



HAL
open science

New insights into the organ-specific adverse effects of fumonisin B1

Nicolas Loiseau, Arnaud Polizzi, Aude Dupuy, Nicole Therville, Mirindra Rakotonirainy, Jennifer Loy, Jean-Luc Viadere, Anne Marie Cossalter, Jean-Denis Bailly, Olivier Puel, et al.

► **To cite this version:**

Nicolas Loiseau, Arnaud Polizzi, Aude Dupuy, Nicole Therville, Mirindra Rakotonirainy, et al.. New insights into the organ-specific adverse effects of fumonisin B1. Archives of Toxicology, 2015, 89 (9), pp.1619-1629. 10.1007/s00204-014-1323-6 . hal-03331305

HAL Id: hal-03331305

<https://ut3-toulouseinp.hal.science/hal-03331305v1>

Submitted on 1 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

NEW INSIGHTS INTO THE ORGAN-SPECIFIC ADVERSE EFFECTS OF FUMONISIN B1: COMPARISON BETWEEN LUNG AND LIVER.

Running Title: New insights into FB1 effects on organs

Nicolas LOISEAU^{1,2*}, Arnaud POLIZZI^{1,2}, Aude DUPUY³, Nicole THERVILLE⁴, Mirindra RAKOTONIRAINY^{1,2}, Jennifer LOY^{1,2}, Jean-Luc VIADERE^{1,2}, Anne-Marie COSSALTER^{1,2}, Jean-Denis BAILLY^{1,2}, Olivier PUEL^{1,2}, Martine KOLF-CLAUW^{1,2}, Justine BERTRAND-MICHEL³, Thierry LEVADE⁴, Hervé GUILLOU^{1,2}, Isabelle P. OSWALD^{1,2}

¹ INRA, UMR1331 Toxalim, Research Centre in Food Toxicology, F-31027 Toulouse, France;

² Université de Toulouse, ENVT, INP, UMR1331 Toxalim, F-31076 Toulouse, France;

³ INSERM, U1048, MetaToul-Lipidomic Core Facility, Université Paul Sabatier, Toulouse, France;

⁴ INSERM, UMR1037, Centre de Recherches en Cancérologie de Toulouse, Toulouse, France ;

* Corresponding author: Dr. Nicolas Loiseau, ToxAlim, UMR1331, INRA/INPT/UPS, Integrative Toxicology & Metabolism group, 180 Chemin de Tournefeuille, BP 93173, 31027 Toulouse Cedex 3, France. Tel.: +33 561285003; fax: +33 561285310; e-mail: Nicolas.Loiseau@toulouse.inra.fr

Abstract

Fumonisin B1 (FB1) is a well-known inhibitor of *de novo* sphingolipid biosynthesis, due to its ability to inhibit Ceramide Synthases (CerS) activity. In mammals, this toxin triggers broad clinical symptoms with multi-organ dysfunction such as hepatotoxicity or pulmonary edema. The molecular mechanism of CerS inhibition by FB1 remains unknown. Due to the existence of six mammalian CerS isoforms with a tissue specific expression pattern, we postulated that the organ specific adverse effects of FB1 might be due to different CerS isoforms. The sphingolipid contents of lung and liver were compared in normal and FB1-exposed piglets (gavage with 1.5 mg FB1/kg body weight daily for 9 days). The effect of the toxin on each CerS was deduced from the analysis of its effects on individual ceramide (Cer) and sphingomyelin (SM) species. As expected, the total Cer content decreased by half in the lungs of FB1-exposed piglets, while in contrast, total Cer increased 3.5-fold in the livers of FB1-exposed animals. Our data also indicated that FB1 is more prone to bind to CerS4 and CerS2 to deplete lung and to enrich liver in d18:1/C20:0 and d18:1/C22:0 ceramides and to interact with CerS1 to enrich liver in d18:1/C18:0 ceramides. Cer levels were counterbalanced by those of SM. In conclusion, these results demonstrated that the specificity of the effects of FB1 on tissues and organs is due to the effects of the toxin on CerS4, CerS2 and CerS1.

Keywords

Fumonisin, Ceramide, Sphingolipids, Lung, Liver, *Fusarium verticillioides*

Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

Fumonisin B1 (FB1) is a polyketide mycotoxin consisting of two tricarballic acids, a twenty carbon chain length and an alpha-amino alcohol polar head. FB1 is the most prevalent member of a fusariotoxin family called fumonisins. This family of toxins contains approximately 30 members (Wan Norhasima et al. 2009). FB1 is mainly produced by *Fusarium verticillioides* and *F. proliferatum*. These fungi species are natural corn and sorghum pathogens. Thus, FB1 presence in feed and food is mainly restricted to maize-based products. As reported by several studies (Dutton 1996; SCOOP 2003), whatever the country, the occurrence and the level of FB1 contamination in maize and maize-based products remains high. Indeed, Dutton reported that more than three quarters of the world's maize production contained FB1 levels from 1ppm to dozens of ppm. More recently, the European SCOOP task reported that whatever the country in Europe, a third of food analyses show an FB1 contamination from 300 ppb to 10 ppm. Thus, in 2007, the European Union regulated to set the strict maximum levels of FB1 in human nutrition according to type of food, usage and consumers (Commission Regulation (EC) No 1126/2007). Similarly, in 2006, the European Union gave recommendations to its member states in order to judge the acceptability of feed compounds cereals and cereal products for animal feed according to the susceptibility of the main farm animal species (Commission Recommendation 2006).

FB1 provokes severe mycotoxicosis in animals through very different clinical symptoms (FAO/WHO Expert Committee on Food Additives 2002; Haschek et al. 2001). In most mammalian species, FB1 elicits nephrotoxicity, hepatotoxicity (Bolger et al. 2001) and disturbances of the intestinal barrier function (Bouhet and Oswald 2007). More specific clinical symptoms have also been reported: leukoencephalomalacia in horses, pulmonary edema and cardiac dysfunction in pigs (Haschek et al. 2001), neural tube defects through the effects of FB1 on folate transport in rodents and humans (Marasas et al. 2004; Missmer et al. 2006), and carcinogenesis in rodents (FAO/WHO Expert Committee on Food Additives 2002). It is also a contributing factor in human oesophageal cancers in association with other mycotoxins such as ochratoxine A, deoxynivalenol and/or zearalenone (Shephard et al. 2013). Consequently, FB1 has been classified by the International Agency for Research on Cancer (IARC) in the Group 2B as a chemical potentially carcinogenic to humans. Hepatotoxicity, nephrotoxicity, immunotoxicity and other FB1 clinical symptoms that initially appeared to be species-dependent have also been observed in other species. The neurodegeneration observed in Equine LeukoEncephaloMalacia (ELEM) induced by fumonisin food intoxication has been reproduced in BALB/c mice after intracerebroventricular infusion (Osuchowski et al. 2005) or in pigs after experimental acute fumonisin toxicosis (Fazekas et al. 1998). Similarly, lung edema and cardiac dysfunction have been observed in mice after FB1 exposure (He et al. 2002). FB1 alters sphingolipid metabolism and tumor necrosis factor α expression in mouse heart and lung. Therefore, it seems to be more important to focus on FB1 tissue-specificity than on species-specificity to investigate the large clinical panel of this toxin's activity.

