Selective impairment of drug-metabolizing enzymes in pig liver during subchronic dietary exposure to aflatoxin B1

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Abstract

Consequences of subchronic exposure to aflatoxin B1 (AFB1) on liver monooxygenase and transferase enzymes were compared in control pigs and pigs given 385, 867 or 1807 µg AFB1/kg of feed for 4 weeks. Animals exposed to the highest dose of toxin developed clinical signs of aflatoxicosis, like liver fibrosis, hepatic dysfunction and decreased weight gain. This group had significantly lower levels of liver cytochrome P450, ethoxyresorufin O-deethylase (EROD) activity, testosterone metabolism, P450 1A and P450 3A protein expression. By comparison, mild degenerative hepatic changes, no hepatic dysfunction but a similar pattern of liver P450 enzymes activity without changes in P450 3A expression were observed in pigs exposed to 867 µg AFB1/kg of feed. Benzphetamine and aminopyrine N-demethylase activities were increased in pigs exposed to 867 or 1807 µg AFB1/kg of feed. Pigs exposed to 385 µg AFB1/kg of feed had low levels of EROD activity and all other biotransformation and clinical parameters remained at control levels. Aniline hydroxylase activity, P450 2C protein expression, UDP-glucuronosyl and glutathione S-transferase activities were unaffected at all doses of AFB1. In conclusion, P450 1A and P450 3A appear to be specific targets of AFB1 even if pig did not display clinical sign of liver toxicosis.

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Keywords: Aflatoxin B1; Swine; Liver; Cytochrome P450; P450 1A; P450 3A

1. Introduction

Mycotoxins are secondary metabolites produced by fungi. They are not essential to mold growth but they sporadically contaminate crops, causing major economic losses every year. Most of the mycotoxins of concern are produced by Aspergillus, Penicillium, Fusarium, Claviceps and Stachybotrys. The consumption of food or feed contaminated by mycotoxins is a potential health hazard for both humans and animals (Council of Agricultural Science and Technology, 2003; Oswald et al., 2005). Aflatoxins are a group of hepatotoxic, carcinogenic and immunotoxic mycotoxins produced principally by Aspergillus flavus and Aspergillus parasiticus (Steyn, 1995; Meissonnier et al., 2006). Aflatoxin B1 (AFB1) is the most prevalent and most toxic of these toxins. It is also one of the most common mycotoxins found in foods processed for human consumption, such as peanuts,

Abbreviations: AFB1, aflatoxin B1; ALP, alkaline phosphatase; AST, aspartate transaminase; ALT, alanine transaminase; CDNB, 1-chloro-2,4-dinitrobenzene; EROD, ethoxyresorufin O-deethylase; GST, glutathione S-transferase; γ-GT, γ-glutamyl transpeptidase; H&E, haematoxylin and eosin; PAS, periodic acid-Schiff reagent; PNP, p-nitrophenol; P450, cytochrome P450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UGT, UDP-glucuronosyl transferase.

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corn, cotton seeds, Brazil nuts, pistachios, spices and dry fruits (Council of Agricultural Science and Technology, 2003). Epidemiological studies have clearly concluded to a relationship between aflatoxins ingestion, hepatitis B or C virus infection and liver cancer incidence, so AFB1 was classified as carcinogenic for humans by the International Agency for Research on Cancer (Grootman and Kensler, 2005). Very recently, in 2004, an outbreak of acute human aflatoxicosis in eastern Kenya resulted in 317 cases of acute hepatitis and 125 deaths (Azziz-Baumgartner et al., 2005).

The liver is the first organ targeted during aflatoxicosis, due to its considerable bioactivation capacity. The phase I oxidative pathway, catalyzed by cytochrome P450 (P450) dependent monooxygenases, bioactivates AFB1 to generate toxic forms, which are then detoxified by glutathione S-transferase or epoxide hydrolase (Eaton et al., 1994; Meissonnier et al., 2005). Data obtained in vivo and in vitro have demonstrated for the enzymes of the P450 1A subfamily, a high affinity for AFB1 and the involvement in the formation of the carcinogenic intermediate AFB1 8,9-epoxide, and of aflatoxin M1. The enzymes of the P450 3A subfamily have a lower affinity for AFB1 and are involved in the formation of aflatoxin Q1 and AFB1 8,9-epoxide (Gallagher et al., 1994, 1996; Wang et al., 1998; Kuilman et al., 2000). Moreover, in human exposed to dietary AFB1, epidemiologic investigations demonstrated that CYP3A5 polymorphism is associated with increased level of the mutagenic AFB1 8,9-epoxide, particularly in individuals with low CYP3A4 (Wojnowski et al., 2004).

