

Possible Role of Phosphatidylcholine and Sphingomyelin on Fumonisin B1-mediated Toxicity

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A major corn-related mycotoxin, fumonisin B1 (FB1), continues to attract attention of researchers as well as risk-assessors due to the diverse toxicological characteristics, including distinct target tissues in different animal species and opposite susceptibility in males and females in mice and rats. More than thirty years passed since the structure identification as a sphingoid-like chemical, but the causal mechanism of the toxicity remains obscure in spite of extensive studies. Considerable amounts of knowledge have been accumulated on the biochemical/toxicological actions of FB1, but the influence on lipid dynamics and mobilization in the body has not been focused well in relation to the FB1-mediated toxicity. Considerable influences of this toxin on mobilization of sphingolipids and phospholipids and also on adaptive changes in their compositions in tissues are implicated from recent studies on FB1-interacting ceramide synthases. Accumulated patho-physiological data also suggest a possible role of hepatic phospholipid on FB1-mediated toxicity. Thus, a mechanism of FB1-mediated toxicity is discussed in relation to the mobilization of phospholipids and sphingolipids in the body in this context.

Key words: mycotoxin in foods, phospholipid mobilization, ceramide, sphingomyelin, species difference in target tissues, sex-related difference in susceptibility

1. Introduction

Fumonisin B1 is isolated as mycotoxins from cultures on corn of *Fusarium verticillioides* (*moniliforme*)¹⁻³). Fumonisin B1⁴ (FB1*, **Fig. 1**) has an IUPAC name of (2R,2'R)-2,2'-[[[(5R,6R,7S,9S,11R,16R,18S,19S)-19-amino-11,16,18-trihydroxy-5,9-dimethyl-6,7-icosanediyl]bis[oxy(2-oxo-2,1-ethanediy)]]]disuccinic acid (C₃₄H₅₉NO₁₅, Mw 721.83).

This chemical is soluble in water and stable at room temperature and to light. FB1 has values of LogP is 2.20 and LogD at pH 7.4 is -3.88. The acid part, also called as carballylic acid, has a chelating capability with metals of divalent cations^{5,6}. This chemical is known to inhibit sphinganine/sphingosine N-acyltransferase (ceramide synthase/CerS), causing an accumulation of sphingoid bases (IC₅₀ ~75 nM)⁷, and also inhibits protein phosphatases (PP); IC₅₀ values are 80, 300,

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; C1-unit, one carbon unit; CCT α , CTP:phosphocholine cytidyltransferase α ; CDE, choline-deficient; ethionine-supplemented; CerS, ceramide synthase; DAG, diacylglycerol; ED, embryonic day; FB1, fumonisin B1; GD, gestational day; GPI, glycosylphosphatidylinositol; HDL, high-density lipoprotein; LC/MS, liquid-chromatography mass-spectrometry; LPA, lysophosphatidic acid; Mrp, multidrug resistance-associated protein/ABCC transporter; NTD, neural tube defect; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pemt, phosphatidylethanolamine N-methyltransferase; P-gp, p-glycoprotein/MDR1/ABCB1 transporter; PLA2R, phospholipase A2 receptor; PP, protein phosphatase; SD, Sprague-Dawley; sPLA2, secretory phospholipase A2; SMS, sphingomyelin synthase

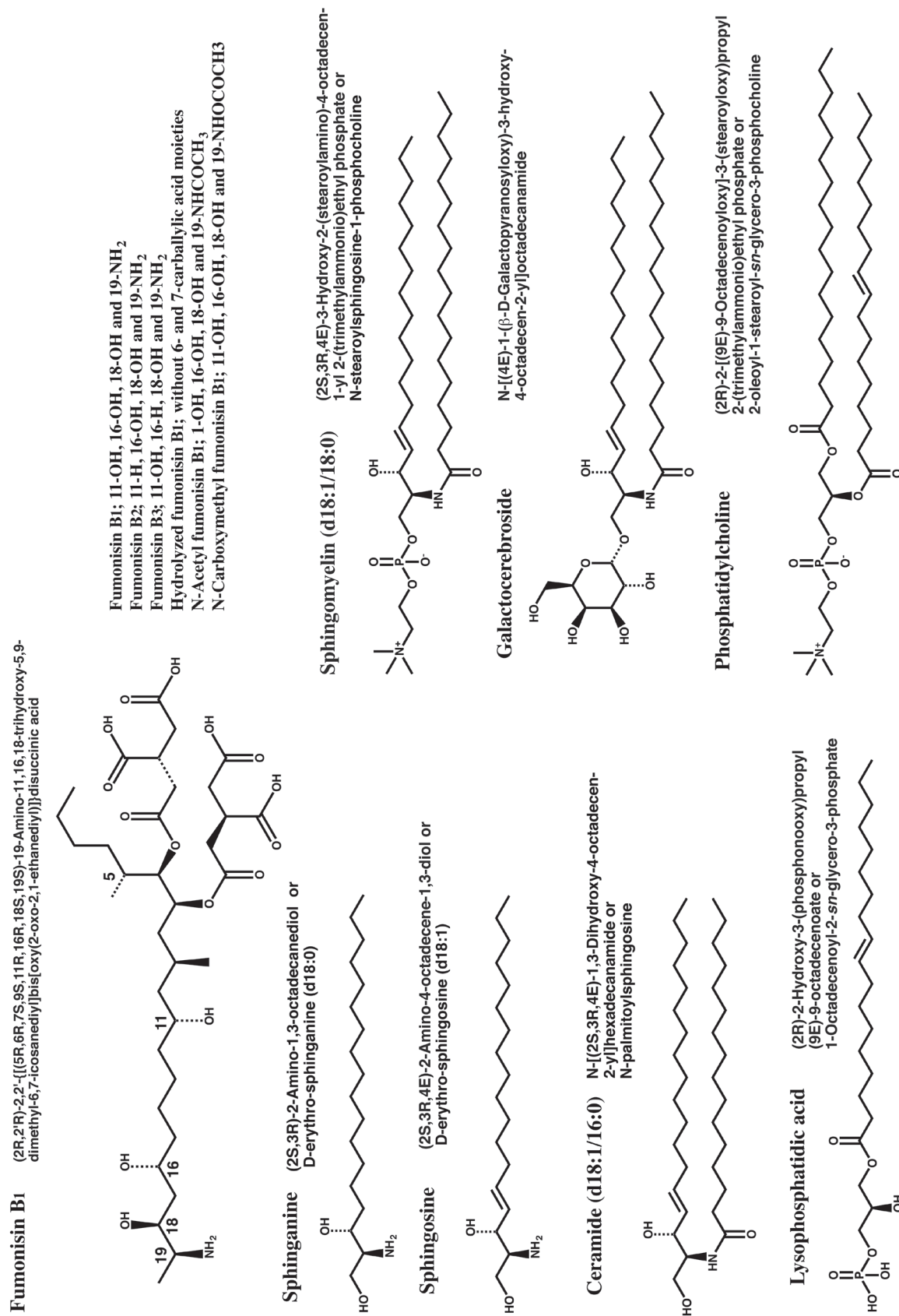


Fig. 1. Structures of typical fumonisin derivatives, sphingoids and phospholipids. Sphingoids, ceramides and phospholipids are shown with their IUPAC names and synonyms. Only a typical fatty acid conjugate is shown for ceramide, sphingomyelin, galactocerebroside, lysophosphatidic acid and phosphatidylcholine. D18:1, dihydroxysphingoid base with 18 carbons and one double bond^[8,19]. *Sr* (stereospecific numbering): Secondary hydroxyl group is shown to the left of C-2 in a Fischer projection. The carbon atom above C-2 is called C-1, the one below C-3; the use of this “stereospecific numbering” is indicated by the prefix “*sr*” before the stem-name of the compound^[7].

400, 500 and 3000 μM for PP5, PP2C α , PP2A, PP1 γ 2 and PP2B, respectively⁸). FB1 is also reported to interact with arginosuccinate synthase, a urea cycle enzyme⁹).

Wide ranges of food contamination and distinct toxicity targets in different animal species of this toxicant continue to attract attentions of regulatory authorities as well as academic researchers^{10–12}). Several reports are available on the toxicity of FB1 and the related chemicals^{12–16}). These studies suggested interactions of ceramides/sphingoids on FB1-mediated toxicity. Ceramides, sphingoids^{17–19}) and their metabolites have been discussed as rheostats of cellular signals^{20–22}). In fact, several reports are available on FB1-mediated alterations in cellular signals accompanying the changes in ceramide and sphingoid levels^{23–25}). In spite of extensive elaborative researches, causal mechanisms of FB1 toxicity remain obscure *in vivo* in animal species.

There are couples of unanswered queries on the mechanisms of FB1-mediated toxicity. The major queries include 1) species differences in target organs, 2) sex-related difference in liver and kidney toxicities in rodents, and 3) whether sphingosine is an etiological factor or a surrogate marker. These queries are linked profoundly to a safety assessment of fumonisin intakes from foods. Previous studies on FB1 toxicity mostly dealt with the influence on biological/pharmacodynamic actions of sphingolipids and their metabolites, and poorly focused on the influence on mobilization of individual lipids. Liver is the common target of FB1-mediated toxicity throughout experimental and domestic animals, and cholestatic signs are invariably detected in the exposed animals as described in details below. These data suggested a possible role of hepatic phospholipid supply for the bile excretion on FB1-mediated toxicity. Therefore, the possible mechanism of FB1-mediated toxicity is discussed focusing on the mobilization of phospholipids and sphingolipids in FB1-exposed animals.

*FB1 preparations isolated from cultured materials are used in the biochemical and toxicological studies. The preparation is often noted to contain FB2 and/or other derivatives. Here, FB1 refers to preparations of more than 90% pure.