In 1988, Gelderblom characterized the chemical structure of FB1 (Gelderblom et al. 1988). Due to its structural similarity with sphinganine (Sa), sphingosine (So), fatty acids and ceramide (Cer), it was natural to postulate that FB1 was able to influence sphingolipid metabolism (Wang et al. 1991). Indeed, Merrill et al. (Merrill et al. 1996; Merrill et al. 2001) showed that the inhibition of ceramide synthases (CerS) by FB1 induces a major increase in free sphinganine levels and a depletion in complex sphingolipids and ceramides. Under these conditions, an increase in the ratio of free sphingoid bases (Sa/So) has been observed in several tissues (Grenier et al. 2012), in plasma (Riley et al. 1993) and in cultured cell lines (Bouhet et al. 2004; Loiseau et al. 2007). Whereas the plasma or urine ratio of Sa to So is considered as the reference biomarker to document an FB1 effect, Sa and/or Sphinganine 1-Phosphate (Sa-1P) levels can also be used as efficient markers.

During the last decade, several groups showed that CerS proteins belong to a family composed of six enzymes distributed differentially in tissues, each of them using a preferential subset of fatty acyl-CoAs (e.g. CerS1/C18; CerS2/C22-24; CerS3/C26; CerS4/C20; CerS5-6/C16) (Pewzner-Jung et al. 2006; Mullen et al. 2012); Table 1 in Online Resource 1. We postulated that the effects of FB1 may be dependent on the tissue specific expression profile of ceramide synthase isoforms and thereby explain the tissue-specific effects. To address our hypothesis, we analyzed the sphingolipid contents of lung and liver from control piglets and those exposed to FB1 per os (1.5mg FB1/kg body weight daily for 9 days). The activity of CerS and sphingomyelin synthases regulate respectively Cer and sphingomyelin (SM) levels. This study was focused on the analyses of these latter compounds.

Materials and methods

Mycotoxin preparation

The mycotoxin extract was obtained after *in vitro* culture of the high-FB1-producing *F. verticillioides* strain NRRL 3428, as previously described (Bailly et al. 2005). Sterilized yellow maize was inoculated with *F. verticillioides* and was incubated for 4 weeks at 21°C. The culture was reduced to powder that was rich in FB1 (9.26 mg/g). It also contained low levels of fumonisin B2 and fumonisin B3 (2.26 and 1.34 mg/g, respectively) but no detectable levels of zearalenone, deoxynivalenol, fusarochromanone, or tricothecenes.

Animals and experimental design

Animals were acclimatized for 2 weeks in the animal facility of the INRA ToxAlim Unit (Toulouse, France) prior to being used in experimental protocols. They were given free access to water and were fed ad libitum with a basal diet formulated according to the energy and amino-acid requirements for piglets, as previously described (Grenier et al. 2011). Six castrated male piglets [Pietrain x (Landrace x Large-White)] were weaned at 28 d. of age and were randomly allocated to either the control or treated group. The initial body weights were (mean ± SD) 10.2 ± 0.6 and 9.4 ± 1.6 kg for the control and extract-treated group respectively. During the 9-day experimental period, treated piglets received an oral bolus daily of fumonisin extract (corresponding to 1.5 mg FB1/kg body weight) diluted in a 200g/L solution of glucose (used as a sweetener) to reach a volume of 0.65mL/kg body weight. This dose corresponds to the ingestion of feed containing approximately 25 to 30 mg of FB1 per kg of body weight. Such contaminated feed has been shown to influence pig jejunal glycolipid distribution and the Sa/So ratio (Loiseau et al. 2007). Control piglets received the glucose solution alone. Pigs were killed by electronarcosis followed by exsanguination. All animal experimentation procedures were carried out in accordance to the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Five authors have an official authorization from the French Veterinary Services for animal experimentation.

Tissue and blood sampling

At day 0 and day 9, blood samples were aseptically collected from the left jugular vein. Blood was collected in tubes containing sodium heparin for biochemistry, or citrate for fibrinogen measurement (Vacutainer®, Becton-Dickinson, USA). Plasma samples were obtained after centrifugation of blood and stored at -20°C for later analysis. Upon termination of the experiment, corresponding to 9 days of dietary exposure to treatments, immediately after electrical stunning, pigs were killed by exsanguination, and liver and lungs were removed, perfused with PBS, cut into small pieces, flash-frozen in liquid nitrogen and stored at -80°C until lipid analysis.

Chemicals and reagents

Liquid chromatography solvents [methanol (MeOH), chloroform (CHCl₃) (HPLC-grade) and ammonium acetate] were purchased from Sigma-Aldrich. Synthetic internal lipid standards (Cer d18:1/18:0, Cer d18:1/15:0, SM d18:1/18:0 and SM d18:1/6:0) were from Avanti Polar Lipids (Alabaster, USA).

Preparation of ceramide and sphingomyelin stock and working standard solutions

A 1 mg/mL stock solution of each of Cer d18:1/18:0, Cer d18:1/15:0, SM d18:1/18:0, and SM d18:1/6:0 was prepared in MeOH. All stock solutions were stored at -20°C. Calibration solutions were made from the stock solution of analyte and of the internal standard, and prepared in eluent 20%B/80%A (see below). The calibration curve consisted of ten calibration points with Cer d18:1/15:0 and SM d18:1/6:0 as internal calibration standards. The range was between 0.488 and 250 pg/μL, and 8 pg/μL as fixed concentration internal standard.

Ceramide and sphingomyelin extraction

A piece of liver was homogenized in 1mL of MeOH/5mM EGTA (2:1 v/v) with FAST-PREP (MP Biochemicals). Lipids corresponding to 1 mg of tissue were extracted according to a procedure adapted from Bligh and Dyer (Bligh and Dyer 1959) in dichloromethane/MeOH (2% acetic acid)/water (2.5:2.5:2 v/v/v), in the presence of the internal standards Cer d18:1/15:0 (400 pg) and SM d18:1/6:0 (400 pg). The solution was centrifuged at 1500 rpm for 3 min. The organic phase was collected and dried under nitrogen and then dissolved in 50 μL of eluent 20%B/80%A. The extract was then stored at -20°C prior to analysis.

