfatide metabolism, myelin structure, and behavior functions.

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Keywords

Myelin; Phylloquinone; Vitamin K; Menaquinone-4; Sulfatides; Diet

Introduction

Neurological disorders, and to a lesser extent, normal aging are associated with decline in motor and cognitive behavior [1,2,3]. Disruption of brain myelin has been implicated as an important contributor to cognitive deficit in humans [2] and animals [4,5,6,7]. Myelin, which is an axonal insulator, is formed in the CNS by oligodendrogliaocytes [8,9,10,11]. A high concentration of lipids is essential for proper myelin functions. Myelin membranes are particularly enriched with glycolipids, including galactosylceramide (GalCer) and its sulfated form, sulfatide [8, 12,13,14,15]. Concentrations of sulfatides increase during brain development, parallel to an increase in brain myelination [16,17,18]. Earlier studies *in vivo* and *in vitro* demonstrated differential rates of myelination among brain regions [19,20]. Decreases in myelin sulfatides content and/or changes in their molecule structure have been implicated as important factors in the disruption of myelin morphological structure, with a subsequent attenuation of myelin efficiency as an axonal insulator [6,21]. Furthermore, dysregulation in brain sulfatide metabolism, and decreases in the content of myelin sulfatides with age, has been implicated as a significant risk factor for behavioral deficits observed in normal aging [7,22], and age-associated neurological disorders [23,24,25].

Numerous studies suggest that exogenous factors, including nutritional factors, can modulate myelination [11,26,27]. Vitamin K, a fat-soluble vitamin, has been implicated in regulation of brain sphingolipid metabolism, including sulfatide metabolism [28,29,30]. Furthermore, a recent study reported a positive correlation between sulfatides and vitamin K, which is present almost exclusively in the form of menaquinone-4 (MK-4) in the brain [31,32]. However, there are inconsistencies on the putative role of vitamin K on sulfatides, as reviewed elsewhere [30]. To the best of our knowledge the dietary effect of vitamin K on brain myelin was not investigated. Under normal dietary conditions, there is a tissue-specific conversion of vitamin K from the dietary form (primarily phylloquinone; K1) to MK-4. The hydrogenated form of vitamin K (2', 3'-dihydrophylloquinone; dK) does not convert to MK-4 to the same extent that K1 is converted to MK-4 [33,34]. The objective of this study was to investigate the age- and brain region-specific effects of different dietary forms of vitamin K on myelin sulfatides. This is the first attempt to establish and characterize a novel animal model that exploits the inability of dietary dihydrophylloquinone to convert to brain menaquinone-4 to study the dietary effects of vitamin K on brain sulfatide in brain regions controlling motor and cognitive functions.

Material and methods

Reagents

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific (Houston, TX, USA). AIN-93G diet (cat.# TD.94045), AIN-93-VX vitamin mix (cat.# TD. 94047), AIN-93G-MX mineral mix; vitamin K deficient powdered diet (cat.# TD. 97053), and AIN-76 mineral mix (cat.# CA.170915) were purchased from Harlan Teklad (Madison, WI, USA). A purified source of vitamin K (phylloquinone; K1) was purchased from Sigma (Sigma Chemical Co., St. Louis; cat.# V-3501). The 2',3'-dihydrophylloquinone (dK) was a gift from Dr. J. Pyrek (University of Kentucky, Mass Spectrometry Facility, Lexington, KY). Protein Assay kits were obtained from Bio-Rad Laboratories (Hercules, CA, USA; cat #500-0116). Sulfatide standards were purchased from Sigma (Sigma Chemical Co., St. Louis; cat.# S1006).

Diets

In the current study, we used a standard rat diet, AIN-93G; and two experimental diets: (a) vitamin K deficient diet supplemented with phyloquinone (K1 diet), and (b) vitamin K deficient diet supplemented with 2', 3'-dihydrophyloquinone (dK diet). The concentrations of the two dietary forms of vitamin K (i.e., phyloquinone, K1; and its hydrogenated form, dihydrophyloquinone, dK) were experimentally determined (K1 diet: $198 \pm 9.0 \mu\text{g}/\text{kg}$ diet; dK diet: $172 \pm 13.0 \mu\text{g}/\text{kg}$ diet). We selected amounts of dK or K1 that were adequate to sustain coagulation based on our preliminary studies (data not shown). Diets were stored at 4°C until used. Aliquots of the diets were analyzed by HPLC for both forms of vitamin K (i.e., K1 and dK) to confirm their stability and concentrations [35].