2. Profiles of FB1 pharmacokinetics in Typical Animal Species

2.1 Pharmacokinetics in Rats

Early studies on FB1 using fluorescent derivatization for the detection showed the rapid disappearance from blood circulation and the minimal elimination to urine. Plasma half-life of FB1 quantified as the fluorescent was shown as 3.15 hr after the p.o. administration of 10 mg/kg b.w. to male Wistar rats, whereas it was calculated 1.03 hr after the i.v.

administration of 2 mg/kg b.w.²⁶). Plasma half-lives were calculated as 1.164 and 1.69 hrs after the subcutaneous injection of FB1 0.8 and 8 mg/kg b.w., respectively, in new born male Sprague-Dawley (SD) rats²⁷). In the oral study, FB1 stayed for longer periods in liver (t_{1/2}, 4.07 hr) and kidney (t_{1/2}, 7.07 hr) than in the plasma. Plasma half life of FB1 is reported to be 18 min after the i.p. administration in male BD IX rats²⁸). Experimental data described above showed the discordance on the disappearance rates of blood FB1 between the oral and parenteral administrations²⁶). Due to the invariability of the elimination kinetics of an identical chemical among different routes of the administration, the terminal portion of the curves observed in the oral experiments is rather likely to represent the absorption rate (in the so-called a flip-flop model). Continued absorption of FB1 is thus expected after the oral administration, although the extent of the absorption is limited in rats.

Studies using 5,9-dimethyl-¹⁴C-enriched FB1²⁹) confirm the major role of bile excretion and the minimal role of urinary excretion. The biliary excretion recovered in 24 hr shares 67% of the radioactivity given i.p. to male Wistar rats at a dose of 7.5 mg/kg b.w. of ¹⁴C-FB1³⁰).

Soon after the intravenous injection of ¹⁴C-FB1 (4.5 μg /head), the blood level dropped rapidly and the levels in liver and kidney exceed the blood level between 0.5 hr and 96 hr after the administration³¹). These data suggest the sequestration of FB1 in tissues including kidney and liver. In addition, a high level of ¹⁴C-FB1 is also continuously detected in the gastrointestinal tract.

The blood level of ¹⁴C-FB1 was increased rapidly after the intragastric administration (1 mg/head after addition of unlabeled FB1) and then the low but stable ¹⁴C-FB1 level was maintained until 96 hr. In the three consecutive p.o. administrations of ¹⁴C-FB1 (1 mg), levels of ¹⁴C-FB1 radioactivity in liver and kidney are somewhat higher at 24 hr after the third administration than at the point just after the third administration³¹). These data suggest the temporal stationary state of ¹⁴C-FB1 in both tissues during the repeated administration.

Cumulative urinary excretion of unchanged FB1 was 2.3, 0.8 and 0.4%, respectively, until four days after gavage administrations of 0.69, 6.93 or 69.3 $\mu\text{mol/kg}$ FB1 (corresponding to 0.5, 5 or 50 mg/kg b.w.) to male F344 rats. After hydrolysis of the urine, 7.4, 1.2 and 0.5% of the respective doses were recovered³²). Thus, no dose-dependent increases were observed in urinary excretion of FB1 in rats.

2.2 Pharmacokinetics in Pigs

After an intravenous administration of ¹⁴C-FB1 (0.4 mg/kg b.w.), the plasma disappearance rate was calculated 10.5 min in pigs³³). Total 21.2% and 58.3% of the radioactivity are

recovered in urine and feces for three days, respectively. High levels of the radioactivity remain in the kidney and liver among the tissue examined.

Following the intragastric administration of ^{14}C -FB1 (0.5 mg/kg b.w.), the radioactivity appeared in the blood after 30 min and most was eliminated in the feces (87 to 95% of the dose) within 72 hr³³). Less than 1% of the dose was excreted in urine. The oral bioavailability was estimated at around 4% in pigs.

In an oral study using bile duct-cannulated animals, most of the recovered radioactivity was found in the feces (86 to 94% of the dose) after 72 hr³⁴). Only small amounts of the radioactivity were recovered from the bile (1.7% of dose) and urine (1.2% of dose).

Fusarium-cultured materials 26 g containing 125 mg of FB1 (corresponding to 5 mg/kg) was given to male piglets (5 mg/kg b.w.) orally, and plasma FB1 levels are monitored by high-performance liquid-chromatography³⁵). Highest plasma concentration (C_{max}) of FB1 appears at 2 hr (T_{max}) after the administration. The sharp decline after T_{max} suggested the rapid equilibrium with tissue/organ compartment and the half-life ($t_{1/2}$ of β -phase) was calculated from the plasma profile as around 12 hr. The value is likely to represent the half-life of the absorption in similar to the case with rat pharmacokinetics described above. FB1 was excreted in urine (0.93%) and feces (76.5%) until 84 hr.

Absorption of FB1 was estimated 4% in pigs fed the 45 mg/kg diet for 10 days, and the total urinary excretion was 1.5% using liquid-chromatography mass-spectrometry (LC/MS)³⁶). Mono-carballylic ester hydrolyzed (24%) and di-hydrolyzed metabolites (aminopentol, 16%) were detected together with FB1 (ca 65%) in the urine. FB1 was detected in liver, kidney, lung and spleen (noted as the decreasing order) even in 10 days after the withdrawal of the FB1-contaminated feed.

After feeding of 100 mg/kg diet of FB1 for 5 to 11 days, tissue FB1 levels of pigs euthanized or died were determined on serum, bile, lung, liver, kidney, brain, spleen, pancreas, heart, muscle, eye, and fat by LC/MS³⁷). High levels of FB1 were detected in kidney, liver, lung, spleen and pancreas.

2.3 Pharmacokinetics in Velvet Monkey

Plasma disappearance rate of FB1 was calculated 40 min after the i.v. administration of 1.6 mg/kg b.w. in velvet monkey. Significant amounts (5–20%) were eliminated into the urine, although fecal excretion was the major route of the elimination³⁸). More than 70% of the radioactivity was recovered within three days after an oral ^{14}C -FB1 administration (8 mg/kg b.w.) in velvet monkeys. Fecal excretion also shared the main route of the elimination. Considerable

amounts of radioactivity were detected in the intestines, while the urinary excretion ratios were slightly less in the oral study than in the parenteral study.

2.4 Urinary Excretion of FB1 in Human

Daily urine output of volunteers consuming 206 g/day of tortillas and biscuits prepared from masa and maize flour were determined for FB1 and the derivatives³⁹). Only intact FB1 was detected in urine and the amount was estimated as 0.5% of the intake. Within five days after the cessation of consumption, urinary FB1 disappeared and thus suggests an apparent half-life of less than 48 hr in humans.

2.5 Metabolism and Transport

No enzymatic formation of FB1 metabolite was detected in rat hepatocyte and microsomal systems, suggesting FB1 as a poor substrate of cytochrome P450 forms⁴⁰). Although the urinary excretion of hydrolyzed FB1 without carballylic acid side chain was detected in urine, hydrolysis of ester bonds at C6 or C7 was not observed in hepatocyte system. Thus, no substantial data is available on the enzyme responsible for FB1 hydrolysis.

Experiments with male *Mdr1a/1b* double knockout mice indicate that the cellular or brain transport of FB1 appears to be independent of the P-glycoprotein-mediated transporting system⁴¹). Using cell culture systems with or without verapamil or probenecid (P-gp and Mrp inhibitors respectively), involvement of P-gp in the influx/efflux mechanisms of aminopentol (hydrolyzed FB1), but not FB1, was suggested in the intestinal cells⁴²).

To pregnant rats, ^{14}C -FB1 (0.101 mg) was given i.v. on gestational day (GD) 15 and the distribution to fetus was examined 1 hr after the injection⁴³). No apparent radioactivity was detected in the fetus. The sphinganine level was also not altered, suggesting the scarce distribution of FB1 in rat fetus. A report, however, noted the presence of radioactive FB1 in both placental and embryonic tissues after the intraperitoneal injection at GD 10.5 to pregnant mouse dams of LM/Bc strain, which accompanied the increase in their sphinganine levels⁴⁴).

Although no clear mechanism was available on the selective retention of FB1 in liver and kidney, there are possible contributions of transporters. Interactions of FB1 with organic anion transporter of rat (rOAT1)⁴⁵) and of human (hOAT3)⁴⁶) were reported in the inhibition study using typical substrates. These data suggest the possibility of efficient uptake in liver and reuptake in kidney of FB1 through OAT family of transporters.

Prolonged retention of ^{14}C -FB1 radioactivity in intestine and limited excretion in bile of pigs may imply at least partly

the contribution for intestinal excretion of FB1 through transporters such as ABCG2 (BCRP) known to express in jejunum and liver^{47,48}), although no substantial data is yet available on FB1.

Information on the tissue distribution of FB1 other than liver and kidney is limited in the pharmacokinetic studies described above, but the distributions of FB1 to thymus, adrenal, pancreas are suggested from acute or sub-acute toxicity studies in pigs and other species. These data suggest the sustained absorption at intestines and continued sequestration in susceptible tissues after the oral administration of FB1 in experimental animals.

3. Profiles of Species- and Sex-related Differences in Toxicity Target Tissues

3.1 Toxicity in Mouse

Liver apoptosis and hepatocellular hypertrophy are detected in female B6C3F1 mice fed 52 mg/kg diet or more of FB1 (corresponding to 11.5 mg/kg b.w./day or more) for 28 days¹⁰). Pathological changes and altered serum parameters indicative of hepatotoxicity are found in female B6C3F1 mice fed 81 mg/kg diet (corresponding to 28.9 mg/kg b.w./day) of FB1 for 90 days⁴⁹). No apparent changes in clinical parameters are detected in male B6C3F1 mice after the FB1 administration.

In another FB1 feeding study for 28 days⁵⁰), increases in blood alanine aminotransferase (ALT), alkaline phosphatase (ALP) cholesterol and total bile acids are detected in B6C3F1 female mice fed 163 mg/kg diet (corresponding to 41 mg/kg b.w./day) or higher doses of FB1. Bile duct hyperplasia as well as apoptosis is found in female mice fed 234 mg/kg or 484 mg/kg diets (corresponding to 62 or 105 mg/kg b.w./day). Changes in hepatotoxic parameters such as ALT, ALP, aspartate aminotransferase (AST), cholesterol and total bile acids are also found in male B6C3F1 mice fed FB1 at a high dose (484 mg/kg diet corresponding to 93 mg/kg b.w./day). These feeding studies are consistently indicative of higher susceptibility of female mice than the males to FB1. Similar differences were observed in mice fed FB1 by the gavage administration for 14 days⁵¹). Liver necrosis was detected in female B6C3F1 mice at 15 mg/kg b.w./day, whereas it was detected in the males at 35 mg/kg b.w. or higher dose. Both the sexes showed the increases of hepatotoxic parameters such as serum cholesterol and ALT.

