S-4 FUMONISIN B_1 IN DEVELOPING RATS ALTERS BRAIN SPHINGANINE LEVELS AND MYELINATION.

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Fumonisin B_1 in developing rats alters brain sphinganine levels and myelination. KWON, O. S., SCHMUED, L. C., and SLIKKER, W., JR. *Neurotoxicology*. --- Objectives of this study were to test the hypothesis that fumonisin B_1 (FB_1) alters sphinganine (Sa) levels and myelin synthesis in the central nervous system of developing rats. FB_1 (subcutaneous, 0.4 or 0.8 mg/kg/day) from postnatal days (PND) 3 to PND 12 resulted in a significant reduction of body weight gain and decreased survival rates. Both Sa levels and Sa/sphingosine (So) ratios were significantly increased in the brain of rats given 0.8 mg FB_1/kg/day. To confirm the effect of limited nutrition on changes in the Sa levels and myelinogenesis, rats given 0.8 mg FB_1/kg/day or treated by limited nutrition (temporary removal from dam during postnatal period) were compared to those in saline controls. Sa levels and Sa/So ratios were increased significantly in the 0.8 FB_1-treated, but were not altered in the limited nutrition group. Myelin deposition in the corpus callosum and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) activities were decreased significantly in both nutritionally limited and FB_1-exposed rats. These data indicate that sphingolipid metabolism in the central nervous system of developing rats is vulnerable to FB_1 exposure. The hypomyelination associated with FB_1-treatment may be mediated by limited nutrition.

Key Words: Fumonisin B_1, Mycotoxin, Sphinganine, Sphingosine, Hypomyelination, 2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase; E.C.3.1.4.37).

INTRODUCTION

Fumonisins are produced predominantly by the fungus *Fusarium moniliforme* throughout the world and contaminate dietary staples such as corn (Sydenham *et al.*, 1990; Nelson *et al.*, 1991; Visconti and Doko, 1994). Fumonisin B_1 (FB_1), the major toxic metabolite, has been shown to have cancer-promoting effects in rats (Gelderblom *et al.*, 1988; Marasas *et al.*, 1984), to cause swine pulmonary edema (Harrison *et al.*, 1990), and to be associated with human esophageal cancer (Marasas *et al.*, 1988a; see Norred, 1993, for review).

Equine leukoencephalomalacia, the most common toxicity caused by *Fusarium moniliforme* in horses and donkeys, is a neurotoxic disease that is characterized by multifocal liquefactive necrosis of predominantly the white matter of the brain (Wilson *et al.*, 1973; Wilson and Maronpot, 1971; Marasas *et al.*, 1988b). A horse intravenously administered FB_1 showed clinical signs of neurotoxicity including severe edema and focal necrosis in the brain, providing evidence that FB_1 causes equine leukoencephalomalacia (Marasas *et al.*, 1988b).

FB_1 has been reported to be a naturally occurring inhibitor of ceramide synthase in rat liver and mouse brain microsomes (Wang *et al.*, 1991; Merrill *et al.*, 1993). This enzyme plays a

pivotal role in *de novo* sphingolipid biosynthesis and turnover. Free sphinganine (Sa) and sphingosine (So) levels were increased in lung, kidney, liver, serum, and urine from pigs and rats (Riley *et al.*, 1993, 1994) and in pony serum after FB_1 exposure (Wang *et al.*, 1992). Brain So and Sa levels, however, were not reported in pigs or rats exposed to FB_1. Recently, Goel *et al.*(1995) showed that Sa levels were elevated in horse brain tissues. A comprehensive study from prenatal FB_1 treatment in rats suggested that of the many behaviors examined, only a minor depression in acoustic startle response during the first or second block of 9 trials in male rats, but not females, was observed (Ferguson *et al.*, in press). These data support that the hypothesis that systemic absorption and/or placental transport of FB_1 may be low after maternal oral administration (Voss *et al.*, 1996; Ferguson *et al.*, in press).