Ceramide and sphingomyelin analysis by liquid chromatography mass spectrometry

Ceramide/Sphingomyelin extracts and standards were dissolved in 50 μ L of solvent 20%B/80%A. Standards and sample solutions were analyzed using an Agilent 1290 UPLC system coupled to a G6460 triple quadrupole spectrometer (Agilent Technologies) and using MassHunter software for data acquisition and analysis. A UPLC Silica Synchronis column 100 x 2.1 mm; 1.7 μ m (Interchim) was used for LC separation. The column temperature was controlled at 40°C. The mobile phase A was CHCl₃/MeOH (80:20, v/v), and B was CHCl₃/MeOH/water, 5mM ammonium acetate pH 5.6 (60:34:6, v/v). The gradient used was: between 0 to 10 min, the proportion in B increased from 20% to 100%; 10-13 min, 100% B; and then back to 20% B at 13.5 min until 15 min for column re-equilibrium prior to the next injection. The flow rate of the mobile phase was adjusted to 0.3 mL/min and the injection volume was 5 μ L. QqQ MS analysis was performed in the positive ion mode of electrospray ionization (ESI), under the following conditions: capillary voltage, 4000 V; nozzle voltage, 1000 V; source temperature, 325°C; desolvation temperature, 400°C; cone gas flow rate, 10 L/min; desolvation gas flow rate, 12 L/min; nebulizer, 20 psi (SM) or 30 psi (Cer); MS collision energy (EC), 25 V. Nitrogen was used as collision gas. Precursor ion scan mode was used, with a 25V of energy collision, in order to quantify: ceramides (d18:1) following the precursor ion with a m/z of 264.2 using 120V as fragmentor voltage; and sphingomyelins following the precursor ion with a m/z of 184 using 160V as fragmentor voltage. The mass range (m/z 450–750 for Cer; 555-850 for SM) was scanned with a scan time of 500 ms. Data analysis was performed with QqQ Quantitative and Qualitative software B.04.00 (Agilent Biosystem).

Gene expression studies

Total RNA was extracted with Ribozol™ reagent (Amresco, Solon, USA). For real-time quantitative polymerase chain reaction (qPCR), total RNA samples (2 μ g) were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Primers for SYBR® Green assays are presented in Table 2 (in Online Resource 1). Amplifications were carried out on a Stratagene Mx3005p™ Real Time PCR System (Agilent Technologies, Santa Clara, USA), using SYBR® Premix Ex Taq™-Tli RNase H Plus reagent (Takara Bio Inc., Shiga, Japan). qPCR data were normalized to Ribosomal protein L32 (Rpl32) messenger RNA (mRNA) levels and analyzed with LinRegPCR

Statistics

The results are shown as mean \pm SD. All statistical analyses were performed using R (www.r-project.org). For comparison between two independent groups, the Student's t-test was used. For time-course dependent data, differential effects were analyzed by analysis of variance (ANOVA) followed by Student's t-tests with a pooled variance estimate. A *P*-value < 0.05 was considered significant and a *P*-value < 0.01, very significant.

RESULTS

Influence of FB1 on the ceramide and sphingomyelin content

The influence of FB1 on dihydroceramide synthesis has been evaluated *in vitro* using regular fatty acyl chains length containing 16 or 18 carbons. We showed that whatever the fatty acyl-CoA or the tissue, 200nM of FB1 was able to significantly reduce the dihydroceramide synthesis (Online Resources 1 and 2). In the presence of only 20nM FB1, dihydroceramide synthesis activity was inhibited in liver using either palmitoyl-CoA or stearoyl-CoA, while this activity was enhanced in lung in the presence of stearoyl-CoA suggesting that FB1 could have dual and opposite effects on CerS. In cells, the chain length of fatty acids present in dihydroceramides and ceramides varies from 16 to 26 carbons; moreover, specific CerS isoforms are involved in the synthesis of these various molecular species. As fatty acyl chains longer than 18 carbons are difficult to manipulate, the effect of FB1 on all CerS isoforms was analyzed directly *in vivo* on the ceramide and the sphingomyelin contents of liver and lungs from control and FB1-exposed animals.

After 9 days of treatment, the tissues showed no pathological signs. In order to evaluate the effect of the FB1, the animals were weighed along with whole liver and lungs. For each animal, the ratio of tissue/body weights was calculated and is plotted in Fig. 1a. These results show a significant increase in both liver and lung weight for animals in the exposed group as compared to the control group. The C18 sphingoid bases (Sa/So) ratio that is considered as a good biomarker of FB1 exposure, was determined (Fig. 1b.). As expected, animals from the exposed group showed a significant increase in the ratio in both liver and lung. Some plasma biochemical markers were also quantified: total cholesterol (TC), triglycerides (TG), total proteins (TP), albumin (ALB), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), bilirubin (BIL), fibrinogen (FIB) and haptoglobin (HAP). Among these plasma biomarkers, only TC and AST showed a significant elevation in FB1-exposed pigs (Fig. 1c. and 1d.).

Influence of FB1 on sphingolipid alteration in liver

We next investigated the effect of FB1 exposure on the quantity of liver ceramides and sphingomyelins using liquid chromatography mass spectrometry analysis. Surprisingly, the total quantity of ceramides increased significantly by about 3.5 fold (Fig. 2a). Analysis of the gene expression of the six CerS isoforms showed that CerS6 gene expression significantly decreased by half in the presence of FB1 (Fig. 2h). This significant decrease in CerS6 was confirmed by investigating the relative proportion of CerS genes in the liver in the presence of FB1 (Fig. 2g). We also observed a trend in FB1 exposure to increase the relative proportion of CerS4 and to decrease CerS2. However, the increase in total ceramides might be due to the extensive hydrolysis of sphingomyelin (even if a significant decrease is not shown, due to the different scale of the range between ceramides and sphingomyelins) in order to compensate for the lack of *de novo* ceramide synthesis.

The relative expression of genes related to the synthesis and the hydrolysis of sphingomyelins (Sgms1, Sgms2, Smpd1, Smpd2, Smpd3, Smpd4) and to the hydrolysis of ceramides (Acer1, Acer2, Acer3) was also investigated, but no significant modulation of expression of these genes in presence of FB1 was observed.

The modulation of the acyl chain distribution of the ceramides was analyzed in detail. The global amount of ceramides increased (Fig. 2c) significantly due to the increase in the quantity of ceramides containing C18:0 to C24:1 fatty acids with the most significant increase ($p < 0.01$) for Cer (d18:1/C22:0). In terms of the proportion of different molecular species (Fig. 2d), FB1 exposure significantly depleted the liver in Cer (d18:1/16:0) and elevated Cer (d18:1/C20:0). Whereas the ceramide content of liver increased in pigs exposed to FB1, the total amount of liver sphingomyelins was halved (Fig. 2b). However this change in total sphingomyelin was not significant, but the quantity of some species decreased significantly (Fig. 2e) such as SM (d18:1) with acyl chains of C16:0, C18:0, C22:1, C24:1, C24:2 and SM (d18:0) with acyl chains of C18:0 or C20:0. In the presence of FB1, the sphingomyelin distribution (Fig. 2f) shows both a significant decrease in SM (d18:0/C18:0), SM (d18:1/C22:1), SM (d18:1/C24:2) and a significant increase in SM (d18:0/C20:0), SM (d18:1/C22:0), SM (d18:0/C24:0). These results could be attributed to an enhancement of CerS4 activity, to an increase in its protein expression or to an inhibition of CerS5 and/or CerS6 activities or to a reduction of their protein expression.