Although no obvious damage in kidney was reported in FB1 feeding study, slight increases in the incidence of single epithelial cell necrosis in cortical and medullary renal tubules with vacuolation of the cytoplasm in epithelial cells in the outer medulla, were found in females after the gavage

administration at 15 to 75 mg/kg b.w. of FB1. These effects were not detected in the male mice. In addition, adrenal cortical cell vacuolation was observed at the 15 mg/kg b.w./day in the females and at the 35 mg/kg b.w./day in the males.

Observed higher susceptibilities of female, rather than male, B6C3F1 mice to FB1 in the dysfunctions of kidney and adrenal as well as liver suggest the systemic distribution of a mutual etiological factor.

Female B6C3F1 mice were fed diets containing FB2, FB3, hydrolyzed-FB1, N-acetyl-FB1, or N-carboxymethyl-FB1 (approximately 0, 14, 70, and 140 $\mu\text{mol/kg}$ diet, **Fig. 1**) for 28 days. None of these diets caused a decrease in body weight gain over the 28 days¹⁰).

3.2 Toxicity in Rat

Hepatocellular apoptosis was detected in female F344 rats treated with 99 mg/kg diet of FB1 or the higher doses (corresponding to 12–56 mg/kg b.w./day) for 28 days⁵²). Bile duct hyperplasia was also detected in the females fed 234 or 484 mg/kg diets (corresponding to 28 or 56 mg/kg b.w./day). Hepatocellular apoptosis and bile duct hyperplasia were also observed in the male animals, but at the higher doses, 234 and 484 mg/kg diets, respectively. Kidney apoptosis was observed in both sexes of the animals treated with 99 mg/kg diet of FB1 or the higher doses.

In a different feeding study of FB1 for 28 days⁵⁰), liver apoptosis was detected in female F344 rats treated with the 163 mg/kg diet (corresponding to 20 mg/kg b.w./day). Increases of serum cholesterol and total bile acid levels were found after feedings of the 234 mg/kg diet (corresponding to 28 mg/kg b.w./day) or the higher dose in the female rats. Liver apoptosis was also observed in the male rats treated with the 234 mg/kg diet, and increases of serum cholesterol, total bile acid and ALP are detected at the 484 mg/kg diet (corresponding to 56 mg/kg b.w./day). Kidney apoptosis was detected in the female at the 163 mg/kg diet group and in the male at the 99 mg/kg diet group. Kidney weight loss was found at the 234 mg/kg diet in both sexes of rats.

Nephrosis involving the outer medulla was found in male F344 rats fed diet containing 9 mg/kg diet of FB1 (corresponding to 0.21 mg/kg b.w./day) or the higher dose for 13 weeks, and, to a lesser degree, in the females fed FB1 containing the 81 mg/kg diet⁴⁹). Renal weight decreases were found in both sexes of rats fed at dietary levels of the 9 mg/kg diet (corresponding to 5.66–6.35 mg/kg b.w./day) or the higher levels. In this study, hepatotoxicity was not found in rats fed at dietary levels of up to the 81 mg/kg diet for 90 days.

In a two-year NTP study⁵⁰), renal tubule apoptosis was observed in male F344 rats fed 15 mg/kg diet of FB1 (corre-

sponding to 0.76 mg/kg b.w./day) from six-week evaluation, and renal tubule epithelial hyperplasia is detected in the groups fed the 50 and 150 mg/kg diet (corresponding to 2.5 and 7.5 mg/kg b.w./day) at the final evaluation. No significant increases in the incidence of neoplasms and non-neoplastic lesions were detected in the females fed 5 to 100 mg/kg diet of FB1.

These diet studies using F344 rats indicate the following points; 1) kidney is the most susceptible target of FB1, 2) liver damages are also occurred at the higher doses, and 3) female F344 rats are rather resistant than the male rats to FB1.

Experiments using both sexes of SD rats also indicate hepatic lesions after the feeding of 150 mg/kg diet of FB1 (corresponding to 13 or 13.6 mg/kg b.w./day) for four weeks⁵³. Cortical nephrosis appeared in the males fed 15 mg/kg diet of FB1 (corresponding to 1.4 mg/kg b.w./day), and in the females fed the 50 mg/kg diet (4.1 mg/kg b.w./day).

Susceptibility to FB1-mediated nephropathy was also higher in male SD rats than the females in FB1 feeding study⁴³. In a diet study using male SD strain of rats, liver and kidney apoptosis were detected in the groups fed 53 and 6.9 mg/kg diet of total FBs (corresponding to 5.3 and 0.69 mg/kg b.w./day), respectively, for three weeks⁵⁴. Increases in serum ALP and urea nitrogen were also found in male SD rats exposed to 53 mg/kg diet of total FB. Increases in apoptosis score in liver was detected at the 88.6 mg/kg diet (8.86 mg/kg b.w./day) for 10 days and in kidney at the 13.5 mg/kg diet (corresponding to 1.35 mg/kg b.w./day)⁵⁵.

Significantly depressed body weight and food consumption were observed in female SD rats treated with gavage administration of FB1 at 35 and 75 mg/kg b.w./day for 11 days⁵⁶. Reduced liver weight, elevated serum ALT and histopathological changes suggest hepatic dysfunction at doses of the 15 mg/kg b.w./day and the higher. Altered renal morphology, together with changes in urine osmolality and urine enzyme levels, was observed at the 5 mg/kg/day and the higher doses. In addition, transient increase in cellular vacuolation was detected in bone marrow at a dose of 5mg/kg b.w./day. Increased cytoplasmic vacuolation of adrenal cortex cells occurred in rats treated with the 15 mg/kg b.w./day. Elevated serum cholesterol was observed at 5 mg/kg b.w./day of FB1 and the higher.

In male SD rats treated with gavage administration of FB1, glomerular and tubular damages were suggested at 5 mg/kg b.w./day from the alteration of clinical parameters⁵⁷. Single cell necrosis is increasingly numerous in the liver from 15–75 mg FB1/kg b.w./day. Thus, clear sex-related difference exists on FB-induced renal apoptosis, but faint in the hepatic apoptosis, in SD rats. Consistent with F344 rats, Female SD

rats are more resistant than the males on FB1-induced kidney dysfunctions.

The doses yielding liver and kidney damages are equivalent between the diet study for four weeks and gavage studies for 11 days using both sexes of SD rats. These results among sub-chronic studies are consistent with the view that sustained absorption in intestine of FB1 is a key factor in oral toxicity studies.

Reductions in body weight, food consumption and feces production with polyuria without a compensatory increase in water consumption were observed in male SD rats treated in parenteral (i.p.) with 7.5 or 10 mg/kg b.w./day for four consecutive days⁵⁸. In addition to dehydration, decreased absolute organ weights of liver, kidney, spleen and thymus were detected. Elevated levels of serum blood urea nitrogen (BUN) and altered kidney morphology indicate nephrotoxicity. Serum levels of ALP, ALT, AST, cholesterol, total bilirubin, and calcium were elevated, and of glucose and amylase are reduced in these animals. These data suggest the dysfunctions of pancreas as well as kidney and liver.

Damages of lung and intestine, in addition to liver and kidney, are reported in male BDIX rats treated p.o. with 4.7 mg/kg of FB1 for 12 days and 7 mg/kg for the successive 9 days of FB1¹.

3.3 Toxicity in Rabbit

Single or multiple doses of FB1 (less than 0.5 mg/kg i.v.) caused renal proximal tubular damage in rabbits. Serum levels of urea nitrogen and creatinine were increased and urinary glucose and protein concentrations were also altered⁵⁹. These findings suggest that FB1 targets the proximal tubular epithelia, especially at the cortico-medullary junction, in rabbit kidney. The increased levels of protein in the urine may be indicative of the extensive tubular epithelial necrosis.

3.4 Toxicity in Swine

Swine showed pulmonary edema and hydrothorax after the feeding of fumonisin-contaminated corn⁶⁰ or intravenous injection of FB1 (0.174 to 0.4 mg/kg b.w.) daily for 4 to 7 days⁶¹. Pancreatic lesions were present in all pigs developed pulmonary edema/hydrothorax. Liver changes were observed in all pigs fed FB1 diet for the feeding study⁶¹. The animal shows typical lesions including interlobular edema of lung and pancreatic lesions⁶².

Feeding of 20 mg FB1/kg b.w./day for four days resulted in the pulmonary edema and hepatotoxicity in pigs⁶³. Pigs developed pulmonary edema beginning on day 3; none survived beyond day 4. Progressive elevations in hepatic parameters, including serum enzymes, bile acids, total bilirubin, and histologic changes, began on day 2. Early histological

changes in the lung consisted of perivascular edema followed by interlobular and peribronchial edema without the sign of inflammation. Ultrastructurally, alveolar endothelial cells contained unique accumulations of membranous material within pulmonary intravascular macrophages beginning on day 2. Marked elevations in sphinganine, sphingosine, and their ratio (sphinganine/sphingosine Sa/So) began on day 1 for all tissues, irrespective of being morphologically (lung, liver) or not (kidney, pancreas)⁶³. Thus, FB1 induces early elevations in sphingolipids and hepatic damage, followed by alveolar endothelial damage in pigs^{63,64}. In an experiment using pulmonary endothelial cells, increased albumin permeability was observed in the presence of 30 to 50 mM FB1⁶⁵. Neither toxicity nor ultrastructural changes were, however, detected after the exposure at 5–20 µg/ml (corresponding to 7–28 µM), relevant to *in vivo* exposure levels, for seven days⁶⁵. These data may favor the idea that pulmonary dysfunction is an indirect consequence of the damage on primary target tissue(s).