Animals

Sixty male Fischer 344 rats 2- (young), 12- (adult), and 24- months- old (old) ($n = 10$ per age group) were obtained from the National Institute of Aging (Harlan Sprague Dawley, Indianapolis, IN). All experimental animals were provided with *ad libitum* water and AIN-93G diet for 14 days of the acclimatization period, followed by the experimental dietary intervention for 28 days (Figure 1). Animals were group pair fed (by age and diet). The amount of diets provided to each animal was adjusted daily to accommodate consumption patterns (in accordance with the amounts of food remaining from any animal or, in the case of consumption of all of the previous day's diet, incrementally increased). This procedure ensures that all age-matched animals have comparable caloric and nutrient daily food consumptions. Body weights were recorded weekly. All health or other related observations were also recorded. Animals were used in compliance with all applicable laws and regulations and principles expressed in the National Institutes of Health, USPHS, Guide for the Care and Use of Laboratory Animals. The study was approved by the Animal Care and Use Committee of the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston, MA).

Sample preparation

Upon completion of the 28-day dietary intervention animals were sacrificed and brain regions (i.e., striatum, hippocampus, and cortex) were dissected on ice, immediately frozen in liquid nitrogen, and stored at -80°C until further analyses. Myelin fractions were isolated from individual brain regions.

Isolation of myelin fractions

Myelin fractions were isolated from the brain regions by differential centrifugation using a modified procedure as described previously [36]. Briefly, individual brain regions (cortex, striatum and hippocampus) were homogenized in 0.32 M sucrose buffer containing protease inhibitors to prevent protease activation and protein cleavage. Crude membrane fractions (P-2) were separated from the crude nuclear fractions in each of the brain regions by low speed centrifugation, followed by ultra centrifugation on a Ficoll gradient (7% and 14%) for separation of myelin fractions. The purity of the isolated myelin fractions was confirmed previously by electronmicroscopic and biochemical analyses [37,38]. Collected myelin fractions were assessed for protein, sulfatide and vitamin K concentrations.

Analysis of protein

The concentration of protein in myelin homogenates was determined by using a Bio-Rad Protein Assay (cat #500-0116) for 96 well plate, as described previously [39].

Analysis of sulfatides

Sulfatide concentration in myelin membranes was assessed using a spectrophotometric method [31,40]. Briefly, sulfatides were purified from the myelin lipids prior their quantitative assessment. Specifically, glycolipids, including gangliosides and other water-soluble myelin constituents, were removed by the chloroform-methanol (2:1 vol/vol) Folch procedure as described previously [36]. The potentially remaining traces of glycolipids and other water-soluble myelin constituents were further removed by washing the lower phase with “pure solvents upper phase” prepared with 0.1M KCl. Sulfatides were then purified from the other classes of myelin lipids (i.e., phospholipids, cholesterol, etc.) by two dimensional high performance thin layer chromatography [36]. Sulfatides were identified using sulfatide standards (Sigma Chemical Co., St. Louis; cat.# S1006). The absorbance of the colored sulfatide-Azure A complex was measured at 645nm by using Perkin-Elmer Lambda 7 spectrophotometer. The concentration of sulfatide was calculated based on calibration curve by using sulfatide standard (0–30 nmol). The results for sulfatide concentration in myelin fractions were adjusted per gram of myelin protein and expressed as micromole sulfatide per gram protein ($\mu\text{mol/g pr.}$).

Analysis of vitamin K

Aliquots of myelin were assessed for concentration of vitamin K (i.e., K1, MK-4 and dK) by reversed-phase HPLC [34]. Results were adjusted per myelin protein, and expressed as picomole of MK-4 per g protein (pmol/g pr.).