The metabolic pathways involved in AFB1 bioactivation and detoxication have been described in detail, but very few studies have investigated the impact of aflatoxins on the activities of drug-metabolizing enzymes in the liver. It has been shown that AFB1 has limited effect on conjugating enzymes, but this toxin specifically impairs the microsomal hepatic P450 system during acute or subacute aflatoxicosis (Galtier et al., 1984; Gawai et al., 1992; Raisuddin et al., 1994). Guerre et al. (1996a,b, 1999, 2000) showed in a sensitive animal species – the rabbit – that the decrease in P450 activity could be linked, at least partly, to microsomal oxidative damage and an increase in heme oxygenase activity (catabolism of heme-containing proteins). The aim of the present study was to investigate the impact of subchronic exposure to low doses of AFB1 on drug-metabolizing enzymes in the liver, monooxygenases and transferases. Hepatic drug-metabolizing activities in pigs and humans are very similar (Anzenbacher et al., 1998; Donato et al., 1999; Soucek et al., 2001), so we studied pigs because this species resembles humans sufficiently well to constitute a good model of aflatoxicosis in humans. Moreover, pigs are particularly likely to be exposed to AFB1 due to the composition of their feed. The effects of three dietary doses of the mycotoxin were compared, to facilitate the detection of any dose-dependent effect.

2. Materials and methods

2.1. Chemicals

AFB1 (purity >98%) was purchased from Alexis Corporation (Lausanne, Switzerland). The bicinechonic acid protein assay kit and SuperSignal West Pico Chemiluminescent Substrate were obtained from Pierce (Rockford, IL). 6β-hydroxytestosterone and testosterone were obtained from Steraloids Inc. (London, UK). Bovine serum albumin, acryl/bisacryl solution and glycine were purchased from Euromedex (Souffelweyersheim, France). Anti-human P450 1A1/2 (Daichi, Pure Chemical Co., Tokyo) and anti-human P450 3A (Gentest TM, Woburn, USA) antibodies were obtained from Becton Dickinson (Le Pont de Claix, France). Polyclonal anti-human P450 2C antibodies were purchased from Abcam (Cambridge, UK). Polyclonal anti-human UGT 1A antibodies (Santa Cruz Biotechnology Inc.) were obtained from Tebu-bio (Le Perray-en-Yvelines, France). All other chemicals and reagents were of the highest grade available from Sigma (St. Louis, MO). Distilled deionized water was used in all studies.

2.2. Feed manufacture and analytical controls

Four feed batches contaminated with various doses of AFB1 were manufactured at the INRA Toulouse (UR66) and INRA Rennes (UMR SENAH, St Gilles France). Briefly, AFB1 was weighed, mixed with vitamins and minerals and then incorporated into the cereal mixture, which was then granulated. AFB1 was handled according to the safety conditions recommended by the National Institute of Research and Safety (INRS, France). Feed composition is detailed in Table 1. The raw materials used in feed production and the final feed were tested for natural or artificial contamination with mycotoxins. Aflatoxins B1, B2, G1, G2, deoxyxynivalenol, fumonisin B1, ochratoxin A and zearalenone were analyzed, using standard HPLC techniques coupled with fluorimetric detection, or UV detection for deoxynivalenol, whereas T-2 toxin was

Table 1

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
</tr>
<tr>
<td>Soybean meal</td>
</tr>
<tr>
<td>Wheat</td>
</tr>
<tr>
<td>Barley</td>
</tr>
<tr>
<td>Calcium phosphate</td>
</tr>
<tr>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>Vitamin and mineral premix</td>
</tr>
<tr>
<td>Vegetable oil</td>
</tr>
<tr>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
</tbody>
</table>

Composition:

- Crude fiber (g): 39.9
- Starch (g): 482.0
- Crude protein (g): 218.8
- Lysine (g): 13.3
- Threonine (g): 9.0
- Methionine (g): 4.0
- Ca (g): 10.9
- P (g): 7.1
- Net energy (MJ): 11.2

*a* Vitamin A, 2,000,000 IU/kg; vitamin D3, 400,000 IU/kg; vitamin E 4000 mg/kg; vitamin C, 8000 mg/kg; vitamin B1 400 mg/kg; vitamin K3, 400 mg/kg; iron, 20,000 mg/kg; copper, 3993 mg/kg; zinc, 20,000 mg/kg; manganese, 8000 mg/kg.