3.5 Toxicity in Horse

Leukoencephalomalacia is a typical toxicity of FB1 in horses^{66,67}. Gavage administration of cultured materials containing FB1 (2.5 g cultured material/kg b.w./day for seven days) to a horse caused severe hepatotoxicity and mild edema of the brain⁶⁸. In a similar experiment with the cultured material of 1.25 g/kg b.w./day, another horse developed mild hepatic disorder and moderate edema of the brain. In both animals the brain edema was particularly noticeable in the medulla oblongata.

A horse given intravenously 7 times from day 0 to day 9 with 0.125 mg of FB1/kg b.w./day showed clinical signs of neurotoxicity, which appeared on day 8. Euthanasia was performed on the horse on day 10 while the animal was in a tetanic convulsion. The principal lesions were severe edema of the brain and early, bilaterally symmetrical, focal necrosis in the medulla oblongata⁶⁸.

In horses treated intravenously with 0.05–0.20 mg/kg of FB1 for up to 28 days, hindlimb ataxia, delayed forelimb placing, and decreased tone and movement of tongue are observed⁶⁹. These results indicate that FB1 induced dose-dependent changes in the cerebrospinal fluid and nerve system.

3.6 Developmental Toxicity

Pregnant CD1 mice were treated orally with semi-purified extracts of *F. moniliforme* containing FB1 at a dose of 0, 12.5, 25, 50 or 100 mg/kg b.w./day between GD 7 and 15. At GD 18, litters were examined for gross abnormalities and for skeletal or visceral examinations⁷⁰. Significant

maternal mortality was observed in mice treated with the 50 and 100 mg/kg b.w./day. Maternal body weight gains, live-offspring number per litter, and mean body weight of the offspring were decreased at FB1 doses of 25 mg/kg b.w./day or the higher. The frequency of resorption of implants increased at all the doses in a dose-dependent manner. The incidence of ossification deficits also increased from the 25 mg/kg b.w./day dose. Cleft palate was observed at the highest FB1 dose group. Maternal intoxication manifested as ascites associated mainly with increased histopathological scores reflecting hepatocellular damage. Concomitant increases in serum ALT on GD 12 was also observed at all doses above 12.5 mg/kg b.w./day of FB1. Authors⁷⁰ suggest based on these results that FB1 is developmentally toxic in mice, and that this toxicity may be mediated by maternal hepatotoxicity.

In addition, extracts from *Fusarium* cultures and purified FB1 have been shown to be embryotoxic to hamsters^{71,72}, mice^{70,73}, rats^{74,75}, chicken^{76,77}, and rabbits⁷⁸. With exception of studies with hamsters and chicken, embryo toxicities *in vivo* appeared in association with signs of maternal toxicity^{73–75,78}.

FB1 was toxic, especially to pre-somite stage, in a whole embryo system of rat. Embryo growth were retarded, and the development was inhibited without specific abnormalities. Addition of ceramide, N-acetylsphingosine or raft ganglioside Gm1 did not reverse FB1-mediated toxicity, suggesting that inhibition of sphingolipid synthesis was not the cause of the toxicity. Further, the main metabolite of FB1, aminopental (AP-1), inhibited embryonic growth, but was 100-fold less toxic than FB1⁷⁹.

Due to the possible association of FB1-contaminated foods with an outbreak of neural tube defects (NTD) occurred in 1991 in south Texas county bordering Mexico^{80,81}, attentions were focused on risks of FB1 exposure to NTD.

Both *in vitro*⁸² and *in vivo* studies treated FB1 parenterally⁴⁴ showed the association of FB1 exposure with NTD in mice.

4. Currently Known Biochemical Alterations in FB1-exposed Animals

Considerable amounts of data have been accumulated for the biochemical changes in FB1-mediated toxicities. These may be summarized and subdivided into bile acids, sphingolipids and phospholipids from the point of endobiotics involved.

4.1 Overflow of Bile Acids in Systemic Circulation

As described above, liver is the common target of FB1-mediated toxicity throughout the experimental and domestic animals examined, and cholestatic signs, including increased levels of serum bile acid, are consistently detected in the exposed animals.

The predominant bile acids in control mouse liver are reported to be taurocholic acid (86.1 ± 18.5 nmol/g liver) and cholic acid (4.7 ± 0.9), tauro- β -muricholic acid (34.2 ± 8.8) and β -muricholic acid (35.1 ± 5.0), and tauro- ω -muricholic acid (18.1 ± 4.5) and ω -muricholic acid (11.8 ± 2.1)⁸³. These are excreted in bile, but in situations of the restricted bile excretion, bile acids are excreted in urine through kidney. The hepatic levels of major bile sulfates, taurocholic acid 7-sulfate, cholic acid 7-sulfate, and taurochenodeoxycholic acid 7-sulfate were higher in male mice than in female mice⁸³. This indicates the male-dominant sulfation of cholic acid and chenodeoxycholic acid, and thus in turn suggests that hepatic elimination of hydrophobic bile acids is delayed in female mice. The observed sex-related difference in bile acid excretion is consistent with higher susceptibility of female mice to FB1.

Bile acid exists within enterohepatic circulation (>96%), and clearance through the plasma-renal system occurs selectively at the over-limiting capacity of liver to send bile acids into bile as described above. Obstruction of the common bile duct leads to cholestatic liver. Cholestasis results in the accumulation of hydrophobic bile acids in liver. As a possible cause of liver injury, direct action of bile acids has been proposed from apoptosis of hepatocyte cell line⁸⁴, which is prevented by a pan-caspase inhibitor, z-VAD-FMK⁸⁵.

Unlike in mice, urinary excretion of taurocholate is higher in female rats than in male rats due to the higher renal expression of a specific Oatp1 transporter⁸⁶. An activity of MRP3 transporter mediating bile acid effluence to plasma is higher in female rats than the males^{87–89}. Further, hepatic sulfating activity of bile acids, which is mediated by hydroxysteroid sulfotransferase, is higher in female rats than the males^{90,91}. These sex-related differences on bile acids are accorded with the higher susceptibility of kidney of male rats to FB1.

Thus, data on plasma bile acids are in good agreement with the idea of bile acid-involvement on FB1-mediated kidney toxicity. No morphological changes with apoptosis or activation of caspases are, however, evident *in vivo* in bile duct ligated rats and mice^{92–94}. In addition, serum pro-apoptotic bile acid levels are approximately 1,000-fold less than needed to stimulate apoptosis *in vitro*⁹⁵. Instead, neutrophil-induced liver injury was proposed in bile-duct ligated animals^{96,97}. Another study also implies that a hydrophobic lithocholic

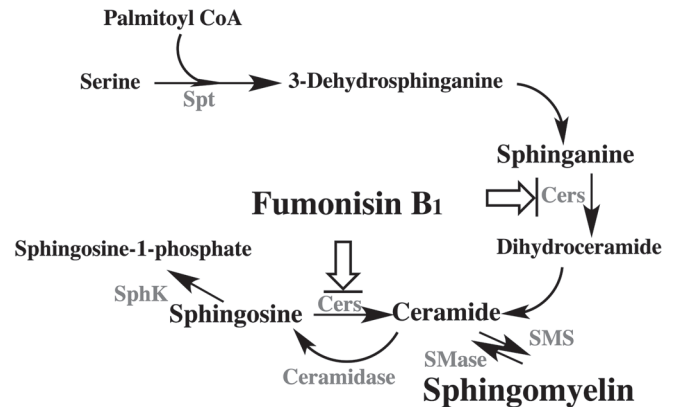


Fig. 2. Biosynthesis of sphingomyelin and interaction of fumonisin B1

Interaction points of fumonisin B1 are shown as arrows with bar. Enzymes are shown in gray color. CerS; ceramide synthase (sphinganine/sphingosine N-acyltransferase), SMS; sphingomyelin synthase, SMase; sphingomyelinase, SphK; sphingosine kinase, and Spt; serine palmitoyltransferase.

acid leads to cholestasis through the disruption of phospholipid and sphingolipid homeostasis in mice⁹⁸.

Certain amounts of bile acids are also excreted in urine of pig. Hydrophilic γ -muricholic acid, but not cholic acid, is excreted in urine and bile in this species^{99,100}.

Therefore, the role of bile acid effluence into systemic circulation for FB1-mediated kidney toxicities is yet unclear, although the chronic exposure to bile acids is possible to exacerbate functions of liver in mice and kidney in rats.

4.2 Alteration of Sphingoid and Ceramide Levels

FB1 is known to influence several distinct signaling pathways of sphingoids including ceramide synthesis, sphingosine-1-phosphate receptor and glycosylphosphatidylinositol anchoring^{19,101–103}.

4.2.1 Sphingoid

FB1 inhibits ceramide synthase, a key enzyme in the *de novo* biosynthesis of sphingolipids (**Fig. 2**). The increase of free sphinganine (and also the ratio with sphingosine) in tissue after the administration of FB1 is used as a biomarker for fumonisin exposure. A single subcutaneous injection of FB1 caused an increase in free sphingoid bases in male BALB/c mice. A significant time-dependent increase in sphingoid bases occurred in the intestine and liver peaking at 4–8 hr and declining to control levels by 24 hr. The level of free sphinganine remained high in the kidney¹⁰⁴. This persistence in renal cells was rapidly reversed in the presence of the inhibitor (ISP-1/myriocin)¹⁰⁵ of serine palmitoyltransferase, which catalyzes sphinganine biosynthesis.

The associations of sphingoid levels with FB1 toxicities were also detected in kidney and liver of rats⁵⁵) and in lung of pigs⁶³). However, no clear mechanistic clues have been provided between FB1-mediated changes in sphinganine levels and organ toxicities *in vivo* in these animal species.

Calmodulins (CaM1-4) are small, ubiquitous adaptor proteins that amplify calcium cation's diminutive size to the scale of proteins¹⁰⁶). Sphingosine is a potent inhibitor of several calmodulin-dependent enzymes including multifunctional Ca²⁺/calmodulin-dependent protein kinase, Ca²⁺/calmodulin-dependent phosphodiesterase, and smooth muscle myosin light chain kinase¹⁰⁷). Inhibition of each of the enzymes is competitive with calmodulin, suggesting that sphingosine may be a calmodulin antagonist.