Influence of FB1 on sphingolipids in lung

Mass spectrometry analysis of the contents of lung ceramides and sphingomyelins showed that in the presence of FB1, the total amount of ceramides decreased by half (Fig. 3a), whereas the total amount of SM almost doubled (Fig. 3b). These changes, however, were not significant. Analysis of the gene expression of the six CerS isoforms, showed that CerS6 and CerS5 gene expression was significantly decreased (respectively 3- and 5-fold) in the presence of FB1 (Fig. 3h). The significant decrease in CerS6 was confirmed by the relative proportion of CerS genes in lung in the presence of FB1 whereas the decrease in the relative proportion of CerS5 was a trend. However, the relative proportion of CerS1 and CerS4 was significantly increased in lung after FB1 exposure (Fig. 3g). The FB1 exposure also significantly increased Smpd1 in lung (Fig. 3h). Given that this protein is involved in the hydrolysis of sphingomyelins, the increase might compensate for the inhibition of the *de novo* synthesis of ceramides.

A detailed analysis of the molecular species of ceramides and sphingomyelins revealed significant alterations. In terms of quantity (Fig. 3c), d18:1-Ceramides containing C18:0 and C20:0 acyl chains decreased significantly in lung under FB1 exposure with the most significant change being a decrease in Cer (d18:1/C22:0) and Cer (d18:1/C24:0). In terms of relative abundance (Fig. 3d), only Cer (d18:1/C20:0) and Cer (d18:1/C22:0) showed a significant decrease. For sphingomyelins, a significant increase in quantity (Fig. 3e) was observed for some SM (d18:1) species like those with C16:0, C22:1, C24:1, C24:2, and SM (d18:0) with acyl chains of C16:0 or C18:0. In the presence of FB1, the SM distribution (Fig. 3f) showed both a significant increase in SM (d18:0/C16:0) and SM (d18:0/C18:0) with the same trend for all sphingomyelins, and a significant decrease in SM (d18:0/C20:0), SM (d18:1/C22:0), SM (d18:0/C24:0), opposite to the global trend. These results thus show a ceramide depletion in lung after FB1 treatment that could be attributed to an inhibition of CerS4, CerS2 and to a lesser extent, CerS1 activities, or to a decrease of their protein expression. Moreover, FB1 exposure induced mainly C16:0 and C24:1-sphingomyelins. Altogether, these results indicate that the depletion in C24-Ceramide is counterbalanced by an increase in C24-Sphingomyelin, minimizing the role of CerS2, but also, that the C16-Sphingomyelin increase could be attributed to an enhancement of CerS5 and/or CerS6 activities or of their protein expression.

DISCUSSION

Fusarium verticillioides is a common cereal pathogen and FB1 is highly produced on maize. We postulated that this toxin may interact with specific CerS. Thus, we first focused on the effect of FB1 on dihydroceramide synthase in the tissues of interest (liver and lung) using as donors the four major fatty acids contained in maize (i.e., palmitoyl-CoA [C16:0], stearoyl-CoA [C18:0], oleoyl-CoA [C18:1] and linoleoyl-CoA [C18:2]). Using these four fatty acyl-CoA, FB1 interaction with dihydroceramide synthase was studied through Lineweaver-Burk analysis, as shown in Online Resources 1 and 2. Whereas no effect of FB1 was observed on the dihydroceramide synthesis using linoleoyl-CoA and oleoyl-CoA (data not shown), a competitive inhibition of FB1 with sphinganine was observed for dihydroceramide synthesis using palmitoyl-CoA or stearoyl-CoA. This effect of FB1 on dihydroceramide synthases occurred as low as 20 μ M. Moreover, these results show that FB1 can inhibit or activate dihydroceramides synthesis depending on the tissue (liver or lung) and the mycotoxin concentration. Indeed, palmitoyl-dihydroceramide synthesis was consistently inhibited by FB1 using either liver or lung microsomal extracts while only stearoyl-dihydroceramide synthesis was inhibited by FB1 using liver microsomal extracts. Using lung extracts, stearoyl-dihydroceramides synthesis was activated by the toxin at 20nM and inhibited at 200nM. Our *in vitro* results show for the first time that according to the tissue and dose, FB1 can have opposite effects. Despite the fact that maize contains the four fatty acids indicated above, in mammals dietary fatty acids can be further elongated (Guillou et al. 2010). Thus, an *in vivo* study exploration was considered essential and more relevant to investigate the effects of FB1.

In order to validate under field conditions our observation of the dual effect of FB1 when low doses and/or short time exposure apply, piglets were exposed to a low dose (1.5 mg of toxin /kg b.w.) for only 9 days. Our results show that after that period, the sphingoid base ratio Sa/So was significantly increased, which confirms the sensitivity of this biomarker of exposure (Loiseau et al. 2007). Liver toxicity is usually associated with a decrease in liver weight relative to the whole body weight and an increase of the AST serum level. The liver dysfunction was confirmed by increased plasma AST and TC, but the tissue weight relative to the body weight increased significantly in animals exposed to FB1 (Fig. 1) as previously described by Gumprecht *et al.* in 1998. The increase in the relative weight of the liver might be related to a potent steatotic and/or inflammatory state in the tissue of exposed animals (Grenier et al. 2012; Halloy et al. 2005; Siloto et al. 2013).

Considering the ceramide and sphingomyelin contents and their distribution in piglet liver and lung, our results (Fig. 2 and 3) confirm the *in vitro* data suggesting a dual and opposite actions of FB1 on the synthesis of these sphingolipid species. Both stimulatory and inhibitory effects of FB1 have been reported. Indeed several studies show that FB1 treatment decrease the level of ceramides in cell culture (Merrill et al. 1993; Mullen et al. 2011) and in animals (Loiseau et al. 2007; Kim et al. 2007; Pewzner-Jung et al. 2010b). However, an increase in ceramide levels by FB1 has been reported in other studies using HEK cells over-expressing Uog1 (also called CerS1) or transfected with Trh1 (also called CerS4) or Trh4 (also called CerS5) (Venkataraman et al. 2002, Riebeling et al. 2003). The present study reports increased ceramides in liver and decreased in lungs in animals exposed to FB1. According to our results, FB1 most likely impairs ceramide synthesis through CerS4 and to a lesser extent through CerS2 and CerS1. Indeed, based on the levels of ceramides containing a fatty acyl chain length from 18 to 24 carbons, whereas the “regular effect” of the inhibition of ceramides synthesis by FB1 was observed in lung, an increased synthesis of ceramides was measured in liver for the same animals. Regarding the relative abundance, Cer (d18:1/C20:0) is the only species for which a significant increase was found in the liver whereas a decrease was seen in lung. These results confirm how important CerS4 activity is in the tissue specificity of FB1 toxicity. In addition to this common feature, in the liver, the Cer (d18:1/C16:0) proportion decreased significantly in the presence of FB1, which is the opposite trend to the rest of the ceramide species but it is in good agreement with the significant decrease in CerS6 expression. As shown *in vitro*, this result suggests that FB1 is not able to enhance CerS5/6 activity. In lung, the proportion of Cer (d18:1/C16:0) was not significantly modulated even if the gene expression of the two isoforms decrease significantly in the presence of FB1. At least, the proportion of Cer (d18:1/C22:0) decreased significantly, possibly implicating CerS2 in lung. It should be noted that dimer formation or other post-translational events may explain the lack of correlation between CerS activity and CerS mRNA (Laviad et al. 2012; Mesicek et al. 2010)