Sphingolipid biosynthesis has been shown to be inhibited in cultured neurons and a cell line treated with FB_1 (Merrill *et al.*, 1993; Yoo *et al.*, 1992). Alterations of sphingolipid metabolism and myelin synthesis in the central nervous system may be caused by FB_1 exposure and the direct disruption of brain enzyme activity involved in sphingolipid synthesis and/or damage to myelinating cells. In support of this theory it has been reported that: (1) 35% of the myelin lipid in both the rat and human brain is glycosphingolipid (cerebroside and sulfatide) and sphingomyelin (Norton and Cammer, 1984), the key components in the myelination of axons in the brain and spinal cord, (2) FB_1 is considered a potent inhibitor of ceramide synthase, the rate-limiting step in the synthesis of sphingolipid (Merrill *et al.*, 1993), (3) sphingolipid biosynthesis and axonal outgrowth in cultured hippocampal neurons are inhibited by FB_1 (Harel and Futerman, 1993), and (4) FB_1 (>35 μM) and Sa have been reported to be cytotoxic (Yoo *et al.*, 1992; Stevens *et al.*, 1990). The purpose of the present work was to test the hypothesis that FB_1 causes alterations of brain sphingolipid metabolism and myelin deposition.

MATERIALS AND METHODS

Reagents. FB_1 (as a free acid) was obtained from the Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration (FDA), Washington, D.C. Purity of the FB_1 as analyzed by the Division of Chemistry of the National Center for Toxicological Research (NCTR) was found to be >91% FB_1 (FB_1-related compounds such as FB_1 methylester and dimers are known major impurities, while the toxicant fumonisin B_2 (FB_2) is not an impurity). D-Sphingosine, DL-*erythro*-dihydrosphingosine (C_{18}-sphinganine, defined as sphinganine), DL-*threo*-dihydrosphingosine, psychosine, o-phthaldialdehyde, and FB_2 were purchased from Sigma (St. Louis, MO). C_{20}-sphinganine was obtained from Dr. A. H. Merrill, Jr. (Atlanta). Gold chloride (hydrogen tetrachloroaurate trihydrate) was purchased from

Aldrich (Milwaukee, WI). The HPLC grade of methanol and chloroform were obtained from J. T. Baker (Phillipsburg, NJ). The other agents used for sphingoid bases and FB_1 analysis were of analytical grade.

Animals. Plug-positive Sprague-Dawley rats were obtained from the NCTR breeding colony and housed individually in polycarbonate cages with woodchip bedding (Northeastern product, Warrensburg, NY). The colony room was maintained at a constant temperature (23 ± 3°C) and humidity (50 ± 20%). A 12-hr light/12-hr dark cycle was maintained beginning at 6:00 am. Millipore-filtered tap water and feed (NIH-31) were provided *ad libitum*. Pups born between 7:00 am and 7:00 pm were considered the same age (Postnatal day 0).

Chronic FB_1 administration. Pups were weighed each day to monitor changes in body weight. Brain wet weight was measured at the time of sacrifice.

Experiment 1: Postnatal day 2(PND2) male rats were culled, footmarked with black ink (color code 9053 Black, Spaulding Color Corp., Voorheesville, NY) for identification, and weighed. The rats were randomly divided into saline control, 0.1, 0.4 and 0.8 mg FB_1/kg groups (in 5 ml saline/kg). Each dam was given 10 pups. FB_1 was administered subcutaneously once a day (9:00 am) from PND 3 to PND 12 using a 100-250 μl Luer-lock microsyringe (Hamilton Co., Reno, Nevada; for example, 5 μl saline or FB_1 solution/g rat). The rats were sacrificed 24 hr after the last dose.

Experiment 2: In order to determine if nutritional deficiencies in developing rats may have an effect on Sa levels and myelin deposition, three groups of rats treated with saline, limited nutrition, or FB_1 (0.8 mg/kg/day) were maintained in the same condition as described above. Limited nutrition was produced as described by Krigman and Hogan (1976) by separating pups from the dam for 6 to 7 hrs per day (from PND 3 to PND 12).

Brain dissection. Animals were sacrificed by decapitation. Whole brain were removed rapidly, dissected and placed on dry ice. Whole brain was dissected into brainstem and cerebellum; remaining portions of the brain were divided into forebrain (including olfactory bulb, olfactory tubercle, caudate nucleus, and frontal cortex) and hindbrain (including hippocampus, occipital and entorhinal cortex). The dissected regions were stored at -70°C until analysis. The forebrains and brainstems were used for the analysis of sphingoid bases; hindbrains were stored for the analysis of FB_1.