*b* Corresponding to 880 g DM/kg.
determined by the standard GC–MS method (INZOLaboratory, Chateau-Thierry, France). Detection limits were 0.05 μg/kg for aflatoxins B1 and B2, 0.1 μg/kg for aflatoxins G1 and G2, 10 μg/kg for fumonisin B1, 0.2 μg/kg for ochratoxin A, 20 μg/kg for deoxynivalenol and T-2 toxin and 3 μg/kg for zearalenone. Aflatoxins B2, B1, G1, G2, fumonisin B1, ochratoxin A and zearalenone levels were below the detection threshold in both raw material and feeds. T-2 toxin and deoxynivalenol were found to be naturally present in the cereals used, resulting in concentrations of 30 μg and 350 μg/kg of feed, respectively for these toxins. Concerning AFB1, levels of 1 μg, 385 μg, 867 μg and 1807 μg AFB1/kg of feed were measured in control feed and the three artificially contaminated batches.

2.3. Animals

All animal experimentation procedures were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. Twenty 3-week-old weaned castrated male piglets were obtained locally. These piglets were identified with ear tags and allowed to acclimatize for 2 weeks in the pigsty, with free access to water and a commercial starter diet. Five piglets were then allocated to each experimental group on the basis of body weight (initial weight 13.7 ± 0.5 kg). The piglets were housed in earthen pens and were removed immediately and weighed. Representative sections of liver were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5–6 μm and stained with haematoxylin and eosin (H&E), period acid-Schiff (PAS) reagent, or with trichrome reagent. Histopathology followed by exsanguination, in accordance with the American Veterinary Medical Association panel recommendations (2001). Livers were removed immediately and weighed. Representative sections of liver were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5–6 μm and stained with haematoxylin and eosin (H&E), period acid-Schiff (PAS) reagent, or with trichrome reagent.

2.4. Biochemical analysis of plasma

Blood samples were collected into heparinized tubes by jugular venipuncture. Plasma samples were obtained by liver centrifugation and stored at −20 °C until analysis. Plasma concentrations of glucose, triglycerides, cholesterol, albumin, total proteins, and plasma activities of transaminases, γ-glutamyl transpeptidase (γ-GT) and alkaline phosphatase (ALP) were determined with an automatic serum analyzer (Konelab, Thermo-electron Corp., France) and the necesary reagents.

2.5. Histopathology

At the end of the experimental period, the piglets were killed by electrocution followed by exsanguination, in accordance with the American Veterinary Medical Association panel recommendations (2001). Livers were removed immediately and weighed. Representative sections of liver were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5–6 μm and stained with haematoxylin and eosin (H&E), period acid-Schiff (PAS) reagent, or with trichrome reagent.

2.6. Preparation of liver microsomes and cytosol

Livers were washed with ice-cold saline solution. Liver sections were prepared, frozen in liquid nitrogen and stored at −70 °C. Liver microsomes and cytosols were prepared by differential centrifugation, as previously described (Galtier et al., 1983). Microsomal and cytosolic protein contents were determined using the bicinechonic acid protein assay kit (Pierce), with bovine serum albumin (fraction V) as the standard.

2.7. Phase I and phase II enzyme assays

Lever microsomal cytochrome P450 content was determined with the spectrophotometric method of (Omura and Sato, 1964).

The ethoxyresorufin O-deethylase (EROD) activity of liver microsomes was determined by resorufin release, with fluorometric quantification, as described by Lake (1987).

