Warfarin administration reduces synthesis of sulfatides and other sphingolipids in mouse brain

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Abstract The modulation of phosphosphingolipid synthesis by vitamin K depletion has been observed in the vitamin K-dependent microorganism, *Bacteriodes levii*. When cultured briefly without the vitamin, a reduction occurred in the activity of the first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine synthase. In this report, 16-day-old mice were treated with the vitamin K antagonist, warfarin. Brain microsomes from these animals showed a 19% reduction in synthase activity. Mice treated with warfarin for 2 weeks showed a major reduction in sulfatide level (42%), with a lesser degree or no reduction in levels of gangliosides and cerebrosides. In further experiments, mice were treated with warfarin for 2 weeks and a group was then injected with vitamin K_1 (aquamephyton) for 3 days. Enzyme activity returned to a normal level within 2–3 days. Sulfatide levels had increased 33% in the vitamin K-injected group and ganglioside levels also increased, whereas levels of cerebrosides and sphingomyelin declined. Sulfatide synthesis determined by [^{35}S] sulfate incorporation, showed a 52% increase in incorporation following administration of vitamin K for 3 days. These results suggest a role for vitamin K in the biosynthesis of sulfatides and other sphingolipids in brain. This putative role could be by post-translational protein modification analogous to the role of vitamin K in other systems.—**Sundaram, K.S., and M. Lev.** Warfarin administration reduces synthesis of sulfatides and other sphingolipids in mouse brain. *J. Lipid Res.* 1988. **29:** 1475–1479.

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The discovery of γ-carboxyglutamic acid (Gla) in prothrombin (1) and the participation of vitamin K in the carboxylation of glutamic acid residues in certain proteins is not only relevant to the mechanism of blood clotting but has been found to have application to the biochemistry of other Gla-containing proteins such as osteocalcin (2). The mechanism by which vitamin K participates in the carboxylation of Gla has yet to be clarified (3).

The mode of action of vitamin K in *B. levii* was studied by growing the bacterium in vitamin K-free medium (4), determining the biochemical defect, and reversing the defect by vitamin K supplementation. Limited growth in the absence of vitamin K had no effect on macromolecular synthesis, (DNA, RNA, or protein synthesis). When lipid synthesis was examined in the bacterium, a profound effect of vitamin K depletion was observed. In the absence of vitamin K, synthesis of the sphingolipids virtually ceased whereas other phospholipids, phosphatidylethanolamine and phosphatidylserine, were synthesized normally. The addition of vitamin K to a depleted culture resulted in the recovery of sphingolipid synthesis before a general increase in cell metabolism occurred (4).

The first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine (3-KDS) synthase (serine palmitoyltransferase, EC 2.3.1.50) catalyzes the condensation of palmitoyl-CoA and L-serine to form 3-ketodihydrosphingosine.

This enzyme requires pyridoxal phosphate as a cofactor (5–7). The bacterial 3-KDS synthase has been partially purified (7).

Extracts of *B. levii* grown in the presence of vitamin K showed high activity of this enzyme, whereas in extracts of vitamin K-depleted cultures only traces of activity were found. The addition of vitamin K_1 to a vitamin K-depleted culture resulted in the recovery of enzyme activity during a 15–90 min period (6). The stimulation of sphingolipid synthesis by vitamin K in *B. levii* suggested that the vitamin might play a role in the synthesis of sphingolipids in animals. Here we report evidence indicating that vitamin K has a role in the synthesis of sphingolipids in mice. Such a role may be due to post-translational protein modification of biosynthetic enzymes or regulators of the pathway.

Abbreviations: Gla, γ-carboxyglutamic acid; 3-KDS, 3-ketodihydrosphingosine; C–M, chloroform-methanol; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Male Swiss mice (ICR) weighing 8–12 g were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN and used at 16 days of age (postweaned) for all experiments. Food and water were given ad libitum. The mice were injected intraperitoneally on alternate days with Na warfarin (10 mg/kg body weight) dissolved in 0.1 ml saline. No spontaneous bleeding was found in these mice after warfarin treatment. The mice were killed under anesthesia and blood was collected by cardiac puncture. Prothrombin times were determined according to Quick (8). Brains were removed and homogenized with 20 vol (20 ml/g wet tissue) of chloroform-methanol (C–M) 2:1 (v/v).