HPLC instrumentation. HPLC analysis was performed with a Waters' model M-6000A pump connected to a 16 μl flow cell fluorescence detector (Waters 470, Waters, Milford, MA). A Waters 746 integrator was used. A C_{18} Nova-Pak column (60 Å, 4 μm, 3.9 x 150 mm) was obtained from Waters. The mobile phase used for sphingoid base analysis consisted of methanol/5 mM potassium phosphate buffer (9:1, v/v) at the flow rate of 1.1 ml/min, and the detector was set at excitation and emission wavelengths of 340 and 440 nm, respectively. Attenuation and gain of the detector were set at 4 and 1000, respectively. Attenuation of the integrator was fixed at 16.

HPLC analysis of sphingoid bases in brain tissues. Two regions of brain were homogenized by ultrasonification (Cell Disruptor W-350 Sonifier, Branson Ultrasonics Corp., Danbury, CT) for 10 sec in 4 volumes of 50 mM potassium phosphate buffer (pH 7.0). One hundred μl of the forebrain and brainstem homogenates was used. C_{20}-sphinganine (10 μM, 20 μl) was added to the homogenate in a screw-cap culture tube (13 x 100 mm, with screwed Teflon cap) and vortexed. One half ml of borate buffer (0.4 M, pH 13.1) and 4 ml of chloroform/methanol (9:1, v/v) were added. The layers were agitated on a shaker (Eberbach Corp., Ann Arbor, MI) for 20 min, centrifuged (Hermle Z360K, Woodbridge, NJ) for 8 min at 2000 rpm (600 x g) at 4 °C. The chloroform layer was evaporated in an evaporator (Savant, Savant Instruments Inc., Farmingdale, NY). The culture tubes were shaken for 5 min and centrifuged after the addition of 0.5 ml of borate buffer (0.4 M, pH 4.4) and 3 ml of n-hexane to the residue. The hexane layer was then removed by suction. 40 μl of 10 N potassium hydroxide followed by 4 ml of chloroform/methanol (2:1, v/v) was added to the aqueous layer. The organic layer was rinsed with 3 ml of borate buffer (0.4 M, pH 10.5) and evaporated. Finally, samples were derivatized with o-phthaldialdehyde (OPA) and were detected by fluorescence. The residue was dissolved in 200 μl of methanol and 100 μl of borate buffer (0.4 M, pH 10.5). 20 μl of OPA solution (10 mg OPA dissolved in 1 ml methanol containing 100 μl of 0.4 M borate buffer (pH 10.5) and 10 μl of 2-mercaptoethanol; total volume=1.11 ml) was added. After 10 min, the solution was filtered by centrifuging with a 0.2 μm nylon membrane (Bioanalytic system, Westlafayette, IN), and 5 μl of the brain tissue solution was injected onto the HPLC. Retention times for sphingosine, sphinganine, and internal standard were 6.1, 8.3 and 14.5 min, respectively. Relative recoveries were 89.3 ± 4.9% (CV, 9.5%; n=3) for sphingosine and 94.3 ± 1.2% (CV, 1.1%; n=3) for sphinganine. From these determinations, the Sa/So ratios were also calculated.

Histochemical myelin stain and quantification. PND 13 rats were anesthetized (320 mg/5 ml/kg; ip) with sodium pentobarbital for a surgical preparation. The rats used for myelin histochemistry were perfused with 10% formaldehyde in 0.1 M phosphate buffer (40 ml; pH 7.3) after pre-washing with saline solution (10 ml) through the left ventricle or the ascending aorta at the perfusion rate of 4 ml/min. The whole brain was removed and post-fixed for one week in 10% formaldehyde in 0.1 M potassium phosphate buffer containing 20% sucrose and subsequently sectioned on a freezing microtome (Shandon Lipshaw, Pittsburgh, PA) at 35 μm. The staining procedure for floating sections in baskets was followed as described in Schmued (1990). The stained sections were mounted on gelatin-covered slides and the optical density of the myelin stain was digitized by means of graphic density display for densitometric analysis using a microcomputer imaging device (MCID, Imaging Research Inc., Ontario, Canada). The optical density in bilaterally symmetrical regions of forebrain corpus callosum was averaged.