All these results generally fit with the literature. Indeed, even if it has not been reported in liver, CerS4 has been identified as a potent target responding to modulators of the endogenous lipid metabolism like leptin (Bonzon-Kulichenko et al. 2009), insulin or changes in phospholipid transfer protein activity (PLTP α) (Rosenthal et al. 2011). The critical role of CerS2 has also been reported in liver growth and lipid metabolism (Pewzner-Jung et al. 2010a; Pewzner-Jung et al. 2010b). Moreover, this CerS isoform is also involved in the formation of an N-C24-FB1 acylated derivative in HEK cells over-expressing CerS 1-6 in the presence of 20 μ M of FB1 (Harrer et al. 2013). Probably due to the low dose of FB1 oral exposure, none of the FB1 acylated derivatives were

detected in our samples. Furthermore, deficiency of this ceramide synthase isoform predisposes inflammatory airway and airspace diseases (Petrache et al. 2013).

Based on these *in vivo* results, on the low distribution of this mycotoxin, on the specific tissue-species targeting and on the broad pharmacological spectrum of FB1, we assume that mycotoxin toxicity not only depends on its cellular level but also on the level of individual ceramide synthase isoforms (more particularly CerS4, CerS2 and CerS1) in the tissue of interest.

As shown in Figures 2 and 3, and because of the known effects of FB1 on sphingolipids (Loiseau et al. 2007), ceramides, dihydroceramides, sphinganine and sphingosine are not the only sphingolipid species modulated by this mycotoxin. Indeed sphingomyelins are also modulated by FB1 in the opposite way to ceramides. Sphingolipid metabolism is closely interconnected with fatty acid metabolism. Recently, it was shown that ELOVL fatty acid elongase 1 (ELOVL1), which is particularly involved in saturated and monosaturated C20- and C22-CoAs synthesis, is essential for the production of C24 sphingolipids (Ohno et al. 2010). Indeed, these authors established that ELOVL1 activity is regulated by ceramide synthase CerS2, also implicated in C24-sphingolipid synthesis. This knowledge fits perfectly with gas chromatography analysis of the lipid extracts from liver or lung showing a general increase in esterified fatty acids in animals receiving a diet contaminated with FB1 (data not shown). In the liver, this increase affected all fatty acid species leaving their relative proportions almost constant. On the contrary, in lung, saturated fatty acids increased two-fold whereas the mono- and polyunsaturated ones did not change. A detailed analysis showed that in the presence of FB1, new species of C20:0; C22:0 and C24:0 appeared in the liver whereas these same species disappeared in lung. According to these results, increased C16 and C18 saturated fatty acids in lung can explain pulmonary oedema by Toll-like receptor 4 (TLR4) activation and inflammation response signalling (Zanotti et al. 2009). Moreover, sphingomyelins have been implicated in the modulation of sterol regulatory element-binding protein (SREBP) through inhibition of SREBP cleavage-activating protein (SCAP) (Scheek et al. 1997). Indeed, SCAP is able to activate SREBP by cleavage or to scavenge it under its native form in the endoplasmic reticulum. Therefore, given the results of the effects of FB1 on ceramides and sphingomyelins containing a long chain fatty acyl moiety (>C20), and considering that FB1 contributes to steatosis (Grenier et al. 2012), we suggest that this toxin is not only able to modulate the content of sphingolipids but also able to modulate that of the neutral lipids and fatty acids (Data not shown).

CONCLUSION

This study shows that FB1 exposure induces a tissue-specific effect most likely modulated by CerS4, CerS2 and CerS1, through either inhibition or activation of their activities. These three ceramide synthase isoforms seem to drive the fumonisin toxicity and its selectivity for the tissues most enriched in them. Furthermore, the pharmacokinetics of FB1 show a volume of distribution corresponding to a similar concentration of this toxin in each tissue. Thus, we can assume that the toxicity of FB1 is not simply due to a specific accumulation of this toxin in any given tissue. The differences lie in the ratio between the level of protein expression of the CerS isoforms, in particular CerS4, CerS2 and CerS1 in each tissue, and in the affinity of FB1 for these enzymes. These differences may explain partially the dual and opposite effect of this mycotoxin on the liver and lungs. Nevertheless, further experiments are necessary to confirm these results.

As shown in this study, the changes in ceramides and sphingomyelins are associated with changes in the content of long-chain fatty acids (\geq C20). This seems to be due to an effect of FB1 on CerS4 and CerS2. Indeed, CerS2 has been shown to modulate ELOVL1 activities involved in the elongation of C16 and C18- saturated fatty acyl CoAs to C20 to C24-fatty acyl CoAs.

ACKNOWLEDGMENTS

This study was supported by the Région Midi-Pyrénées, France.

REFERENCES

- Bailly JD, Querin A, Tardieu D, Guerre P (2005) Production and purification of fumonisins from a highly toxigenic *Fusarium verticilloides* strain. *Rev Med Vet-Toulouse* 156 (11):547-554.
- Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37 (8):911-917.
- Bolger M, Coker RD, DiNovi M, Gaylor D, Gelderblom W, Olsen M, Paster N, Riley RT, Shephard G, Speijers GJA (2001) Fumonisin. In: Safety Evaluation of Certain Mycotoxins in Food, WHO Food Additives Series 47, FAO Food and Nutrition Paper 74, Prepared by the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Geneva.