The lipids were extracted and partitioned by the procedure of Folch, Lees, and Sloane Stanley (9). The upper phase was dialyzed, lyophilized, and dissolved in C–M 85:15, and applied to an Iatrobeads column (2 g suspended in C–M 95:5). The column was eluted with 30 ml C–M 85:15, followed by 80 ml C–M 1:2 for elution of gangliosides. Total gangliosides were estimated by the resorcinol method of Svennerholm (10) as modified by Miettinen and Takki-Luukkainen (11) using free N-acetyl neuraminic acid as standard. From the Folch lower phase, cerebrosides and sulfatides were separated by TLC using chloroform-methanol-water 65:25:4 (v/v/v) (12). The bands cochromatographing with standard cerebroside and sulfatide were visualized with iodine vapors, removed separately, and used for galactose estimation (13). Glucocerebroside and galactocerebroside were estimated together. Phospholipids were separated by chloroform-methanol-water-NH_4OH 65:25:4:0.5 (v/v/v/v). The band cochromatographing with standard sphingomyelin was visualized with iodine vapors, removed, and used for phosphate determination (14).

Microsomes were prepared from the brains of control and warfarin-treated mice (15,16). 3-KDS synthase activity was assayed in a reaction mixture containing palmitoyl-CoA (0.4 mM), DL-[3-^{14}C] serine (4 mM, 2 μCi; 18 mCi/nmol), phosphate buffer (0.05 M, pH 7.4, containing 1 mM dithiothreitol and 1 mM pyridoxal phosphate), and 11.0–11.5 mg microsomal protein. Enzyme reaction mixtures (0.25 ml final volume) were incubated in capped tubes at 37°C with vigorous shaking and were terminated by the addition of chloroform-methanol. Following TLC (6,7) and autoradiography, the 3-KDS formed was determined by scraping the radioactive zones from the plate and transferring the silica gel directly to counting vials. Radioactivity was determined by adding 10 ml Scintiverse (Fisher Scientific) and counting in a scintillation counter. The level of 3-KDS synthesized by these preparations was comparable to that reported by others (15,17).

Brain sulfatide synthesis was determined by intraperitoneal administration of [^{35}S]sulfate (5 μCi/g; sp act carrier-free, 43 Ci/mg S) in 50mM phosphate buffer, pH 7.2. The mice were killed after 48 hr and sulfatides were isolated by TLC. The labeled spots were removed and counted by liquid scintillation spectrometry.

Na warfarin was a generous gift from Dr. J. W. Suttie, University of Wisconsin, Madison WI. DL-[3-^{14}C]serine and [^{35}S]sulfate were purchased from ICN Radiochemicals, Irvine CA; palmitoyl CoA was from P-L Biochemicals, Milwaukee, WI. Iatrobeads were a gift from Dr. R. W. Ledeen, Albert Einstein College of Medicine, Bronx, NY. Aquamephyton, an aqueous colloidal solution of vitamin K_1, was purchased from Merck Sharp and Dohme, Westpoint, PA. Other chemicals were purchased from Sigma Chemical Co., (St. Louis, MO). All the chemicals were reagent grade or the best quality available. Statistical analyses were made using the *t*-test.

RESULTS

To examine the hypothesis that vitamin K depletion could affect sphingolipid synthesis, mice were depleted of vitamin K by the intraperitoneal administration of warfarin, and 3-KDS synthase activity was then determined in brain microsomes. In the warfarin-treated group, 3-KDS synthase activity was reduced by 19% ($P < 0.05$) **(Table 1).** Treatment of these animals with aquamephyton for 3 days fully restored enzyme activity. Warfarin, unlike another inhibitor of this enzyme,

TABLE 1. Changes in 3-KDS synthase activity after treatment of mice with warfarin and vitamin K

Group	Age	3-KDS Synthase Activity
	days	*pmol/min per mg protein*
Control	28	14.17 ± 2.17
Warfarin	28	11.48 ± 1.65[a]
Vitamin K	29	11.09 ± 2.35[a]
Vitamin K	30	13.83 ± 1.52[b]
Vitamin K	32	15.09 ± 2.13[c]

Sixteen-day-old mice (five animals/group) were injected with Na warfarin (10 mg/kg body weight, i.p. on alternate days) dissolved in saline over a period of 12 days. One group of control and experimental animals was killed. Other groups were injected with vitamin K_1 (aquamephyton, 1 mg/day per mouse, i.p.) and killed daily over a 4-day period. Brains were removed and microsomes were prepared and assayed for 3-KDS as described in Materials and Methods. Values are expressed as means ± SD.