Assay of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP; E.C.3.1.4.37) in brain tissues. The activity of CNP, a specific marker enzyme associated with myelin and oligodendrocytes, was

measured by the modified method described in Weissbarth *et al.*(1980). Brain homogenate aliquots used in the Sa analysis were diluted in 1.0% Triton X-100 (0.1 to 0.5 mg protein/ml). A 10 μl aliquot of the homogenate was added to 150 μl of reaction mixture (0.2 M 2-[N-Morpholino]ethanesulfonic acid buffer, pH 6.0; 1 mM β-Nicotinamide adenine dinucleotide 2',3'-cyclic monophosphate; 30 mM $MgCl_2$; 1 mM EDTA; 1.5 mM glucose-6-phosphate; 0.03% bovine serum albumin and 23 μg/ml glucose-6-phosphate dehydrogenase (EC 1.1.1.49; 190 Units/mg, Sigma)). The tissues were incubated for 20 min at 25°C and the reaction was stopped by the addition of 1 ml of 50 mM sodium bicarbonate buffer (pH 10.6). Fluorescence of NADPH produced was determined with a fluorescence spectrophotometer. Protein content was determined with bicinchoninic acid using bovine serum albumin as the standard (Smith *et al.*, 1985).

Statistics. The chronically treated rats were scheduled in 7 replicates. Each replicate consisted of pups from 3 plug-positive dams (one replicate gave an average of 15 male pups from 2 dams). Female pups were added to make 10 pups per dam per cage that were randomly divided into each group. Each pup served as a statistical unit. All data were presented as mean ± standard error (SE). The general linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC) was used for one way analysis of variance. When F values showed significance, Duncan's multiple-range test was applied to evaluate significant difference. Statistical significance was based on $p<0.05$ level.

RESULTS

Body weight gain and mortality on PND 13 after chronic treatment with FB_1. Body weight gain and mortality observed at PND 13 from rats subcutaneously administered FB_1 (0.1 -0.8 mg/kg) from PND 3 to PND 12, are shown in Table 1. The body weight gains were 63% and 52%, respectively, of saline controls after administration of 0.4 or 0.8 mg/kg/day of FB_1. Mortality was 14% at 0.4 mg FB_1/kg/day, and 31% at 0.8 mg FB_1/kg/day. The body weight gain of the limited nutrition group was 72% of the saline controls; no mortality was observed in these rats.

Dose-dependent effects of FB_1 on Sa levels and Sa/So ratios in brains. In the forebrain, 0.8 mg/kg/day FB_1 significantly increased Sa levels 4.8-fold greater than saline controls. FB_1 did not alter So levels in the same tissue (Fig. 1A). Ratios of Sa to So in forebrain showed significant increases of 3.5-fold at this dose (Fig. 1A). In brainstem, Sa levels were significantly increased in rats treated with 0.8 mg FB_1/kg/day by 2.9-fold (Fig. 1B). Significant increases in Sa/So ratios were observed in the brainstem of rats treated with 0.4 mg/kg/day (2.1-fold) and 0.8 mg/kg/day (2.8-fold) of FB_1 (Fig. 1B). Sa levels and Sa/So ratios of the 0.1 and 0.4 mg/kg/day FB_1 group were similar to those of saline controls (except for the Sa/So ratio in brainstem at 0.4 mg/kg/day; Fig. 1).

Effects of limited nutrition and FB_1 on Sa levels and Sa/So

ratios. The PND 13 body weight gain of nutritionally limited pups was 72% of saline controls (Table 1). The plots of body weight gain from PND 2 to PND 13 are showed in Fig. 2A. Body weight gain of limited nutrition and FB_1 groups is significantly different from that of saline control beginning from PND 5 and PND 6, respectively. Fatalities resulting from FB_1 exposure typically occurred in PND 12 or PND 13. PND 13 body weight was 75% and 64% of saline controls (25.5 ± 0.54g, n=10) in the limited nutrition and the 0.8 mg/kg/day FB_1-treated group, respectively (Fig. 2B, left box). Brain weight was 87% and 78% of saline controls (1.13 ± 0.02 g, n=10) in the limited nutrition and the 0.8 mg/kg/day FB_1 treated group, respectively (Fig 2B, right box).

Sa concentrations and Sa/So ratios in the forebrain (Fig. 3A) and brainstem (Fig. 3B) were significantly increased by FB_1 treatment. Limited nutrition, however, did not affect sphingoid base levels or ratios in any of the regions analyzed (Fig. 3).