The activity of acidic sphingomyelinase was enhanced and liver sphingomyelin content was decreased following five daily treatments with FB1 in mice. The expression and activities of serine palmitoyltransferase and sphingosine kinase were increased significantly in liver following FB1 treatment. These data suggest that FB1-mediated inhibition of ceramide synthase stimulates sphingolipid-metabolizing systems to maintain a balance of cellular sphingolipids²³).

4.2.2 Ceramide and ceramide synthase

Ceramide is a lipid-signaling molecule that plays critical roles in regulating cell behavior¹⁰⁸). Six distinct forms of mammalian ceramide synthases (CerS) mediate syntheses of ceramides with restricted acyl chain lengths^{109–111}). CerS1, CerS2, CerS3, CerS4 and CerS5/6 mediate mainly the formation of C18, C22, C26, C18 and C16 dihydroceramides, respectively¹¹²).

The sphingolipid contents of lung and liver were compared in normal and FB1-exposed piglets (gavaged with 1.5 mg FB1/kg b.w. daily for 9 days)¹⁴). The effect of the toxin on each CerS form was deduced from an analysis of its effects on individual ceramide and sphingomyelin species. As expected, the total ceramide content decreased by half in the lungs of FB1-exposed piglets, Total ceramide increased 3.5-fold in the livers of FB1-exposed animals, possibly due to the degradation of sphingomyelin. These data are consistent with the view that FB1 is more prone to bind to CerS4 and CerS2 to deplete lung d18:1/C20:0 and d18:1/C22:0 ceramides¹¹³). These views are consistent with the idea that differences in CerS4, CerS2, and CerS1 expressions in targets are involved in the toxicity of FB1.

CerS2-deficient mice, carrying an insertion (gene trap) in the *CerS2* gene to generate transgenic mice that lack *CerS2* mRNA, lacked ceramide synthase activity toward C24:1 in the brain as well as the liver, and showed only very low activity toward C18:0-C22:0 in liver and also reduced activ-

ity toward C22:0 residues in the brain¹¹⁴). These mice also exhibit strongly reduced levels of ceramide components with very long fatty acid residues (>or=C22) in the liver, kidney and brain. In addition, histochemical staining of myelin is progressively lost from the early adulthood, followed by about 50% loss of compacted myelin and 80% loss of myelin basic protein. Starting around 9 months, both the medullary tree and the internal granular layer of the cerebellum showed significant signs of degeneration associated with the formation of microcysts. Beyond seven months, the CerS2-deficient mice developed hepatocarcinomas with local destruction of tissue architecture and discrete gaps in renal parenchyma.

In another CerS2-deficient mouse model¹¹⁵), liver ceramide and downstream sphingolipids were devoid of very long (C22-C24) acyl chains, consistent with the substrate specificity of CerS2 toward acyl-CoAs. Unexpectedly C16-ceramide levels were elevated *in vivo*, although C16-ceramide synthesis *in vitro* was not increased. Levels of sphinganine were also significantly elevated, by up to 50-fold in the liver.

With the exceptions of glucosylceramide synthase and neutral sphingomyelinase 2, none of the other enzymes tested in either the sphingolipid biosynthetic or metabolic pathways were significantly changed in the liver. Total glycerophospholipid and cholesterol levels were unaltered, although there was a marked elevation in C18:1 and C18:2 fatty acids in phosphatidylethanolamine (PE), concomitant with a reduction in C18:0 and C20:4 fatty acids. These results indicate that CerS2 activity supports different biological functions: maintenance of myelin, stabilization of the cerebellar as well as renal histological architecture, and protection against hepatocarcinomas possibly through crosstalk and regulation between the various branches of lipid metabolic pathways¹¹⁵). These data are consistent with the association of ceramides and sphingomyelin on FB1-mediated liver and brain toxicities.

4.2.3 Sphingolipid signals

On the interaction of sphingolipids with cellular signal molecules, hypoxia-reoxygenation of rat renal tubular epithelial NRK-52E cells resulted in a significant increase in ceramide synthase activity without any significant change in acid or neutral sphingomyelinase. The hypoxia-reoxygenation of NRK-52E cells was also associated with the release of endonuclease G from the mitochondria to the cytoplasm. It further led to the fragmentation of DNA and cell death. Addition of 50 μM FB1 suppressed hypoxia-reoxygenation-induced ceramide generation and provided protection against the release of hypoxia-reoxygenation-induced endonuclease G, DNA fragmentation, and cell death¹¹⁶). FB1 has also been shown to inhibit various inducer mediated-cell deaths in cell

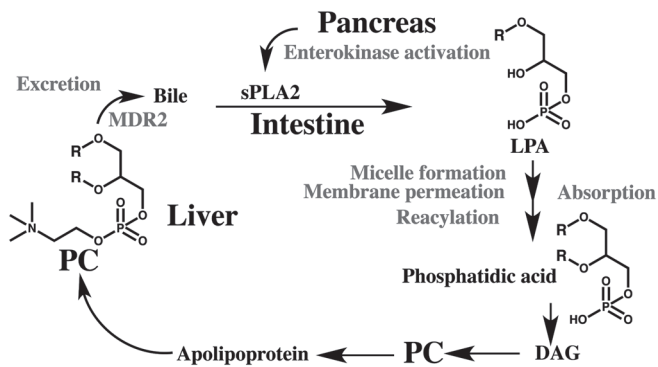


Fig. 3. Enterohepatic circulation of phosphatidylcholine R; fatty acids, DAG; diacylglycerol, LPA; lysophosphatidic acid, MDR2; ABCB4 phosphatidylcholine transporter, PC; phosphatidylcholine, sPLA2; secretory phospholipase A2 (IB form). Specific process and enzymes are shown in gray color.

lines¹¹⁰.

In contrast, sphingolipid metabolites such as ceramide, glycosphingolipids, sphingosine 1-phosphate and gangliosides are involved in β -cell signaling pathways and processes implicated in β -cell diabetic disease such as apoptosis, β -cell cytokine secretion, endoplasmic reticulum-to-Golgi vesicular trafficking, islet autoimmunity and insulin gene expression¹¹⁷. Further, FB1-mediated activation of NF- κ B (nuclear factor-kappa B) and the sequential induction of TNF α (tumor necrosis factor α) expression stimulated in the subsequent increase in caspase 3 activity in porcine renal proximal cell-derived LLC-PK1¹¹⁸. Thus influences of sphingoids on cellular signal molecules may depend on the cell type and experimental conditions to exert the protecting or exacerbating effects. The role of signal molecules derived from sphingoids remain obscure *in vivo* on FB1-mediated toxicity¹¹⁹.

4.3 Supply Circle of Phospholipid

4.3.1 Supply through the intestinal absorption

Similar to xenobiotics, various endobiotics are excreted through bile. Non-esterified cholesterol, bile acids, phosphatidylcholine (PC) and glutathione are the main components of bile. Specific transporters expressed in canalicular membrane are involved in their transfers from liver to bile duct (**Fig. 3**). ATP-binding cassette transporters, ABCG5/8, ABCB10 (BSEP), ABCB4 (MDR2) and ABCC2 (MRP2), mediate the transfer of cholesterol, cholates, PC and glutathione in livers, respectively. Cholesterol, bile acids and PC are forming micelles, holding certain ranges of their ratios in the bile after the membrane permeation to maintain the bile fluidity^{120,121}, and descend to gall bladder and intestine. Insufficient supply of PC, thus, affects the excretion of cholesterol and bile acids. Bile flow is driven mainly by the levels

of bile acids¹²²) and glutathione¹²³) and the deficient supply of PC is able to reduce the rate of bile flow. Prolonged irregular ratios of bile components would thus promote formation of cholestatic conditions.

Dysfunctions in canalicular components are known to associate with enhanced levels of ALP and bilirubin in the body. Observed changes in biochemical parameters, excesses of cholesterol and bile acids compared to triglycerides in FB1-treated mice and rats are consistent with the notion of shortage of phospholipids, particularly PC, to excrete in bile. Liver PC is maintained by supplies from two sources¹²⁴. One is a conversion from triglycerides within hepatocytes. The other is intestinal absorption of phospholipids from bile and diet. Bile supplies the majority (up to 90%) of intestinal phospholipids. Intestinal phospholipids are hydrolyzed to lysophosphatidic acid (LPA) in the presence of secretory phospholipase A2 (sPLA2) released from the pancreas. LPA forms micelles with monoacylglycerol, fatty acids, bile acids and cholesterol, and then passes through the brush border membrane. Within enterocytes, LPA is acylated to phosphatidic acid and then to PC and PE. These phospholipids are delivered as particles like chylomicron and apolipoprotein B to tissues.

4.3.2 Supply through the liver synthesis

Phospholipids are biosynthesized from diacylglycerols in tissues like liver. Liver has two routes of PC biosynthesis. The main route is a sequential pathway starting from choline to phosphocholine to CDP choline and then to PC. Another route is three-consecutive methylations of PE to form PC. Methionine is the donor of the methyl group. The latter route is found only in liver.

CTP:phosphocholine cytidyltransferase α (CCT α) is essential for production of PC of animal cell membranes and is proteolytically sensitive¹²⁵. Calmodulin stabilized CCT α from the calpain-mediated proteolysis. Mapping and site-directed mutagenesis of CCT α uncovered a motif (LQERVDKVK) harboring a vital recognition site, Gln(243), whereby calmodulin directly binds to the enzyme. Thus, calmodulin, through antagonizing calpain, serves as a novel binding partner for CCT α that stabilizes the enzyme under proinflammatory stress¹²⁶. The CDP-choline pathway contributes PC supply for bile secretion. The function of CCT α may be impaired in the presence of an antagonist of calmodulin, sphingosine as described in the section of 4.2.1 (Sphingoid), and also through the direct interaction of CCT α with sphingosine¹²⁷, and indirectly through the repression of CCT gene transcription¹²⁸. Gene knockout of either CCT α (Pcvt1a) or PE N-methyltransferase (Pemt), however, had no strike effect on bile secretion or composition, suggesting

the existence of effective metabolic compensation mechanism^{129,130}).