- Bonzon-Kulichenko E, Schwudke D, Gallardo N, Molto E, Fernandez-Agullo T, Shevchenko A, Andres A (2009) Central leptin regulates total ceramide content and sterol regulatory element binding protein-1C proteolytic maturation in rat white adipose tissue. *Endocrinology* 150 (1):169-178.
- Bouhet S, Hourcade E, Loiseau N, Fikry A, Martinez S, Roselli M, Galtier P, Mengheri E, Oswald IP (2004) The mycotoxin fumonisin B1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicol Sci* 77 (1):165-171.
- Bouhet S, Oswald IP (2007) The intestine as a possible target for fumonisin toxicity. *Mol Nutr Food Res* 51 (8):925-931.
- Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006). vol 2006/577/EC. Official Journal of the European Union.
- Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products (2007). vol 1126/2007. Official Journal of the European Union.
- Dutton MF (1996) Fumonisin, mycotoxins of increasing importance: their nature and their effects. *Pharmacol Ther* 70 (2):137-161.
- Evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives (2002). *World Health Organ Tech Rep Ser* 906:i-viii, 1-62.
- Fazekas B, Bajmocy E, Glavits R, Fenyvesi A, Tanyi J (1998) Fumonisin B1 contamination of maize and experimental acute fumonisin toxicosis in pigs. *Zentralbl Veterinarmed B* 45 (3):171-181.
- Gelderblom WC, Jaskiewicz K, Marasas WF, Thiel PG, Horak RM, Vleggaar R, Kriek NP (1988) Fumonisin - novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl Environ Microbiol* 54 (7):1806-1811.
- Gelderblom WC, Marasas WF, Lebepe-Mazur S, Swanevelder S, Vessey CJ, Hall Pde L (2002) Interaction of fumonisin B1 and aflatoxin B1 in a short-term carcinogenesis model in rat liver. *Toxicology* 171 (2-3):161-173.
- Grenier B, Bracarense AP, Schwartz HE, Trumel C, Cossalter AM, Schatzmayr G, Kolf-Clauw M, Moll WD, Oswald IP (2012) The low intestinal and hepatic toxicity of hydrolyzed fumonisin B(1) correlates with its inability to alter the metabolism of sphingolipids. *Biochem Pharmacol* 83 (10):1465-1473.
- Grenier B, Loureiro-Bracarense AP, Luciola J, Pacheco GD, Cossalter AM, Moll WD, Schatzmayr G, Oswald IP (2011) Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. *Mol Nutr Food Res* 55 (5):761-771.
- Guillou H, Zdravec D, Martin PG, Jacobsson A (2010) The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog Lipid Res* 49 (2):186-199.
- Gumprecht LA, Beasley VR, Weigel RM, Parker HM, Tumbleson ME, Bacon CW, Meredith FI, Haschek WM (1998) Development of fumonisin-induced hepatotoxicity and pulmonary edema in orally dosed swine: morphological and biochemical alterations. *Toxicol Pathol* 26 (6):777-88.
- Halloy DJ, Gustin PG, Bouhet S, Oswald IP (2005) Oral exposure to culture material extract containing fumonisins predisposes swine to the development of pneumonitis caused by *Pasteurella multocida*. *Toxicology* 213 (1-2):34-44.
- Harrer H, Laviad EL, Humpf HU, Futerman AH (2013) Identification of N-acyl-fumonisin B1 as new cytotoxic metabolites of fumonisin mycotoxins. *Mol Nutr Food Res*. 57(3):516-22.
- Haschek WM, Gumprecht LA, Smith G, Tumbleson ME, Constable PD (2001) Fumonisin toxicosis in swine: An overview of porcine pulmonary edema and current perspectives. *Environ Health Perspect* 109:251-257.
- He Q, Bhandari N, Sharma RP (2002) Fumonisin B1 alters sphingolipid metabolism and tumor necrosis factor alpha expression in heart and lung of mice. *Life Sci* 71 (17):2015-2023.
- Kim DH, Lee YS, Lee YM, Oh S, Yun YP, Yoo HS (2007) Elevation of sphingoid base 1-phosphate as a potential contributor to hepatotoxicity in fumonisin B1-exposed mice. *Arch Pharm Res*. 2007 Aug;30(8):962-9.
- Laviad EL, Kelly S, Merrill AH Jr, Futerman AH (2012) Modulation of ceramide synthase activity via dimerization. *J Biol Chem*. 287(25):21025-33.
- Loiseau N, Debrauwer L, Sambou T, Bouhet S, Miller JD, Martin PG, Viadere JL, Pinton P, Puel O, Pineau T, Tulliez J, Galtier P, Oswald IP (2007) Fumonisin B1 exposure and its selective effect on porcine jejunal segment: sphingolipids, glycolipids and trans-epithelial passage disturbance. *Biochem Pharmacol* 74 (1):144-152.
- Marasas WF, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, Missmer SA, Cabrera J, Torres O, Gelderblom WC, Allegood J, Martinez C, Maddox J, Miller JD, Starr L, Sullards MC, Roman AV, Voss KA, Wang E, Merrill AH, Jr. (2004) Fumonisin disrupts sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human

- neural tube defects among populations consuming fumonisin-contaminated maize. *J Nutr* 134 (4):711-716.
- Merrill AH, Jr., Sullards MC, Wang E, Voss KA, Riley RT (2001) Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environ Health Perspect* 109 Suppl 2:283-289.
- Merrill AH, Jr., Wang E, Vales TR, Smith ER, Schroeder JJ, Menaldino DS, Alexander C, Crane HM, Xia J, Liotta DC, Meredith FI, Riley RT (1996) Fumonisin toxicity and sphingolipid biosynthesis. *Adv Exp Med Biol* 392:297-306.
- Merrill AH Jr, van Echten G, Wang E, Sandhoff K (1993) Fumonisin B1 inhibits sphingosine (sphinganine) N-acyltransferase and de novo sphingolipid biosynthesis in cultured neurons in situ. *J Biol Chem* 268(36):27299-306.
- Mesicek J, Lee H, Feldman T, Jiang X, Skobeleva A, Berdyshev EV, Haimovitz-Friedman A, Fuks Z, Kolesnick R (2010) Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell Signal*. 22(9):1300-7.
- Missmer SA, Suarez L, Felkner M, Wang E, Merrill AH, Jr., Rothman KJ, Hendricks KA (2006) Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. *Environ Health Perspect* 114 (2):237-241.
- Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, Obeid LM (2011) Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. *J Biol Chem*. 286(18):15929-42.
- Mullen TD, Hannun YA, Obeid LM (2012) Ceramide synthases at the centre of sphingolipid metabolism and biology. *Biochem J* 441 (3):789-802.
- Ohno Y, Suto S, Yamanaka M, Mizutani Y, Mitsutake S, Igarashi Y, Sassa T, Kihara A (2010) ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis. *Proc Natl Acad Sci U S A* 107 (43):18439-18444.
- Osuchowski MF, Edwards GL, Sharma RP (2005) Fumonisin B1-induced neurodegeneration in mice after intracerebroventricular infusion is concurrent with disruption of sphingolipid metabolism and activation of proinflammatory signaling. *Neurotoxicology* 26 (2):211-221.
- Petrache I, Kamocki K, Poirier C, Pewzner-Jung Y, Laviad EL, Schweitzer KS, Van Demark M, Justice MJ, Hubbard WC, Futerman AH (2013) Ceramide synthases expression and role of ceramide synthase-2 in the lung: insight from human lung cells and mouse models. *PLoS One* 8 (5):e62968.
- Pewzner-Jung Y, Ben-Dor S, Futerman AH (2006) When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis. *J Biol Chem* 281 (35):25001-25005.
- Pewzner-Jung Y, Brenner O, Braun S, Laviad EL, Ben-Dor S, Feldmesser E, Horn-Saban S, Amann-Zalcenstein D, Raanan C, Berkutzki T, Erez-Roman R, Ben-David O, Levy M, Holzman D, Park H, Nyska A, Merrill AH, Jr., Futerman AH (2010a) A critical role for ceramide synthase 2 in liver homeostasis: II. insights into molecular changes leading to hepatopathy. *J Biol Chem* 285 (14):10911-10923.
- Pewzner-Jung Y, Park H, Laviad EL, Silva LC, Lahiri S, Stiban J, Erez-Roman R, Brugger B, Sachsenheimer T, Wieland F, Prieto M, Merrill AH, Jr., Futerman AH (2010b) A critical role for ceramide synthase 2 in liver homeostasis: I. alterations in lipid metabolic pathways. *J Biol Chem* 285 (14):10902-10910.
- Riebeling C, Allegood JC, Wang E, Merrill AH Jr, Futerman AH (2003) Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J Biol Chem*. 278(44):43452-9.
- Riley RT, An NH, Showker JL, Yoo HS, Norred WP, Chamberlain WJ, Wang E, Merrill AH, Jr., Motelin G, Beasley VR, et al. (1993) Alteration of tissue and serum sphinganine to sphingosine ratio: an early biomarker of exposure to fumonisin-containing feeds in pigs. *Toxicol Appl Pharmacol* 118 (1):105-112.
- Rosenthal EA, Ronald J, Rothstein J, Rajagopalan R, Ranchalis J, Wolfbauer G, Albers JJ, Brunzell JD, Motulsky AG, Rieder MJ, Nickerson DA, Wijsman EM, Jarvik GP (2011) Linkage and association of phospholipid transfer protein activity to LASS4. *J Lipid Res* 52 (10):1837-1846.
- Scheek S, Brown MS, Goldstein JL (1997) Sphingomyelin depletion in cultured cells blocks proteolysis of sterol regulatory element binding proteins at site 1. *Proc Natl Acad Sci U S A* 94 (21):11179-11183.
- SCOOP (Scientific Cooperation on Questions to Food). Task 3.2.10 Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states. Subtask III: Fumonisin. (2003). Brussels, Belgium.
- Siloto EV, Oliveira EF, Sartori JR, Fascina VB, Martins BA, Ledoux DR, Rottinghaus GE, Sartori DR (2013) Lipid metabolism of commercial layers fed diets containing aflatoxin, fumonisin, and a binder. *Poult Sci* 92 (8):2077-2083.
- Venkataraman K, Riebeling C, Bodennec J, Riezman H, Allegood JC, Sullards MC, Merrill AH Jr, Futerman AH (2002) Upstream of growth and differentiation factor 1 (uog1), a mammalian homolog of the yeast