Statistical analysis

The results of biochemical measurements were analyzed by analysis of variance (ANOVA) and post hoc Fisher's LSD tests. All statistical analyses, including correlations, were performed using SYSTAT version 10.2.01; (SPSS, Inc. Chicago, IL). Data points collected from two rats (12mo, K1 diet and 24mo, dK diet) were excluded from the analyses as outliers. Three cortical samples from the dK dietary group (two, 12mo and one, 24mo) did not contain a sufficient amount of sample for the assessment of MK-4 (i.e., values were below the minimum detectable concentrations of 0.05nmol/kg tissue). Results were considered statistically significant if the observed significance value was less than 0.05.

Results

A total of 53 rats completed the dietary intervention (Figure 1). One young (2mo) rat died shortly after arrival and prior to the start of the dietary trial; 6 old rats (24 mo; 2 rats from the vitamin K1-supplemented group and 4 rats from the dK-supplemented group) died of apparent age-related pathologies, including neoplasia, which did not appear to result from experimental treatments. There were no significant differences in the weights between two dietary groups of rats within the same age groups (Figure 1).

Sulfatides

To test the hypothesis that lack of conversion of dietary dK to MK-4 in the brain will affect sulfatides in brain myelin, we compared sulfatide content in myelin fractions isolated from the cortex, striatum, and hippocampus of young (2mo), adult (12mo), and old (24mo) rats maintained on dK or K1 diets. Results showed a significant diet *vs* age effect on sulfatides in the hippocampus [$F(2,47)=6.47$; $P=0.003$] and cortex [$F(2,47)=5.48$; $P=0.007$], whereas in the striatum this effect was not significant ($P=0.408$) (Table). Young rats fed with dK diet had lower compared to K1 diet sulfatide content in the hippocampus (K1 *vs* dK diet: $P=0.014$) and cortex (K1 *vs* dK diet: $P=0.012$), whereas in the striatum there were no significant differences between the two diets ($P=0.242$) (Table). Adult and old rats fed with dK diet showed lower

sulfatide content than K1 dietary groups in the striatum (12 mo; K1 vs dK: $P=0.003$) and cortex (24 mo; K1 vs dK: $P=0.001$) (Table). Age-related changes in the sulfatide content were observed in the hippocampus (young vs adult, $P=0.004$; young vs old, $P=0.001$), striatum (young vs adult, $P=0.001$), and cortex (young vs old, $P=0.037$; adult vs old, $P=0.006$) in rats fed with dK diet (Table). There were no age-related differences in the content of myelin sulfatides in rats fed with K1-supplemented diet.

Vitamin K

In this study we investigated whether the differences in the conversion efficiency of the vitamin K dietary forms (i.e., K1 and dK) to menaquinone-4 (MK-4) would differentially affect MK-4 content in myelin fractions isolated from the hippocampus, striatum, and cortex with age. Results showed that both dietary forms of vitamin K were converted to MK-4 in the brain. However, dietary dK had lower than K1 conversion efficiency as reflected through a lower content of MK-4 in the striatum (2mo, $P=0.001$; 12mo, $P=0.01$; 24mo, $P=0.04$) and cortex (2mo, $P=0.005$; 12mo, $P=0.019$) (Table; K1 vs dK). There were no significant differences in MK-4 content between the two dietary groups in the hippocampus (Table). These findings suggest that the efficiency of the dietary dK conversion to MK-4 in the hippocampus was similar to the dietary K1. There were no significant diet vs age effects on MK-4 content in myelin fractions isolated from the hippocampus ($P=0.61$), striatum ($P=0.81$), or cortex ($P=0.49$).

Association between sulfatides and MK-4

No significant correlations were observed between sulfatides and MK-4 in evaluated brain regions among young (2mo) rats fed with either K1 or dK diets ($P>0.05$; data not shown). However, there were significant positive correlations between sulfatides and MK-4 in the adult (12mo) rats fed with K1 diet (K1: hippocampus, $r=.71$, $P=0.02$; cortex, $r=.90$; $P=0.001$), or dK diet (dK: hippocampus, $r=.83$; $P=0.003$) (Figure 2). These correlations were also significant in old (24mo) rats fed with K1 diet (K1: hippocampus, $r=.87$; $P=0.005$; cortex, $r=.82$, $P=0.014$) (Figure 2A and 2C). There were no significant correlations between sulfatides and MK-4 in old rats fed with dK diet. No significant correlations were observed in the striatum within the same age groups (Figure 2B). Serum MK-4 concentrations (Booth et al., 2008) were significantly positively correlated with sulfatide concentrations in myelin fractions isolated from the hippocampus in the individual animals ($r = 0.601$, $P = 0.001$).