[a] $P<0.05$, comparing warfarin or vitamin K groups with control group.

[b] $P<0.05$, comparing a vitamin K group with the warfarin group.

[c] $P<0.025$, comparing a vitamin K group with the warfarin group.

L-cycloserine, a suicide substrate inhibitor (16), had no effect on synthase activity in vitro (Sundaram, K.S., and M. Lev, unpublished observations) indicating an indirect mode of action in reducing synthase activity.

Effect of warfarin treatment on individual classes of phospho- and glycosphingolipids

The reaction catalyzed by the first enzyme of the sphingolipid pathway is the rate-limiting step (17–19). Reduced activity of this enzyme after warfarin administration must result, therefore, in the lower levels of the end products of this pathway, namely, cerebrosides, sulfatides, gangliosides, and sphingomyelin. In order to determine the degree of inhibition and the type of compounds inhibited, mice were treated with warfarin for 2 weeks, after which they were killed and the brains were analyzed for sphingolipid content. Cerebroside and sphingomyelin levels were reduced in the brains of warfarin-treated mice to a low degree (by 12 and 17%, respectively). However, the level of sulfatides was markedly reduced (by 42%, $P < 0.005$) **(Table 2).** This observation on sphingolipid levels in the brains of warfarin-treated mice was repeated in three successive experiments.

Restoration of glycosphingolipid synthesis by vitamin K administration to warfarin-treated mice

Attempts were made to reverse the reduction in glycosphingolipid levels by the administration of aquamehyton (vitamin K_1) to warfarin-treated mice. Following a 2-week course of warfarin treatment, the administration of aquamephyton for 3 days resulted in *a*) a complete recovery in 3-KDS synthase activity over a 2-3-day period (Table 1), and *b*) a 33% increase in sulfatide levels compared to the warfarin-treated group ($P < 0.05$). The recovery in sulfatide synthesis over the 3-day period of vitamin K treatment was, therefore, not complete since levels did not reach those of the control untreated group. *c*) Ganglioside levels had, however, recovered completely (Table 2). *d*) In contrast to these results, cerebroside and sphingomyelin levels continued to decline following administration of aquamephyton ($P < 0.05$, Table 2).

Sulfatide synthesis was directly examined by determining the incorporation of [^{35}S] sulfate into sulfatides. Mice were given a course of warfarin for 2 weeks and [^{35}S]sulfate was injected intraperitoneally. Incorporation into sulfatides was determined after 2 days. Another group warfarin-treated mice was administered aquamephyton together with [^{35}S]sulfate and incorporation into sulfatides was determined 2 days later. The results of this experiment showed an 11% reduction in incorporation in the warfarin-administered group and a 52% increase in [^{35}S]sulfate incorporation into sulfatides after administration of vitamin K to the warfarin-treated mice.

DISCUSSION

Our results in the study of vitamin K antagonism and restoration on sphingolipid levels show that the antagonism of vitamin K by warfarin causes a significant reduction in the activity of 3-KDS synthase and in the synthesis of sphingolipids in the brains of mice. The mechanism of vitamin K participation in sphingolipid synthesis is unknown; it is possible that the

TABLE 2. Changes in sphingolipid levels after treatment of mice with warfarin and vitamin K

Parameter	Control	Warfarin	Vitamin K
Body weight (g)	26.2 ± 1.8	25.0 ± 1.6	25.4 ± 0.73
Brain weight (g)	0.48 ± 0.01	0.49 ± 0.01	0.49 ± 0.01
Prothrombin time (min)	14	51	13
Sphingolipid level (mg/g wet weight)			
A. Cerebrosides	3.33 ± 0.26	2.92 ± 0.11^{b}	2.53 ± 0.25^{b,A}
B. Sulfatides	1.46 ± 0.24	0.84 ± 0.17^{c}	1.12 ± 0.32^{a,B}
C. Gangliosides	0.40 ± 0.04	0.37 ± 0.04	0.44 ± 0.11
D. Sphingomyelin	2.48 ± 0.94	2.07 ± 0.27	1.72 ± 0.33^{a}
[^{35}-S]Sulfate incorporation into sulfatides (cpm × 10^3/g wet weight)	29.85 ± 2.66	26.00 ± 2.40	39.43 ± 4.31^{b}

Sixteen-day-old mice (five animals/group) were injected with Na warfarin (10 mg/kg body weight dissolved in saline, i.p. on alternate days) for 2 weeks. One group of experimental animals was injected with vitamin K_1 (aquamephyton, 1 mg/animal daily, i.p.) for 3 days before being killed. [^{35}S]Sulfate was injected (5μCi/g body weight, i.p.) 48 hr before the mice were killed. The animals were killed, blood was collected for prothrombin time analysis, and brains were removed. Values are expressed as means ± SD.