PC synthesis is reduced in the liver of *Pemt*-null mice. Thus, treatment of *Pemt* null mice with choline-deficient diets results in abrupt death (within five days)¹³¹. Double knockout mice of *Pemt* and *Abcb4* (*Mdr2*) are somewhat resistant to choline-deficient diets¹³², through knockout of the PC-transporter *Abcb4* (*Mdr2*) to relieve PC loss. The *Mdr2*(-/-)/*Pemt*(-/-) mice also adapt to severe choline deprivation through several mechanisms, including choline recycling by induction of PLA2, by the activity of choline kinase, and CCT α activities and also by a strikingly decreased expression of choline oxidase¹³². These pathological models raise a possibility of uncompensated loss of PC in bile as a main etiological factor in FBI-treated animals, in addition to FBI-mediated inhibition of ceramide synthase^{102,133}.

4.4 Possible Trigger Point of PC-supply Dysfunction

4.4.1 Lipase function

PC is produced from diacylglycerol and CDP-choline. Decreased supply of triacylglycerol from intestine may affect the PC supply in liver. Orlistat is a potent, specific and irreversible inhibitor of gastric and pancreatic lipases¹³⁴. This drug forms a covalent bond with the active serine of both lipases in the lumen of the gastrointestinal tract, which prevents the hydrolysis of dietary fat into absorbable monoacylglycerol. In patients taking orlistat, levels of both cholesterol and LDL are markedly reduced¹³⁵. However, orlistat does not severely affect the plasma triacylglycerol level. The reduction of plasma cholesterol level observed are rather inconsistent with the no clear changes in FBI-mediated animals. These data are rather discordant with the idea that secreted-lipase dysfunction is a main causal mechanism of FBI-mediated liver toxicity.

4.4.2 Pancreatic phospholipase function

Levels of total cholesterol in sera and livers were significantly increased in the rats fed FBI (250 mg/kg diet) for 21 days, while the liver sphingomyelin and PE were significantly decreased and increased, respectively. In a long-term study, only PE was significantly increased in all the FBI-treated animals¹³⁶. In mice treated with FBI for more than one week, changes in biochemical parameters become apparent. Increases in plasma values of ALT, ALP, bilirubin, cholesterol and bile acids are detected. FBI also caused significant cholestasis and liver necrosis in pigs⁶². These data suggest the commonality of hepato-biliary dysfunction, particularly cholestasis, in FBI-treated animals.

As described in the section of 4.3.2 (Supply through liver

synthesis), insufficient supply of PC in bile secretion is likely to participate in the FBI-mediated cholestasis. Enhanced synthesis of PC through the CDP-choline pathway and from PE and methionine is necessary to compensate the loss of PC in the bile.

Studies with an experimental animal model have shown that choline and methionine deficiency causes dysfunctions of liver and pancreas^{137,138}. Young female CD1 mice fed a choline-deficient, ethionine-supplemented (CDE) diet for 24 hr develop hemorrhagic pancreatic necrosis with a 5-day mortality rate of approximately 50%¹³⁹. At the end of the diet administration, the *in vivo* discharge of digestive enzymes is blocked, disappearance of exocytosis took place, and zymogen granules accumulate within acinar cells. Thus, the CDE diet does not affect the general phenomenon of membrane fusion-fission but specifically inhibits the membrane fusion-fission associated with exocytosis. Twenty-four hours after withdrawal of the CDE diet, discharge of zymogen granules into lysosomes (crinophagy) can be observed, and, 24 hr later, autophagocytosis is noted.

In fact, FBI-mediated pancreatic dysfunction is observed in rats⁵⁸) and pigs^{60,64}). The pancreatic disorders may be associated with methionine deficiency as discussed in later.

Young female mice, fed a diet deficient in choline and supplemented with ethionine, develop acute necrotizing hemorrhagic pancreatitis, and the incidence is greater in young and female mice than in adult and male mice^{140,141}). In contrast, female rats are less susceptible than the males to the acute effects of choline deficiency, such as fatty liver and impaired secretion of triglycerides into the blood plasma¹⁴²). In addition, protection conferred by ethinylestradiol in the livers of rats fed a methionine- and choline-deficient diet is consistent with the relative insensitivity of female rats to the hepatotoxicity of dietary methyl donor insufficiency¹⁴³). Observed sex-related differences in CDE diet-mediated liver toxicity are consistent with those in FBI-mediated liver toxicities in mice and rats. These data suggest the possible association of hepato-pancreatic changes on FBI-mediated toxicity.

For pancreatic zymogens, two steps of their activations are required with the exception of trypsinogen. In the first stage, active trypsin is generated from its zymogen by enterokinase, and in the second stage trypsin activates the remaining zymogens giving rise to the well-known activation cascade of pancreatic enzymes¹⁴⁴). Enterokinase is synthesized in the enterocytes of the proximal small intestine¹⁴⁵).

DL-Ethionine has a destructive effect on the acinar tissue of the pancreas of rats¹³⁷). The destructive effects of ethionine on liver and pancreas can be completely prevented by the simultaneous administration of equal molar amounts

of methionine. Ethionine affects protein synthesis through inhibiting the incorporation of methionine and glycine into proteins in the liver¹⁴⁶).

As described above, treatment of *Pemt*-null mice with choline-deficient diets results in severe liver dysfunction and abrupt death within five days. PC deficiency is replenished by a synthesis from PE in livers in the presence of *Pemt*. Methionine is utilized for the methyl donor.

In a state of reduced supply of zymogen from pancreas, decreased digestion capacity of protein at intestine results in the diminished amounts of methionine availability. Methionine supplied through portal vein is consumed extensively within liver for the PC and protein syntheses¹⁴⁷). These situations would link to methionine-deficient conditions in other tissues. Methionine deficiency in the pancreas may promote dysfunctions of pancreas in similar to those in rodents treated with CDE diet. Consistent with this idea, FB1 treatment resulted in the up-regulation of the intestinal brush border transporter B0AT and the basolateral transporter y+LAT1 in newborn chickens, suggesting the demand of an increased intracellular uptake of neutral amino acids such as methionine¹⁴⁸).

In fetal rat pancreas cells, methionine is required for the growth of the exocrine pancreas, and the higher levels are required to achieve basal or maximal differentiation of acinar cells¹⁴⁹). The basal requirement was determined to be 30 mg/L methionine, and a level of 80 mg/L supported maximal differentiation.

4.4.3 Secretory phospholipase

Secretory phospholipase A2s (sPLA2s) are small secreted proteins (14–18 kDa), proteolytically activated after their release from the pancreas. sPLA2s require submillimolar levels of Ca²⁺ to liberate LPA. An sPLA2-deficient mutant showed no clear alteration of phospholipid absorption possibly by the feedback regulation of secretins and partly as a result of compensation by other PLA2 forms¹⁵⁰). Pancreatic PLA2 also has intrinsic secretin-releasing activity^{151,152}). Secretin regulates exocrine secretion as well as gastric motility. A pancreas injured by FB1 may not function well to compensate the secretion. In addition, FB1 is a chelating agent of divalent cations like calcium ion. Considerable retention of FB1 in intestinal cells is expected from the increase of sphingoids even after the s.c. administration in mice¹⁰⁴), suggesting the possible contact of the FB1 with secretory phospholipase A2. These situations would exacerbate the absorption capability for phospholipids at intestine.

In addition to its enzymatic function, sPLA2 can exert various biological responses through the binding to specific M-receptors. Physiologically, sPLA2s play important roles

on the neurotransmission in the central nervous system and the neuritogenesis in the peripheral nervous system. sPLA2 are implicated to link to the neurodegenerative diseases like Alzheimer's disease and cerebrovascular diseases like stroke¹⁵³). Release of sPLA2 in the circulation during pancreatic dysfunction may evoke the signal through M-receptor.

M-type phospholipase A2 receptor (PLA2R) is expressed in podocytes in human glomeruli. One of the ligands, group IB secretory phospholipase A2 (sPLA2 IB), is expressed higher in chronic renal failure patients than in controls. After binding to human podocyte PLA2R *in vitro*, sPLA2 IB form induced podocyte apoptosis in a time- and concentration-dependent manner¹⁵⁴). sPLA2 IB upregulated PLA2R and increased ERK1/2 and cPLA2 α phosphorylation in podocytes, which resulted in enhanced apoptosis. In contrast, PLA2R-silenced human podocytes displayed attenuated apoptosis. These data indicate that sPLA2 IB has the potential to induce human podocyte apoptosis via binding to the PLA2R. No PLA2R1 is expressed in mouse kidney podocytes¹⁵⁵) and thus the role of sPLA2 on FB1-mediated kidney toxicity in mice remains unclear.

4.4.4 Intestinal membrane alteration and zinc-deficiency

Feeding FB-containing diet (FB1 + FB2 25.4 mg/kg feed) for 15 days to chickens resulted in altered glycosylation of mucin and in a decreased level of mucin 2 mRNA in the duodenum¹⁴⁸). In addition, specific mRNA level of basolateral zinc transporter (ZnT1) was reduced in jejunum of FB-treated chickens¹⁴⁸), which may contribute to maintain the zinc ion in brush border.

Marginal zinc-deficiency is known to associate with the decreased absorption of triglyceride in rats¹⁵⁶). Plural mechanisms including apolipoprotein B mRNA editing¹⁵⁷) are known to participate in this phenomenon. FB1 includes two moles of carballylic acid in the molecule, which are chelating agents of divalent cations¹⁵⁸). FB1 is thus possible to contribute intestinal zinc deficiency as a zinc-chelating agent. The primary defect in lipid absorptive processes in zinc-deficient rats occurred in the formation of chylomicrons¹⁵⁹).