Discussion

In this study, the insufficiency of dietary 2',3'-dihydrophyloquinone conversion to menaquinone-4 (MK-4), when compared with the equal intake of phyloquinone, differentially affected sulfatides in myelin fractions isolated from the brain regions of Fischer 344 male rats. Our finding of lower sulfatide content in the cortex (2mo and 24mo), striatum (12mo), and hippocampus (2mo) in the dK dietary group, compared to K1, suggests a potential dietary dK-associated dysregulation in the sulfatide metabolism in oligodendrocytes, brain cells which are responsible for the biosynthesis of myelin constituents in CNS [8,9,10] in these brain regions. Recent studies demonstrated the ability of vitamin K to modify activity of galactocerebroside sulfotransferase (GST), the key enzyme of sulfatide synthesis [41].

The differential activity of oligodendrocytes and the differential rates of myelin formation among brain regions with age [17,18,19,20,42] may also be accountable for the region-specific dietary effects observed in the present study. The myelination of rat brain regions occurs at a different age and continues up to 20 months of age with a higher rate during earlier period of life (i.e., 20 days is the peak of myelin formation); and the lower rate during later period of life (18–20mo) [17,18,19,20,42]. Therefore, our findings of a relatively higher responsiveness to

the dietary intervention among young (2 mo) rats as compared to those among older rats can be attributed to the differential strength of the compensatory reactions among these animals. However, it is also plausible that experimental conditions, including low dietary intake doses of vitamin K and relatively short term of the dietary intervention (i.e., 28 days), chosen for the present study, attenuated our ability to observe dietary effects on myelin sulfatides across all age groups in the evaluated brain regions.

Low sulfatide content in brain myelin has been recently linked with the disruption of myelin integrity [14,21], whereas the disruption of myelin integrity was implicated as an essential contributor to cognitive deficit [6,7,43,44]. Although our findings of dietary-associated decreases in myelin sulfatides suggest a potential disruption in myelin integrity in evaluated brain regions, it is currently unknown whether such disruption would be sufficient to modify motor and cognitive functions controlled by these brain regions.

In the present study both dietary forms of vitamin K were converted to menaquinone-4 (MK-4) in the brain. However, the efficiency of dietary dK conversion to MK-4 compared to K1 was lower in the striatum and cortex, and was similar to K1 in the hippocampus. The location and the mechanism for the conversion of the dietary forms of vitamin K to menaquinone-4 are largely unknown. However, two potential mechanisms were recently proposed [45]. Specifically, it has been suggested that in mice the side chain of phyloquinone side chain can be removed by a currently unknown enzyme to form menadione followed by the geranylgeranylation of menadione to form menaquinone-4 (Figure 3).

The presence of MK-4 in myelin fractions, and the positive correlations between MK-4 and sulfatides in brain myelin, supports recent findings in female rats [31] suggests the physiological role of MK-4 may be independent from its well-known role as a cofactor for the γ -carboxylation reactions [30,34,46]. It is currently not known whether long-term dietary dihydrophyloquinone consumption will modulate these functions through further reduction in dK conversion to MK-4, and whether MK-4 may have a role in myelin formation and maintenance. Our finding of positive correlation between sulfatides in the hippocampus and MK-4 in serum may be potentially important for human studies.

In summary, this is the first study to demonstrate the effect of dietary vitamin K on sulfatides and MK-4 in the purified brain myelin. It remains to be determined whether long-term and/or higher dietary dK consumption would be sufficient to affect brain-region-specific changes in the: (a) number and/or metabolic activity of oligodendrocytes; (b) rate of myelin formation and loss, (c) activity of genes responsible for the synthesis of myelin constituents. Furthermore, the behavioral consequences of altered sulfatide concentrations through manipulation of dietary vitamin K remain to be assessed.

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Figure 1.