Effect of FB_1 on the myelin deposition and CNP activity. Photomicrographs of gold chloride-stained myelin tracts in the dorsal corpus callosum of the forebrain are shown in Fig. 4. The corpus callosum in FB_1-treated rats was narrow relative to that of control. Myelin deposition measured by the optical density of stained sections of comparable thickness in the limited nutrition and FB_1-treated groups is shown in Fig. 5. FB_1 resulted in a significant dose-dependent decrease in myelin deposition in the 0.4 (61% of control) and 0.8 (48% of control) mg/kg/day dose groups. Limited nutrition also significantly decreased myelin deposition (37% of control; Fig. 5). CNP activity in the forebrain exhibited a significant decrease relative to control of 31% in the 0.8 mg/kg/day FB_1 group and of 17% in the limited nutrition group (Table 2). A dose-dependent decrease of the CNP specific activity was also noted in the forebrain (Fig. 6). CNP activity in brainstem was significantly decreased by 22% in the 0.8 mg/kg/day FB_1 group and 11% in the limited nutrition group as compared to the corresponding controls (Table 2).

DISCUSSION

This study focused on the effects of FB_1 on Sa levels and myelin changes in the rat developing central nervous system. These data are relevant because fumonisins have been reported to alter Sa/So ratios in serum collected from ponies with FB_1-induced neurotoxicity (Wang *et al.*, 1992). Similar alterations in Sa/So ratios have also been reported in serum and livers from rats and pigs (Riley *et al.*, 1994, 1993).

The developing nervous system in rats is vulnerable to perturbations by environmental toxicants (Vorhees, 1992; Spyker, 1975). Brain growth achieves its maximum velocity during postnatal development (about PND 7) in the rat (Dobbing and Sands, 1979). It is generally accepted that the immature brain has a greater passive permeability of lipid-insoluble molecules between blood and brain tissue or cerebrospinal fluid than does the mature brain (Saunders, 1992; Saunders and Mollgard, 1984). In rat cerebral cortex, the maximal proliferation of capillary

cells occurs between PND 5 to 9 (Robertson *et al.*, 1985), and some drugs (*e.g.*, phenobarbital, phenytoin) can permeate the neonatal brain more readily than the adult (Cornford *et al.*, 1983). Therefore, it appears that the developing rat may be a more sensitive model to study FB_1 effects than is the adult rat because FB_1 is a hydrophilic, lipid-insoluble compound possessing a relatively large molecular weight (721.8 daltons).

Our results demonstrated that FB_1 treatment altered Sa levels in two regions of the brain. Sa/So ratios were increased significantly in these tissues (Fig. 1). In the same regions of the central nervous system, rats with limited nutrition did not demonstrate altered Sa levels or Sa/So ratios (Fig. 3). These data suggest that sphingolipid metabolism in the central nervous system of developing rats is vulnerable to FB_1 exposure.

Goel *et al.*(1995) reported that brain Sa levels, but not Sa/So ratios, were significantly elevated in horses that developed equine leukoencephalomalacia. It is notable that FB_1 elevates free sphingoid levels in developing rats as does the phenomena of Niemann-Pick disease (type A) in the human fetus (gestational ages; 16-20 weeks). The latter condition, however, causes much more accumulation of So than Sa in cerebral cortex, liver and spleen (Rodriguez-Lafrasse *et al.*, 1994) while the former increased Sa in the brain of developing rats without affecting So levels (Fig. 1 and Goel *et al.*, 1995). This suggests that alterations of brain sphingolipid metabolism may be related to the outcome of animal or human diseases.

Sphingolipid is one of the key components in the myelination of axons in the brain and spinal cord (Norton and Cammer, 1984). Recent studies with cultured mouse cerebellar neurons showed that FB_1 reduced complex sphingolipid synthesis (Merrill *et al.*, 1993). Myelin deposition was significantly decreased in rats treated with 0.4 and 0.8 mg FB_1/kg/day and in the limited nutrition group (Fig. 5). While limited nutrition resulted in reduced myelin deposition and CNP activity with no change in Sa levels, FB_1 treatment reduced these endpoints with increases in Sa levels. Therefore, limited nutrition appears to have no effect on the disruption of Sa N-acyltransferase activity.