- longevity assurance gene 1 (LAG1), regulates N-stearoyl-sphinganine (C18-(dihydro)ceramide) synthesis in a fumonisin B1-independent manner in mammalian cells. *J Biol Chem.* 277(38):35642-9.
- Wan Norhasima WM, Abdulmir AS, Abu Bakar F, Son R, Norhafniza A (2009) The health and toxic adverse effects of *Fusarium* fungal mycotoxin, Fumonisin, on human population. *Am J Infect Dis* 5:273-281.
- Wang E, Norred WP, Bacon CW, Riley RT, Merrill AH, Jr. (1991) Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *J Biol Chem* 266 (22):14486-14490.
- Zanotti G, Casiraghi M, Abano JB, Tatreau JR, Sevala M, Berlin H, Smyth S, Funkhouser WK, Burrige K, Randell SH, Egan TM (2009) Novel critical role of Toll-like receptor 4 in lung ischemia-reperfusion injury and edema. *Am J Physiol Lung Cell Mol Physiol* 297 (1):L52-63.

FIGURES

Fig. 1. Effect of FB1 *in vivo* on physiological parameters after 9 days of exposure at 1.5mg/kg b.w./day: (a) ratio of tissue over whole body weights; (b) C18-sphingoid base ratio (Sa/So); (c) plasma level of total cholesterol; (d) plasma level of AST. These parameters were measured in FB1-exposed animals (solid bars) and in control animals (open bars). In panels a and b, data are plotted according the tissue (means \pm SD of three independent experiments): significant differences were seen using a Student t-test by comparing the FB1-exposed group to the control group. In panels c and d, data are time-dependent (means \pm SD of three independent experiments) by comparing data just before the beginning of the FB1 exposure (D0) and after 9 days of exposure (D9); significance was evaluated using an ANOVA test on paired samples (in each group between D0 and D9). *, **, *** indicate significant differences with a *P*-value < 0.05 , < 0.01 and < 0.005 respectively.