Testosterone 6β-hydroxylase activity was assessed with a modified version of the methods described by Arlott et al. (1991) and Tachibana and Tanaka (2001). Testosterone (150 μM) was incubated for 30 min at 37 °C with 0.3 nmol of cytochrome P450 in 0.1 M phosphate buffer (pH 7.4), MgCl2 (20 μM) and β-NADPH. The reaction was stopped by adding acetonitrile, containing cortexolone (25 μM) as an internal standard. The reaction medium was centrifuged, filtered (Titan syringe filter, 0.45 μm pores) and analyzed by chromatography. Standard curves of testosterone and 6β-hydroxytestosterone containing cortexolone (internal standard) were analyzed by chromatography. Analysis was carried out with a Kontron HPLC unit composed of an HPLC autosampler 465, a Luna C18 Phenomenex (2.5 × 150 mm) 5 μm column coupled to an HPLC pump 422 and a Diode Array Detector 440, for detection at a wavelength of 247 nm. The solvent system consisted of a mixture of solution A (methanol/distilled water 1:9) and solution B (methanol/distilled water/acetonitrile 1:1:8), passed through the system at a flow rate of 0.2 ml min−1. Data from chromatographic runs were processed with Chromasystem 2000 software (Kontron Instrument, Milan, Italy).

Microsomal N-demethylation activities, with aminopyrine and benzphetamine as substrates, were determined by formaldehyde release. Formaldehyde was quantified according to the method of Nash (1953), and modified by Cochin and Axelrod (1959).

Aniline hydroxylase activity in microsomes was determined by quantifying p-aminophenol release as described by La Du et al. (1972).

UDP-glucuronosyl transferase (UGT) activity was determined with p-nitrophenol (PNP) as a substrate and UDP-glucuronic acid, as described by Frei (1970).

Liver cytosolic glutathione S-transferase (GST) activity was determined with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and glutathione (GSH), as described by Habig et al. (1974). Cytosolic proteins were diluted in 0.2 M phosphate buffer (pH 6.5) with CDNB (1 mM) and GSH (reduced form, 1 mM). The glutathione-conjugation of CDNB was determined spectrophotometrically at 340 nm (Lambda 650 spectrophotometer, Perkin–Elmer, Shelton, CT).

2.8. Immunoblot assays

The use of antibodies against the corresponding human forms of P450 is supported by the non availability of antibodies against any pig P450 isoform. On the other hand, there is a close homology in amino acid sequence between both human and pig P450 orthologs. Pig P450 1A1 showed 86% homology with human P450 1A1 (Accession No. NM_214412 and NM_000499), pig P450 3A showed 79% homology with human P450 3A4 (Accession No. NM_214423 and NM_017460) and pig P450 2C showed 82% homology with human P450 2C9/19 (Accession No. NM_214420 and NM_000771 or NM_000769). Moreover, pig P450 iso-enzymes detection has been previously been validated in our laboratory, using liver microsomes from piglets treated for P450 induction (Marvasi et al., 2006). P450 and UGT proteins were detected in solubilized microsomes. Microsomal proteins (10 μg per lane) were separated by SDS-PAGE (Laemmli, 1970) in 10% acryl/bisacyrl gels and were then transferred to nitrocellulose membranes, as described by Towbin et al. (1979). Membranes were incubated with primary antibody diluted 1/200 to 1/500 in a buffer containing 0.5% non-fat milk powder and then with a secondary horseradish peroxidase (HRP)-conjugated antibody (1/10,000 dilution). Antibody binding was detected with a chemiluminescent substrate, after placing the membrane against HyperfilmTM (Amersham Biosciences, Buckinghamshire, UK). The intensity of the immunoreactive signals of the proteins was estimated by densitometry analyses of Western blots, using a Molecular Imager® Gel Doc™ imaging instrument and Quantity One® software (Biorad Laboratories Inc., France) for image acquisition and analysis. The level of expression of the different P450 subfamilies and UGT 1A was estimated by comparing control and treated samples (piglets receiving aflatoxin B1-contaminated diets).

2.9. Statistical analyses

For body and liver weights, and biochemical analyses of plasma samples, data are reported as mean ± SE for five animals. For P450 and UGT protein expression, data are reported as mean ± SE for four animals. These data were analyzed by one-way analysis of variance.
3. Results

3.1. Clinical, histological and plasma biochemical parameters

After 28 days of AFB1 exposure, weight gain and several biochemical plasma parameters relating to hepatic dysfunction were measured (Table 2). Piglets exposed to 385 µg or 867 µg AFB1/kg of feed presented a slight but not significant decrease in body weight gain in comparison to control piglets (90% and 82% of the body weight of the controls, respectively). By contrast, piglets exposed to 1807 µg AFB1/kg of feed had a significantly lower body weight gain (53% of the control value). On autopsy, control piglets and piglets exposed to 385 µg AFB1/kg of feed had a dark red liver, homogeneous in appearance. The liver of one piglet in the group exposed to 867 µg AFB1/kg of feed was marbled in appearance. Two piglets in the group exposed to AFB1 1807 µg/kg of feed had yellow and discolored livers with a pronounced lobular pattern (data not shown).