P values comparing warfarin group to control or vitamin K groups: ^{a}, $P<0.05$; ^{b}, $P<0.025$; ^{c}, $P<0.005$.

P values comparing vitamin K group to control group: ^{A}, $P<0.05$; ^{B}, $P<0.005$.

enzyme(s) involved in one or more biosynthetic steps contain γ-carboxyglutamic acid, which in turn suggests that these enzymes are Ca^{2+}-dependent. This postulated action of vitamin K metabolism has been discussed by Radin (20). The reduction of sulfatide levels after warfarin treatment could be the result of the inhibition of 3-KDS synthase or enzymes involved in the sulfation of galactocerebroside to form sulfatide by the inhibition of post-translational modification of the enzyme. These experiments suggest, therefore, that vitamin K plays a direct role in the synthesis of sulfatides in the brains of mice. There was a significant reduction in the levels of sulfatides following warfarin administration. This was reversed to a large degree after vitamin K was given to the warfarin group. The [^{35}S] sulfate incorporation experiment, however, did not reflect the reduction in levels found in the warfarin group. This may imply that the turnover rate of sulfatides is increased; the administration of vitamin K to the warfarin-treated animals did result in a highly significant increase in incorporation.

The specificity of the warfarin inhibition of sulfatide synthesis was unexpected; it contrasts with results obtained after treatment of mice with L-cycloserine, the potent inhibitor of 3-KDS synthase (16,21). Subcutaneous administration of L-cycloserine results in a specific reduction in cerebroside of brain with little effect on ganglioside, sulfatide, or sphingomyelin levels (16,22).

The continued drop in cerebroside levels after administration of aquamephyton to warfarin-treated mice may suggest that the cerebroside pool is utilized for sulfatide synthesis. Since the synthesis of 3-KDS is known to be rate-limiting (17–19), this would provide a mechanism for the rapid synthesis of sulfatide in the inhibited brain. A similar result was obtained following a course of L-cycloserine administration. After L-cycloserine treatment was terminated, cerebroside levels continued to fall for a period of time before recovery occurred (22). On the other hand, ganglioside synthesis, which had shown a small reduction after L-cycloserine administration, recovered quickly. Therefore, studies with both inhibitors suggest that, after a period of inhibited sphingolipid synthesis, the brain has a distinct preference for the classes of sphingolipids needed.

The ability of vitamin K to control the synthesis of certain sphingolipids might have other possible implications. Many infants are vitamin K-depleted at birth and are therefore supplemented with vitamin K to restore normal prothrombin times. The dosage of vitamin K required to restore the synthesis of sulfatides to normal levels may differ considerably from that required for the restoration of normal prothrombin times, and these infants may be subjected to impaired brain lipid levels even though prothrombin times are normal. In the present experiments for example, sulfatide levels had not completely returned to normal values 3 days after vitamin K administration whereas prothrombin times had.

Sulfatides have been implicated in the binding of thrombospondin (23) and laminin (24) and in the activation of protein kinase C by tumor promoters (25). The ability to obtain sulfatide-depleted mice by warfarin treatment may, therefore, provide a methodology with which to study the metabolic role of this class of glycosphingolipids. It should be noted that glycosphingolipids are synthesized at a high level only in the neonates of man and animals (26), and the major effect of warfarin administration would occur in the young animal where this high biosynthetic activity takes place. Since warfarin is usually administered to adults, it is not surprising, therefore, that the effect of warfarin on brain sulfatide levels was not noted previously, although the teratogenic effects of warfarin are known (27–29).

Vitamin K depletion causes structural reorganization in the membranes of *B. levii* (30) and Gla has been shown to participate in protein-protein interactions (31). It may not be surprising, therefore, that a function of the postulated γ-carboxy regions of the sphingolipid-synthesizing enzymes could also involve membrane organization in addition to enzyme activity.

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