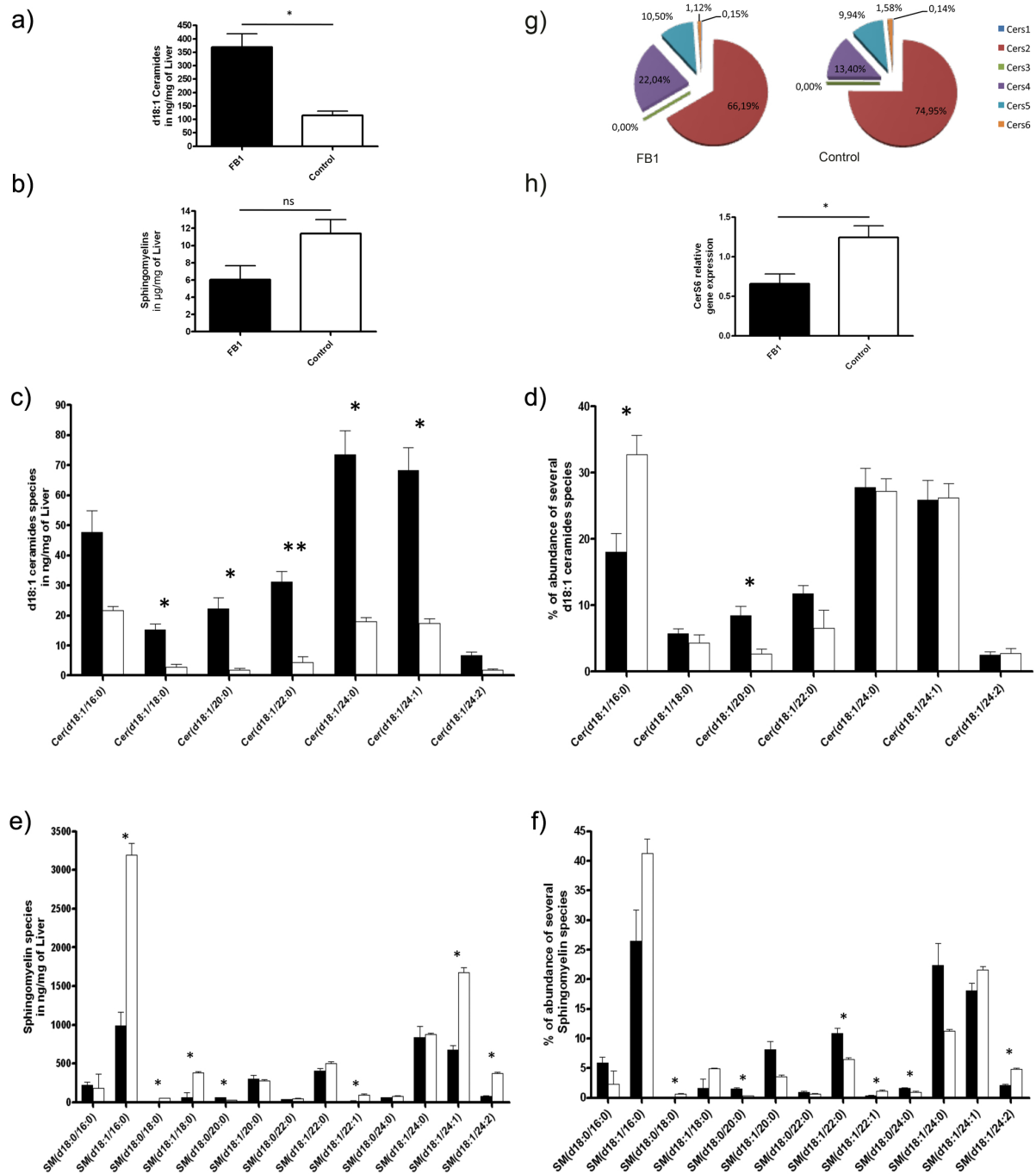


Fig. 2. Modulation of porcine liver ceramide and sphingomyelin content and distribution in control piglets or animals exposed to 1.5 mg of FB1/kg bw/day for 9 days. (a)(b) Total ceramides and sphingomyelins. (c)(d) Changes in the content and abundance of individual ceramides. (e)(f) Changes in the content and abundance of individual sphingomyelins. (g) The distribution of CerS isoforms expressed in the liver. (h) Significant levels of gene expression of CerS, Smpd, Sgms and Acer. These parameters were measured in FB1-exposed piglets (solid bars) and in the reference unexposed group (open bars). Data are means \pm SD from three different experiments in each group. Significance was determined using a Student t-test by comparing of FB1-exposed and reference groups. *, ** indicate significant differences with a P -value < 0.05 and < 0.01 respectively, ns indicates that there was no significant change.

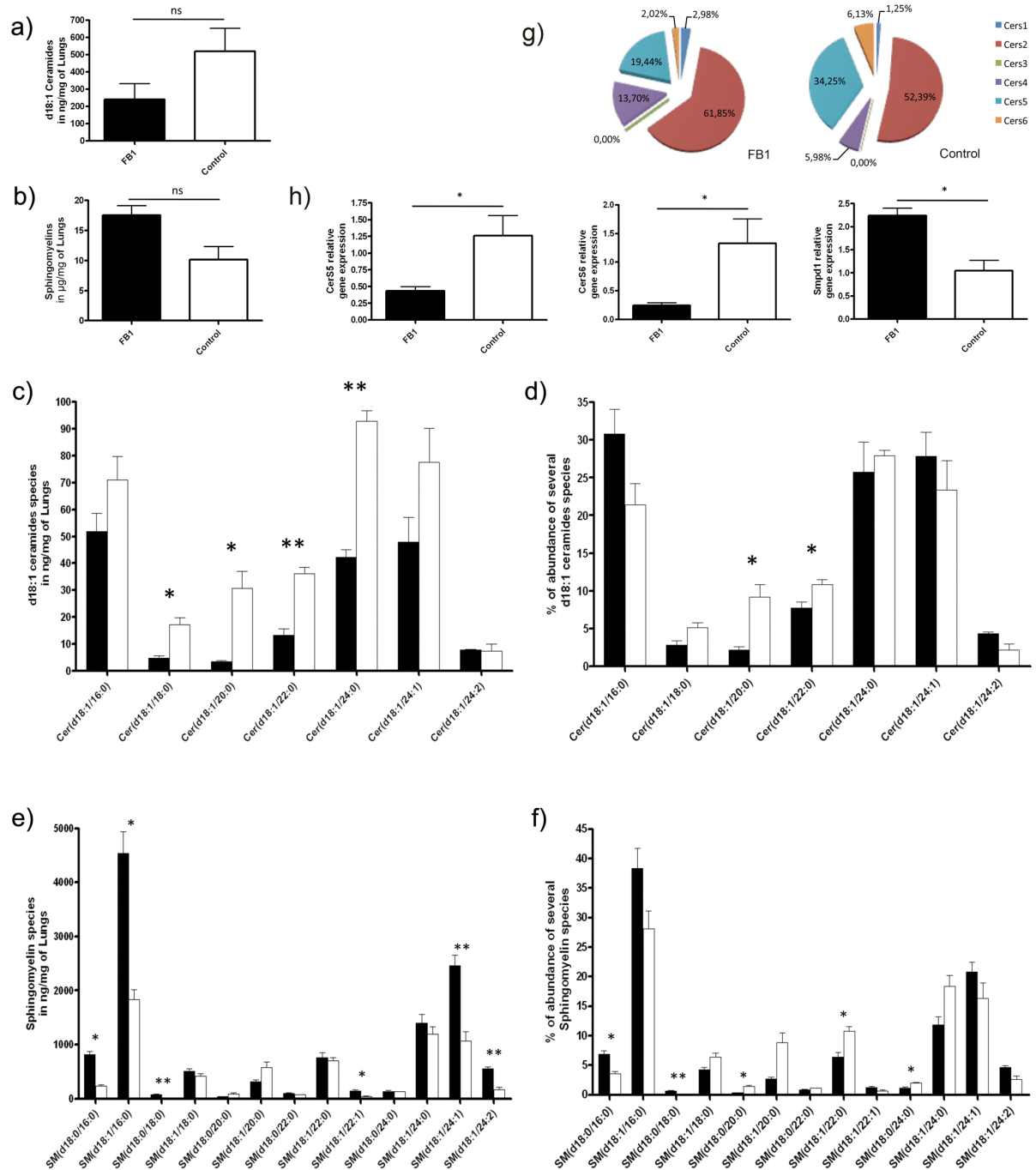


Fig. 3. Modulation of porcine lung ceramide and sphingomyelin contents and distribution in control piglets or animal exposed to 1.5 mg of FB1/kg b.w./day for 9 days. (a)(b) Total ceramides and sphingomyelins. (c)(d) Changes in the content and abundance of individual ceramides. (e)(f) Changes in the content and abundance of individual sphingomyelins. (g) The distribution of CerS isoforms expressed in lung. (h) Significant levels of gene expression of CerS, Smpd, Sgms and Acer. These parameters were measured in FB1-exposed animals (solid bars) and in the reference unexposed group (open bars). Data are means \pm SD from three different experiments in each group. Significance was assessed using a Student t-test by comparing FB1-exposed and reference groups. *, ** indicate significant differences with a P -value < 0.05 and < 0.01 respectively, ns indicates that there was no significant change.