Effects of FB_1 on myelin changes were similar to those in the limited nutrition group, because both 0.8 mg/kg/day FB_1 and limited nutrition resulted in significant reductions in both myelin deposition and CNP specific activity. FB_1 treatment resulted in greater reduction of body weight gain and in decreased survival rates as compared to that observed with limited nutrition, possibly because FB_1 effects on growth could include other factors such as hepatotoxicity. Limited nutrition did not alter Sa levels (Fig. 4). Factors affecting myelin deposition after FB_1 exposure may include (i) nutritional effects, (ii) effects of increased brain Sa levels, and/or (iii) Sa-independent FB_1-induced toxicity. The question of whether Sa itself can cause limited nutritional effects and hypomyelination remains to be answered in further studies. Because postnatal nutritional deprivation may have an effect on reduction of myelin synthesis (Wiggins *et al.*, 1976), the myelin reduction associated with FB_1 treatment in the present study may be due to nutritional

deficiency. FB_1 treatment in developing rats, therefore, appears to cause secondary hypomyelination mediated by limited nutrition.

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TABLE1

Body weight gain and mortality on postnatal day (PND) 13 in fumonisin B_1 (FB_1)-dosed and limited nutrition (LN) groups

Dose of FB_1 (mg/kg/day)	Weight gain (%) at PND 13[a] (% of saline control)	Survived /Total (%)
saline	251 ± 8 (100)	24/24 (100)
0.1	264 ± 12 (105)	12/12 (100)
0.4	159 ± 13** (63)	12/14 (86)
0.8	130 ± 10** (52)	25/36 (69)
LN	181 ± 7** (72)	10/10 (100)

[a]Percentage of body weight (BW) gain was calculated by the equation: ((BW of PND 13 - BW of PND 2)/(BW of PND 2)) x 100.

** Significantly different from saline controls ($p<0.001$).

TABLE 2

2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNP) specific activities in forebrain and brainstem

	Forebrain[a]	Brainstem[a]
Saline (n=7)	463.9 ± 15.3	4052.0 ± 87.1
Limited nutrition (n=6)	387.3 ± 26.4*	3598.3 ± 111.0*
0.8 mg/kg/day FB_1 (n=14)	318.9 ± 15.6**	3176.7 ± 108.6**

[a]CNP specific activity was expressed as nmole of 2'-NADP produced/min/mg protein. Each value was expressed as mean ± SE.

Significantly different from the saline groups (*, $p<0.05$; **, $p<0.01$).

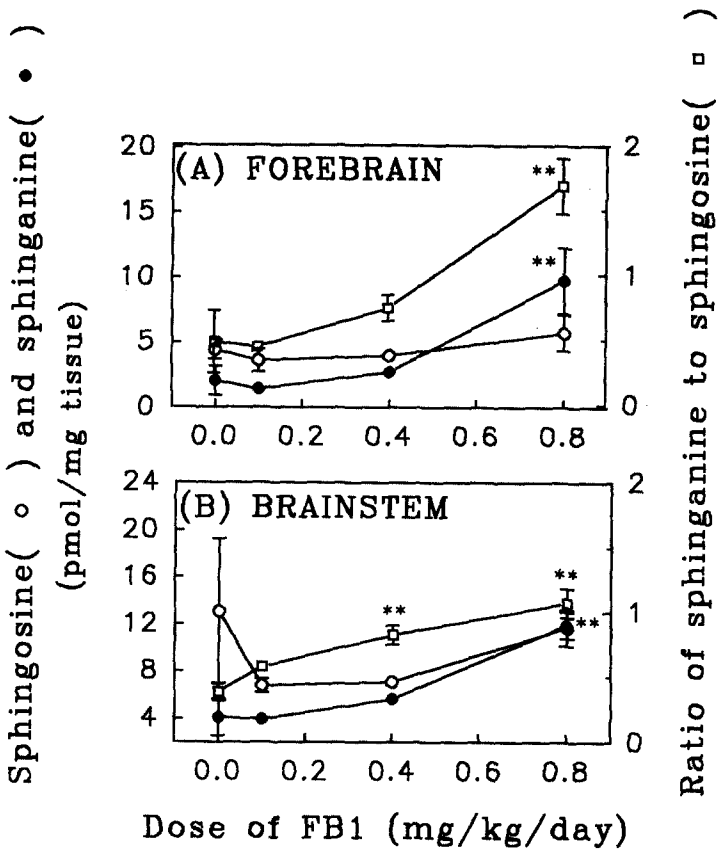

FIG. 1. Dose-dependent changes of FB_1 on sphingosine levels (open circles), sphinganine levels (closed circles) and ratios of sphinganine to sphingosine (open squares) in forebrain (A), brainstem (B). Values are mean ± SE of the saline control (n=7) or FB_1 groups at 0.1 (n=7), 0.4 (n=8), and 0.8 (n=7) mg/kg/day (PND 3 to 12). Significantly different from controls (**, $p<0.01$).