Histopathology analyses demonstrated that livers of the control piglets and three piglets exposed to 385 µg AFB1/kg feed were similar; they displayed a glycogenic overload and normal interstitial tissues (Fig. 1A and B). In two piglets exposed to 385 µg AFB1/kg feed, all piglets exposed to 867 µg AFB1/kg of feed and in two piglets exposed to 1807 µg AFB1/kg feed, changes included moderate to extensive swollen hepatocytes, presence of enlarged nucleus and vacuolation of periportal parenchyma cells (Fig. 1C). In three piglets fed the diet contaminated with 1807 µg AFB1/kg, trichrome staining demonstrated portal tract fibrosis and fibrous parenchyma between portal tracts (Fig. 1D).

Piglets exposed to 385 µg or 867 µg AFB1/kg of feed had plasma transaminase (AST, ALT), γ-GT and ALP activities similar to those of control piglets (Table 2). Moreover, no significant decrease in plasma protein and albumin concentrations was observed. Piglets exposed to the highest level of AFB1 contamination (1807 µg/kg of feed) showed significant alterations, corresponding to hepatic dysfunctions attributable to aflatoxicosis. For these animals, aflatoxin contamination of the feed led to decreases in concentrations of cholesterol, glucose, proteins and albumin, associated with increases in plasma ALP and γ-GT activities, with no change in transaminases activities.

3.2. Liver monooxygenases activity and expression

We evaluated the overall change in total cytochrome P450 enzymes following 28 days of exposure to AFB1, by assessing the total P450 content of liver microsomal preparations. Total P450 levels were similar in piglets exposed to 385 µg AFB1/kg of feed and in the control group (mean value for the 385 µg AFB1/kg of feed group was 93% that of the control value). Conversely, piglets exposed to 867 µg or 1807 µg AFB1/kg of feed had significantly lower total liver microsomal P450 levels than the controls (86% and 55% of the control value, respectively) (Fig. 2).

P450 1A activity was investigated by measuring EROD activity in hepatic microsomes. In piglets exposed to AFB1 (365 µg, 867 µg or 1807 µg/kg of feed), EROD activity was significantly lower than that in the control, at 65%, 59% and 25% of the control value, respectively (Fig. 3).

P450 3A activity was investigated by studying testosterone metabolism, as this enzyme is the major cytochrome P450 involved in testosterone 6β-hydroxylation. Testosterone 6β-hydroxylation activity varied importantly between animals. Lower levels of testosterone 6β-hydroxylation

### Table 2

Effect of AFB1 intake for 28 days on body weight gain, liver weight, plasma biochemical parameters and enzymatic activities

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet</th>
<th>AFB1 385 µg/kg</th>
<th>AFB1 867 µg/kg</th>
<th>AFB1 1807 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (kg)</td>
<td>Control</td>
<td>22.0 ± 0.7a</td>
<td>19.9 ± 1.3a</td>
<td>18.1 ± 1.6a</td>
</tr>
<tr>
<td>Liver (% body weight)</td>
<td>AFB1</td>
<td>2.9 ± 0.1a</td>
<td>3.0 ± 0.2a</td>
<td>3.2 ± 0.1a</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>AFB1</td>
<td>2.7 ± 0.1a</td>
<td>2.6 ± 0.3a,b</td>
<td>2.3 ± 0.3a,b</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>AFB1</td>
<td>0.51 ± 0.07a,b</td>
<td>0.65 ± 0.08a,b</td>
<td>0.76 ± 0.15b</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>AFB1</td>
<td>5.9 ± 0.3a</td>
<td>5.2 ± 0.4a</td>
<td>5.1 ± 0.2a</td>
</tr>
<tr>
<td>Total proteins (g/L)</td>
<td>AFB1</td>
<td>67.4 ± 2.3a</td>
<td>60.0 ± 3.3a,b</td>
<td>61.6 ± 2.7a,b</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>AFB1</td>
<td>40.0 ± 1.3a</td>
<td>35.8 ± 1.2a</td>
<td>35.8 ± 2.3a</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>AFB1</td>
<td>606 ± 64a</td>
<td>482 ± 13a</td>
<td>667 ± 59a</td>
</tr>
<tr>
<td>γ-GT (IU/L)</td>
<td>AFB1</td>
<td>46 ± 4a</td>
<td>50 ± 9a</td>
<td>48 ± 8a</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>AFB1</td>
<td>53 ± 10a</td>
<td>65 ± 10a</td>
<td>67 ± 9a</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>AFB1</td>
<td>44 ± 3a</td>
<td>46 ± 5a</td>
<td>57 ± 3a</td>
</tr>
</tbody>
</table>