5. Interaction of Phospholipids in Tissue Membranes

5.1 Lipid Raft Anchoring and Folate

Glycosylphosphatidylinositol (GPI) functions in the integration of GPI-anchored proteins into lipid microdomains. GPI anchor is biosynthesized from phosphatidylinositol-containing unsaturated fatty acyl chains at the *sn*-2 position, and the lipid moieties of the GPI anchor are exchanged after

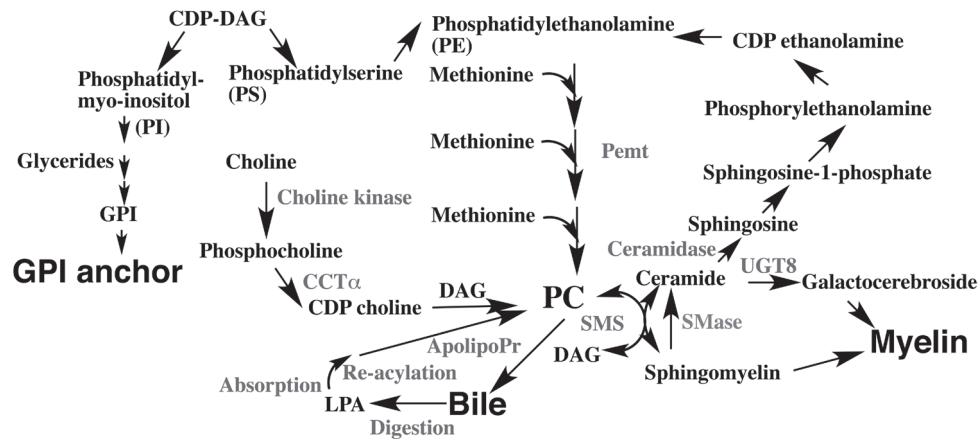


Fig. 4. Interrelationship of phospholipids and sphingolipids on their biosynthesis and recycling. Enzymes and the associated biological processes are shown in gray color.

ApolipoPr; apolipoprotein, CCT α ; CTP:phosphocholine cytidyltransferase α , CDP-choline; cytidine diphosphocholine, DAG; diacylglycerol, GPI; glycosylphosphatidylinositol, LPA; lysophosphatidic acid, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PI; phosphatidylinositol, PS; phosphatidylserine, SMase; sphingomyelinase and UGT8; UDP-galactose ceramide galactosyltransferase.

GPI attachment to proteins in mammalian cells (**Fig. 4**). This lipid remodeling process of GPI-anchored proteins is essential for their association with the lipid microdomains¹⁶⁰.

Granule formation and apical sorting of digestive enzymes in pancreatic acinar cells involves the selective aggregation of a mixture of regulated secretory proteins and the association of these aggregates with specific membrane domains of the trans-Golgi network, which then pinch off as condensing vacuoles.

Isolated membranes of zymogen granules from pancreatic acinar cells are rich in cholesterol and sphingomyelin and formed detergent-insoluble complexes. These complexes contained the GPI-anchored glycoprotein GP-2, the lectin ZG16p, and sulfated matrix proteoglycans. Formation of zymogen granules as well as the formation of detergent-insoluble complexes was reduced after treatment of rat pancreatic lobules with FBI¹⁰³. These results suggested the possible role of GPI-anchor in zymogen processing in the pancreas. GP-2-null mice, however, displayed no gross signs of nutrient malabsorption such as weight loss, growth retardation, or diarrhea¹⁶¹. Zymogen granules in the GP-2 null mice appeared normal on electron microscopy and contained the normal complement of proteins excluding GP-2¹⁶¹. Therefore, the role of GPI anchoring on the FBI-mediated pancreatic toxicity remains obscure.

The folate receptor, like many glycosylphosphatidylinositol-anchored proteins, is found associated with membrane domains that are insoluble in Triton X-100 at low temperature and that are enriched in cholesterol and sphingolipids. The folate receptor-mediated transport of 5-methyltetrahy-

drofolate was almost completely blocked in cells in which sphingolipids had been reduced by approximately 40% in Caco-2 cells after FBI-treatment. FBI had no influence on the receptor-mediated transport or facilitative transport of the folate¹⁶². FBI-treatment was shown to alter recycling of folate receptor in cell systems¹⁶³.

Maternal FBI administration (20 mg/kg of b.w./day, i.p.) to pregnant LM/Bc mice during early gestation resulted in 79% NTDs in exposed fetuses⁴⁴. Sphingolipid profiles were significantly altered in maternal and embryonic tissues following the exposure, and ³H-folate levels and immunohistochemical expression of GPI-anchored folate receptor were reduced. Maternal folate supplementation partially rescued the NTD phenotype, whereas intraperitoneal injections of a lipid raft component, GM1, significantly restored folate concentrations and afforded almost complete protection against FBI-induced NTDs. Maternal FBI exposure is thus proposed to alter folate concentrations in LM/Bc mice, resulting in a dose-dependent increase in NTDs.

Treatment with the same regimen of inbred mouse SWV strain, however, yielded different results. Approximately 15% of the implants were resorbed, and only one exencephalic fetus was observed in the 10 SWV litters examined after the intraperitoneal administration of 20 mg/kg b.w./day on GD 7.5 and GD 8.5. A similar strain difference was also observed between CD1 and LM/Bc mice^{101,164}. CD1 mice exhibit an increased number of early embryonic resorptions and/or late fetal death following FBI exposure, whereas LM/Bc embryos survive, but fail to complete neurulation.

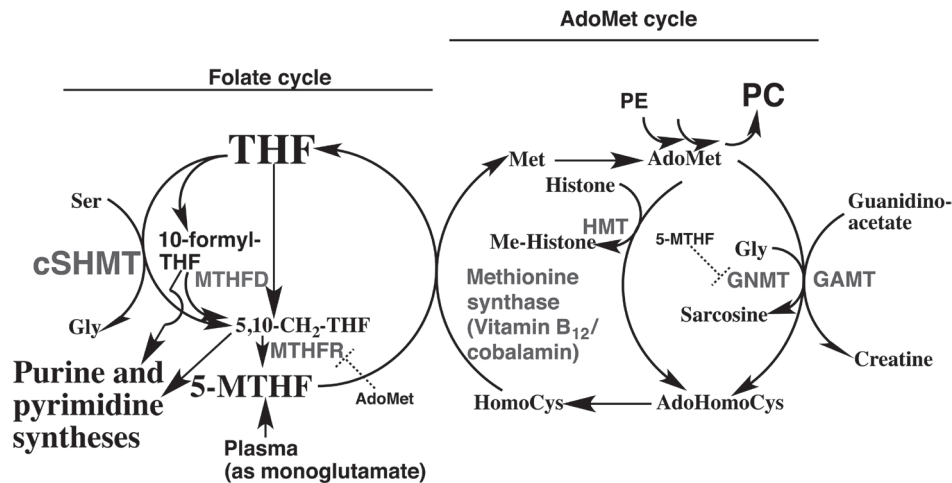


Fig. 5. Folate, cobalamin and methionine and their interactions
 Cys; cysteine, Gly; glycine, Ser; serine, 5-MTHF; 5-methyltetrahydrofolate pentaglutamate, GNMT; glycine N-methyltransferase, HMT; histone methyltransferase, MTHFD; tetrahydrofolate dehydrogenase, MTHFR; methylenetetrahydrofolate reductase, cSHMT; cytosolic serine hydroxymethyl transferase, THF; tetrahydrofolate pentaglutamate, adoMet; S-adenosylmethionine. Dotted half-signs indicate the feedback inhibition. Enzymes are shown in gray color.

5.2 Interaction of Folate, Cobalamin and Methionine

Folate, S-adenosylmethionine and cobalamin (vitamin B12) are key components of the one carbon unit (C1-unit) supply chain for nucleotide biosynthesis¹⁶⁵ (**Fig. 5**). Interruption of this supply chain is known to lead to embryo and developmental toxicities^{166,167}.

Female LM/Bc mice fed folate-sufficient (control) or folate-deficient diet were treated intraperitoneally with 0 (vehicle), 2.5 or 10 mg/kg FB1 on embryonic days 7 (E7) and E8, and their fetuses examined on E16. Dose-dependent NTD induction was found in groups fed the control diet: 3 of 13 low-FB1 dose and 10 of 11 high-FB1 dose litters were affected. Among groups fed folate-deficient diet, NTDs were found only in 4 of 11 high-FB1dose litters. In another trial, consumption of folate-deficient diet also resulted in fewer NTDs at a dose of 10 mg/kg of FB1 and reduced maternal red blood cell folate levels by 80%. In utero death did not fully account for the differences in NTD rates. Thus, folate deficiency does not exacerbate FB1-mediated NTD formation in LM/Bc mice¹⁶⁸.

Cobalamin can bind two carrier proteins in the digestive tract, haptocorrin (R protein) and intrinsic factor, but only the binding to intrinsic factor allows its absorption. Cobalamin is bound almost exclusively to haptocorrin in the acid milieu of the stomach, rather than to intrinsic factor. Cobalamin remains bound to haptocorrin in the slightly alkaline environment of the intestine until pancreatic proteases partially degrade haptocorrin and enable cobalamin to become bound exclusively to intrinsic factor¹⁶⁹.

In the aspirates from patients with exocrine pancreatic dysfunction, cobalamin was found to be coupled >60% to a protein identical to R proteins in terms of molecular mass (125,000), ionic nature (mean pI, 3.51), and also of the reactivity with anti-R protein and anti-intrinsic factor sera. These findings suggest that the formation of unabsorbable cobalamin complexes may be a reason for the impaired cobalamin absorption in exocrine pancreatic insufficiency. Decreased enzyme activity and nondegradation of R proteins may also be due to malactivation of pancreatic zymogens in an acidic pH of the intestinal juice¹⁷⁰. In another study of patients showing malabsorption of cobalamin with exocrine pancreatic insufficiency, a failure to degrade haptocorrin is proposed to prevent the binding of cobalamin to intrinsic factor¹⁷¹. Thus currently plural types of C1-unit dysfunctions are possible causes of developmental toxicity after FB1 exposure¹⁰¹.