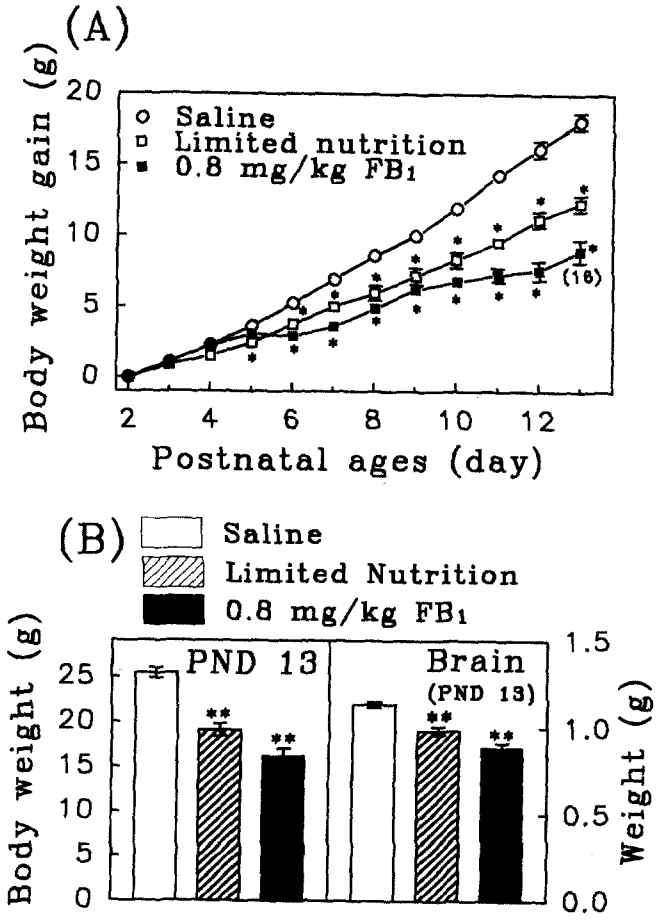

FIG. 2. (A) Effects of FB_1 (0.8 mg/kg/day, PND 3-12) or limited nutrition on body weight gain. Body weight gain was obtained as mentioned in the footnote to Table 1. Values are mean ± SE of saline (n=10), limited nutrition (n=10), and FB_1 (n=21) groups. *Body weight gain of both limited nutrition and FB_1 groups is significantly ($p < 0.001$) different from that of saline controls. (B) Body weight and wet brain weight in saline (n=10), FB_1 (n=16) and limited nutrition (n=10) groups. Values are mean ± SE. **Significantly different from the saline control ($p<0.01$).