Notes: Results are expressed as the mean ± SE for five animals.

ALP, alkaline phosphatase; γ-GT, γ-glutamyl transpeptidase; AST, aspartate transaminase; ALT, alanine transaminase.

a,b One-way ANOVA was performed to compare the treatments. In rows, treatments not sharing a common superscript are significantly different (p < 0.05).
were observed in piglets exposed to 385 μg or 867 μg AFB1/kg of feed than in control piglets, but this difference was not significant. Pigs fed the most highly contaminated feed (1807 μg AFB1/kg of feed) for 28 days had levels of testosterone 6β-hydroxylation significantly lower than those of the controls (36% control values, Fig. 4). Testosterone consumption by hepatic microsomes displayed a lower level of inter-individual variability than testosterone hydroxylation. Levels of testosterone consumption were significantly reduced, to 64% and 56% of the control value, in piglets exposed to 867 μg and 1807 μg AFB1/kg of feed, respectively (Fig. 4).

We investigated changes in other liver P450 activities (Table 3). Benzphetamine and aminopyrine N-demethylase activities were increased by exposure to AFB1. Aminopyrine N-demethylase activity was significantly higher in piglets exposed to 867 μg and 1807 μg AFB1/kg of feed than in control piglets, reaching 123% and 157% of the control value, respectively. A similar pattern was also observed for benzphetamine N-demethylase activity in
these two groups of animals (141% and 182% of the control value, respectively). Aniline hydroxylase activity in hepatic microsomes was not affected by AFB1, regardless of the level of contamination, but higher variability in this activity was observed for piglets exposed to 867 \( \mu g \) or 1807 \( \mu g \) AFB1/kg of feed.

We investigated the effects of 28 days of exposure to AFB1 on the protein expression of P450 1A and P450 3A enzyme in hepatic microsomes (Fig. 5). P450 1A and P450 3A expression levels were similar in piglets given 385 \( \mu g \) AFB1/kg of feed and in control piglets. Conversely, in piglets exposed to 867 \( \mu g \) AFB1/kg of feed, the levels of P450 1A was only 37% these in the control, while P450 3A expression was numerically but not significantly reduced compared to the control values. In piglets exposed to the highest level of AFB1 contamination, P450 1A and P450 3A protein levels were decreased to 25% and 34% of the control values, respectively.

We also investigated the effects of 28 days of exposure to AFB1 on the protein expression of P450 2C enzyme in hepatic microsomes (Fig. 5). The expression levels of this P450 subfamily were similar in piglets for all doses of AFB1 in feed.

### 3.3. Liver transferases activity and expression

We completed our study with the evaluation of enzymes activities implicated in phase II metabolism reactions. Glutathione S-transferase activity was measured in the hepatic cytosol, using CDNB as a substrate. Mean values ranged from 1.04 to 1.23 \( \mu mol \) of CDNB conjugated per minute per milligram of cytosolic protein, depending on the group of piglets (Table 3). No difference in the activity of these enzymes activities was observed between the experimental groups. Microsomal UGT activity was determined with PNP as a substrate; values ranged from 9.82 to 11.88 nmol of PNP conjugated per minute per milligram of microsomal proteins. Both the enzyme activity and the protein expression of UGT 1A subfamily were unchanged in piglets whatever the doses of AFB1 in feed were (Table 3, Fig. 5).