5.3 Myelin

Long-term deficiency of cobalamin or folate causes a demyelinating disease of the brain and spinal cord¹⁷². A reduced supply of methyl groups has been implicated as the mechanism of demyelination from human studies of inborn errors. One child had abnormal methylfolate metabolism, one had abnormal methylcobalamin metabolism, and one had hypermethioninaemia probably caused by methionine adenosyltransferase deficiency. Each patient had abnormal myelination before treatment, suggesting demyelination with low cerebrospinal concentration of S-adenosylmethionine. Treatment with L-methionine or betaine led to substantial

clinical improvement, apparent remyelination, and increases in cerebrospinal S-adenosylmethionine concentration into the normal range¹⁷²). Thus, expected decrease in circulating methionine level in FB1-exposed animals could be linked to the brain dysfunction.

Myelin is a fatty white substance surrounding the axon of nerve cells^{173,174}). The primary lipid of myelin is glycolipid called galactocerebroside. UGT8 enzymes¹⁷⁵) (UDP-galactose ceramide galactosyltransferases) catalyze transfer of galactose to ceramide to form galactocerebroside (Fig. 4). Amounts of compacted myelin as well as myelin binding protein and myelin specific sphingolipids are decreased in CerS2-deficient mice carrying an insertion (gene trap) in the *CerS2* gene¹¹⁴). *CerS2* is localized to white matter of the brain and capable of synthesizing myelin sphingolipids¹⁷⁶) These results suggest the possible involvement of FB1-mediated suppression of brain *CerS2* in myelin dysfunction. Sphingomyelin is also contained in the membranous myelin sheath surrounding nerve cell axons, where it serves as an electrically insulating layer.

Myelination starts at birth in the spinal cord in the mouse, and is achieved in almost all regions of the brain around 45–60 days postnatally¹⁷³). In humans, the peak of myelin formation occurs during the first year postnatally¹⁷⁷), although it starts during the second half of fetal life in the spinal cord. These results may suggest the high susceptibility to FB1 during developing ages. Decreased production of ceramides would lead to reduced levels of galactocerebroside and PC/sphingomyelin, which may be associated with equine leukoencephalomalacia and swine pulmonary edema, respectively.

6. Roles of PC Mobilization and of Methionine-deficiency in FB1-mediated Toxicity

FB1 shows multiple organ toxicities in experimental and domestic animals. Toxicities like leukoencephalomalacia and pulmonary edema are unique and species-specific, but nephrotoxicity occurs in many species and hepatotoxicity appears in all species examined. Profiles of biochemical parameters suggest the suppressed bile excretory function possibly due to the deficiency of PC supply as the initial event of FB1-mediated liver toxicity as described above.

Typical pathological models of liver toxicity are related to phospholipid dysfunctions in rodents. One model is the *Pemt*-null mouse fed a choline-deficient diet¹³¹), and the second is rat and mouse fed choline-deficient diet with ethionine supplementation^{140–142}). The third model is mouse fed a diet deficient of both choline and methionine. The first model

showed severe liver disorders to lead to death within one week. The second model is characterized with both liver and pancreas damages. The third is a typical NASH (nonalcoholic steatohepatitis) model^{98,178}). These models yield consistently deficient conditions of both CDP-choline and methyl donor for PE methylation.

Total phospholipids account for roughly 2–3% wet weight of liver of mouse and the half is PC¹⁷⁹). Thus, total hepatic amount of PC would be around 20–30 mg in 1–2 g of liver of 20 g body weight of mouse. Apparently, the mouse liver secretes the equivalent of its entire hepatic pool of PC into bile daily. Most of the PC is reabsorbed through the intestine as LPA and is returned to the liver and other tissues after the reacylation. Administration of methionine was unable to prevent FB1-mediated liver toxicity in rats¹⁸⁰), although methionine partly prevented an increase of sphingosine, but not sphinganine. Therefore, decreased recycling of PC, excreted in the intestine through the bile, is likely the main mechanism for the FB1-mediated deficiency of hepatic PC. No clear information is available at present on the initial disorder to lead to malabsorption of phospholipids in FB1-treated animals. Plural candidates are discussed in this context. 1) Altered functions of sPLA2 are possible to occur. Functional sPLA2 requires submillimolar concentration of calcium ion for the maximal catalysis, but the chelating of carballylic acid part of FB1 with calcium ion may lower the efficiency of the catalysis as a direct interaction of FB1. 2) Similar to rodents fed CDE diet, FB1 causes lesions of the pancreas as well as liver in rats and pigs. If discharge of digestive enzymes is blocked and zymogen granules accumulated within acinar cells of pancreas in FB1-treated animals, reabsorption of PC would be decreased even considering the compensation through other PLA2 forms. The supply of ethionine is critical in pancreas lesion in the CDE model. Auxiliary synthesis of PC from PE in livers of FB1-treated animals would consume methionine entering through portal vein and thus diminish the amount available through the systemic circulation. This may produce a situation similar to animals treated with CDE diet or diet deficient both choline and methionine as described above.

In addition to the three *in vivo* models described above, administration of thioacetamide is reported to elicit the hepatotoxicity¹⁸¹) and encephalopathy¹⁸²) in rats. Brain levels of sphingomyelin and phosphatidylinositol are decreased in thioacetamide-treated rats. Similar to the CDE diet model, the hepatotoxicity was ameliorated by the coadministration of S-adenosylmethionine intraperitoneally in thioacetamide-exposed animals¹⁸³). Thioacetamide is reported to reduce levels of liver-specific methionine adenylyltransferase and DNA methylation¹⁸⁴). These data also support the idea that

methionine and phospholipid are associated with liver and brain toxicities.

On the association of methionine deficiency with pancreatic disorders, S-adenosylmethionine was shown to suppress autophagy through the action of methyltransferase Ppm1p in yeast¹⁸⁵. The enzyme modifies the catalytic subunit of PP2A. Lysosomal/autophagic dysfunction was proposed to be a key-initiating event in pancreatitis and cancer^{186,187}. CerS2 down-regulation is also reported to lead to autophagy in cell culture systems¹⁸⁸. Autophagosome half-life in mammalian cells is within 10 min, although impaired autophagic flux prolongs the half-life¹⁸⁹. Thus, careful examination may be necessary to detect an alteration of pancreas in FBI-treated animals.

FBI interacts with ceramide synthases (sphingosine N-acyltransferases) to inhibit N-acylations of sphinganine to form dihydroceramides, and of sphingosine to form ceramides (**Fig. 2**). Sphingomyelin synthase (SMS) mediates the biosynthesis of sphingomyelin from ceramides and PC. Human SMS1 and SMS2, rather than functioning strictly as sphingomyelin synthases, are capable of using sphingomyelin and PC as phosphocholine donors to produce PC or sphingomyelin, dependent on the relative concentrations of diacylglycerides (DAG) and ceramide as phosphocholine acceptors, respectively¹⁹⁰. Both SMS1 and SMS2 are ubiquitously expressed¹⁹⁰. SMS1 is associated with the Golgi apparatus, while SMS2 is primarily concentrated at the plasma membrane. The reverse transfer of phosphocholine is detected in various mammalian cells^{191–193}, and syntheses of PC and sphingomyelin are physiologically linked¹⁹⁴. SMS-mediated transfer of phosphocholyl group thus may be an additional pathway for supplying PC in tissues like the liver. Decreased levels of sphingomyelin and increased levels of ceramide or sphingosine are detected in livers of FBI-treated mice²³, rats¹³⁶ and pigs¹⁴, which may be indicative of the adaptive changes to lead to their liver disorders.

PC is a component of high-density lipoprotein¹⁹⁵. Exchanges of PC thus occur through serum lipoproteins among tissues. PC is the predominant phospholipid of alveolar surfactant. Decreased availability of PC as a cellular membrane component, and also as a phosphocholine-donor for sphingomyelin synthesis may be linked to disorders in peripheral tissues such as the kidney and lung in FBI-treated animals. In addition, formation of PC from sphingomyelin would produce ceramides as byproducts. Excess ceramides are metabolized to sphingosine as well as galactocerebroside. Sphingosine and sphinganine are degraded to form ethanolamine phosphate and fatty aldehydes like palmitoic aldehyde. The former is used for the synthesis of PE, whereas the latter may be involved in the oxidative damage that occurs in tar-

get tissues. The high demands for PC supply in liver force the mobilization of PC from tissues through plasma components like high-density lipoprotein (HDL), and also the exchange of phosphocholine moiety between sphingomyelin and PC in tissues. Prolonged shifts in phospholipid flow may trigger the signal for FBI-mediated toxicological events.

Myelin is a fatty white substance surrounding the axons of nerve cells^{173,174}. The primary lipid of myelin is a glycolipid called galactocerebroside. UGT8 enzymes¹⁷⁵ (UDP-galactose ceramide galactosyltransferases) catalyze transfer of galactose to ceramide to form galactocerebroside. Production of galactocerebroside is necessary for myelination and FBI-mediated changes in the composition of myelin is possibly associated with brain disorders. In fact, the amounts of compacted myelin as well as myelin binding protein and myelin specific sphingolipids are decreased in CerS2-deficient mice carrying an insertion (gene trap) in the *CerS2* gene¹¹⁴. CerS2 is localized to white matter of the brain and capable of synthesizing myelin sphingolipids¹⁷⁶. These results suggest the possible involvement of FBI-mediated suppression of brain CerS2 in equine leukoencephalomalacia. In contrast, no embryonic or prenatal abnormalities have been detected in CerS2 null mice^{115,196}.

Sphinganine, sphingosine and the ratio are clearly increased in CerS2-deficient mice in both the liver and kidney, but only the liver toxicity, without kidney pathology, is observed¹⁹⁶. These data may be indicative of sphinganine and sphingosine as surrogate marker of the tissue exposure, but accumulation of both substances themselves is not directly linked to the toxicological events at least in the kidney. Alternatively, subsequent metabolism such as formation of sphingosine-1-phosphate may evoke cellular signals. CerS2, having sphingosine-1-phosphate receptor-like motifs in its sequence¹¹¹, may be regulated *in vivo* by sphingosine-1-phosphate¹¹⁰. Therefore altered signaling through sphingolipids, and the irregular metabolisms may be associated with chronic toxicities of FBI as the studies of CerS2-deficient mice suggested^{114,115}. Progress in quantitative analyses of biochemical consequence of phospholipid flows and signals would help us to clarify the detailed mechanisms of FBI-mediated toxicity.

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