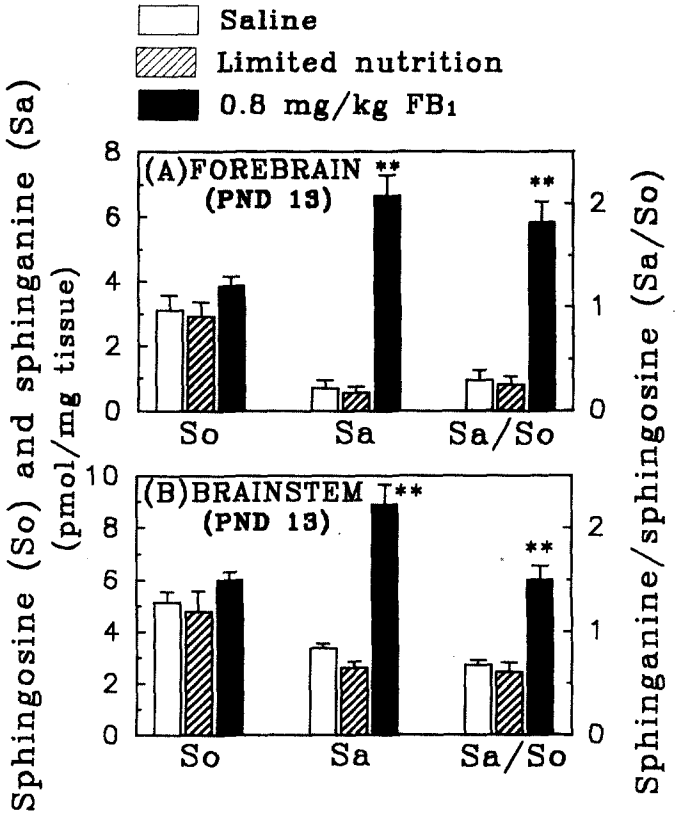

FIG. 3. FB_1 (0.8 mg/kg/day, PND 3-12) increases sphinganine levels and sphinganine/ sphingosine ratios in the forebrain (A), brainstem (B). Numbers of rats in each group: saline (n=7), limited nutrition (n=6), and FB_1 (n=14). **Significantly different from the saline controls ($p<0.01$).

FIG. 4. Photomicrographs of frozen forebrain sections showing myelin tracts in corpus callosum of rats treated with saline (A) or 0.8 mg FB_1/kg/day (B). The major myelin tract (arrow) of the corpus callosum in FB_1-treated rats is narrow as compared to control. Gold chloride stain; scale bar, 250 μm.

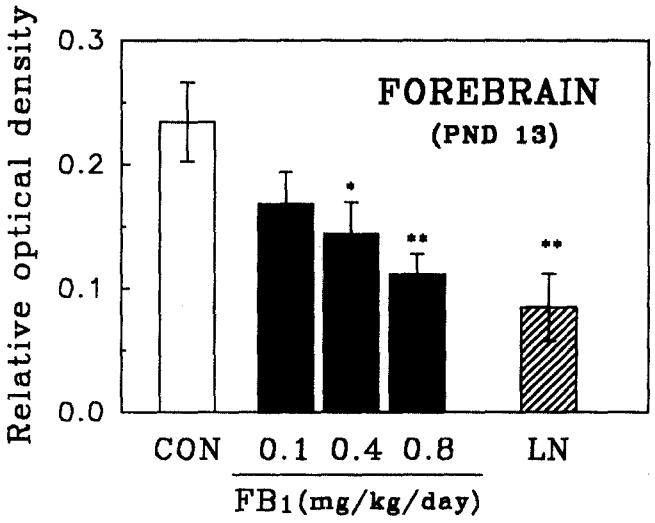

FIG. 5. Dose-dependent effects of FB_1 or limited nutrition (LN) on myelinogenesis in the corpus callosum of forebrain. Numbers of rats in each group: saline (n=11); 0.1 (n=5), 0.4 (n=4), and 0.8 (n=5) mg/kg/day FB_1; and LN (n=4). The optical density in FB_1 treated (0.4-0.8 mg/kg/day; closed bars) and limited nutrition (LN, hatched bar) groups was significantly decreased (*, $p<0.05$; **, $p<0.01$) compared to control (CON, open bar).

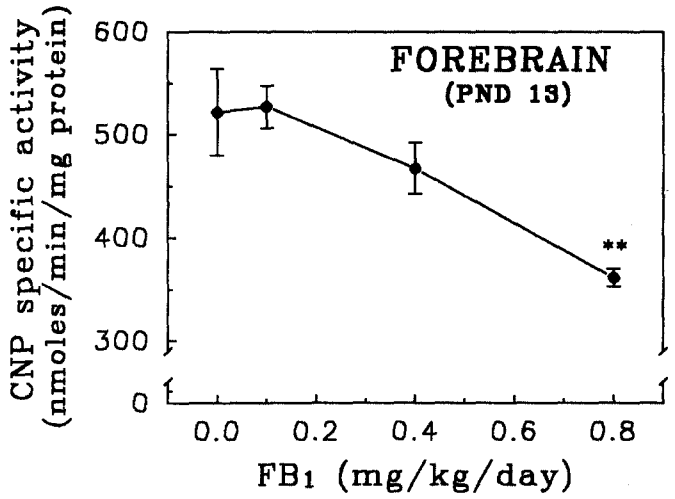

FIG. 6. CNP specific activity in forebrain (performed using an aliquot of the same forebrain homogenate described in Fig. 1). Experimental numbers for dose groups were the same as mentioned in Fig. 1. **Significantly different from control ($p<0.001$).