Twenty 2-month-old (young), twenty 12-month-old (adult) and twenty 24-month-old (old) male Fischer rats were obtained from the National Institutes of Aging (Harlan Sprague Dawley, Indianapolis, IN). Rats were maintained on experimental diets for 28 days. Vitamin K and sulfatide content was determined in myelin fraction isolated from brain regions as described in Material and methods.

Figure 2.

(A, B, C). The association between MK-4 and sulfatides in myelin fractions isolated from the hippocampus (A), striatum (B), and cortex (C) of adult (12 mo) and old (24 mo) rats exposed to dietary phylloquinone (K1 diet) and dihydrophyllloquinone (dK diet). Data points are concentrations of MK-4 and sulfatides from the hippocampus (n per K1 gr.: 12mo-9; 24mo-8; n per dK gr.: 12mo-10; 24mo-5), striatum (n per K1 gr.: 12mo-9; 24mo-8; n per dK gr.: 12mo-10; 24mo-5), and cortex (n per K1 gr.: 12mo-9; 24mo-8; n per dK gr.: 12mo-10; 24mo-4) of rats fed with phylloquinone (K1) or dihydrophyllloquinone (dK) diets. Data points collected from two rats (12mo, K1 diet and 24mo, dK diet) were excluded from the analyses as outliers. Three cortical samples from the dK dietary group (two, 12mo and one, 24mo) did not contain

a sufficient amount of sample for the assessment of MK-4 (i.e., values were below the minimum detectable concentrations of 0.05nmol/kg tissue).

Figure 3.
Potential mechanism of conversion of the dietary forms of vitamin K to menaquinone-4.

Age- and region-specific effects of dietary phylloquinone (K1) and dihydrophyllolquinone (dK) on sulfatides and MK-4 content in brain myelin^l

Table 1

Brain regions	Age	Sulfatides ($\mu\text{mol/g}$ protein)		Menaquinone-4 (pmol/g protein)	
		K1 diet	dK diet	K1 diet	dK diet
Hippocampus	2	31.98 $\pm$ 6.14 ^a	14.05 $\pm$ 2.82 ^{a,j,k}	1049 $\pm$ 244	782 $\pm$ 52
	12	20.81 $\pm$ 2.57	35.35 $\pm$ 4.61 ^l	1089 $\pm$ 115	1065 $\pm$ 180
	24	33.63 $\pm$ 6.06	44.03 $\pm$ 10.63 ^k	1230 $\pm$ 273	859 $\pm$ 226
Striatum	2	23.71 $\pm$ 6.65	15.91 $\pm$ 1.33 ^l	449 $\pm$ 46 ^e	221 $\pm$ 20 ^e
	12	16.91 $\pm$ 1.91 ^c	10.12 $\pm$ 0.70 ^{c,l}	527 $\pm$ 67 ^f	286 $\pm$ 48 ^f
	24	11.95 $\pm$ 0.67	12.79 $\pm$ 1.96	418 $\pm$ 54 ^g	241 $\pm$ 41 ^g
Cortex	2	3.76 $\pm$ 0.37 ^b	2.66 $\pm$ 0.16 ^{b,m}	182 $\pm$ 15 ^h	114 $\pm$ 14 ^h
	12	2.80 $\pm$ 0.20	2.96 $\pm$ 0.27 ⁿ	229 $\pm$ 41 ⁱ	99 $\pm$ 25 ⁱ
	24	3.01 $\pm$ 0.11 ^d	1.85 $\pm$ 0.19 ^{d,m,n}	314 $\pm$ 53	266 $\pm$ 13

^l Values are mean $\pm$ SEM. Values in column with superscripts with a common letter differ by diet (a, b, c, d, e, f, g, h, i), and by age (j, k, l, m, n, o).

^a Dietary effect: $P = 0.014$;

^b $P = 0.012$;

^c $P = 0.003$;

^d $P = 0.001$,

^e $P = 0.001$;

^f $P = 0.01$;

^g $P = 0.04$;

^h $P = 0.005$;

ⁱ $P = 0.019$,

^j Age effect: $P = 0.004$;

^k $P = 0.001$;

$lP = 0.001$;
 $mP = 0.037$;
 $nP = 0.006$.