### 4. Discussion

The aim of the present study was to investigate the impact of AFB1 on hepatic drug-metabolizing enzymes, by subchronic exposure for 28 days in piglets. We looked...
for mild deleterious events occurring during aflatoxicosis, developed in pigs exposed to a diet contaminated with various concentrations of AFB1. Similar exposure (<2000 μg AFB1/kg of feed) was previously reported to cause moderate changes in the metabolic functions of pig liver after a 4-week period of intake (Harvey et al., 1988).

The dose-dependent intoxication of piglets to AFB1 was confirmed by the clinical and biochemical parameters and the liver histological observations. Piglets exposed to 385 μg or 867 μg AFB1/kg of feed displayed only limited clinical effects, and liver metabolic functions were normal after 28 days of toxin intake. The mild degenerative changes observed in hepatocytes from piglets exposed to 867 μg AFB1/kg feed were typical signs of aflatoxicosis as previously described by Tapia and Seawright (1985) in piglets fed a diet contaminated with 750 μg AFB1/kg for 42 days. Previous studies reported weight gain impairment in piglets exposed for three to four weeks to 280 μg AFB1/kg of feed (van Heugten et al., 1994; Marin et al., 2002). The discrepancy between our results and these data may be due to the initial lower weight of the piglets used in these previous studies, resulting in higher sensitivity to AFB1. In our study, only piglets exposed to the highest dose of aflatoxin (1807 μg AFB1/kg of feed) developed liver toxicity associated with metabolic and cholestatic dysfunctions. The fibrosis signed the liver toxicosis due to AFB1, whereas the lack of variability in plasma transaminase activities was in accordance with the absence of marked cytolysis in the livers. These results are consistent with previous data for piglets fed a diet containing 2 mg AFB1/kg of feed for 28 days (Harvey et al., 1988). These animals displayed simultaneous decreases in plasma albumin and cholesterol concentrations, associated with increases in plasma PAL and γ-GT activities, with no change in AST activity. This previous study also reported no change in these plasma parameters in piglets exposed to 1 mg AFB1/kg of feed, as reported here for piglets given 385 μg or 867 μg AFB1/kg of feed.

Total microsomal P450 levels were significantly lower in piglets exposed to 867 μg or 1807 μg AFB1/kg of feed than in the control group. A similar decrease has been reported in several other animal species during acute AFB1 exposure (Gawai et al., 1992; Raisuddin et al., 1994; Guerre et al., 1996a). Conversely, this parameter was not affected in piglets receiving 385 μg AFB1/kg of feed. The normal liver P450 content and the lack of change in plasma parameters observed confirm that exposure to 385 μg AFB1/kg of feed for a period of four weeks causes only mild damage in pig liver. We then examined the mildly deleterious effects of chronic exposure to AFB1 in more detail. We focused on the drug-metabolizing activities of P450 proteins, to determine whether particular subfamilies of P450 enzymes were more sensitive than others to AFB1 toxicity.

Great similarities have been reported for cytochrome P450 between human and pig liver (Donato et al., 1999). P450 1A, 3A and 2C are expressed in pigs at the protein level. P450 1A and P450 3A dependent activities, EROD and testosterone 6β-hydroxylation, respectively, were
found in pig liver microsomes (Desille et al., 1999). Other mixed oxygenase functions, benzphetamine and aminopyrine N-demethylation or aniline hydroxylation were also reported in pigs (Myers et al., 2001; Nebbia et al., 2003).

In piglets exposed to 1807 μg AFB1/kg of feed—the only animals to develop clinical signs of liver toxicosis—significant decreases in EROD and testosterone 6β-hydroxylation activities, and in testosterone metabolism were observed. Western-blot analyses of P450 1A and P450 3A showed that the levels of both these proteins were significantly lower in this group of pigs than in controls. These data, the first to be reported in pigs, are consistent with previous data obtained in rabbits. Exposure to 50 or 100 μg AFB1/kg body weight for five days significantly decreases rabbit liver microsomal P450 content and specifically reduces P450 1A and P450 3A expression and activity (Guerre et al., 1996a). It has been suggested that this specific protein depletion may be related to microsomal oxidative damage resulting from lipid peroxidation during aflatoxicosis, together with the inhibition of protein synthesis (Shen et al., 1994; Guerre et al., 1999).

In piglets exposed to 385 μg AFB1/kg of feed, both P450 1A and P450 3A proteins were expressed in normal amount. EROD activity was significantly decreased whereas testosterone metabolism was not different when compared to the control piglets. In animals exposed to 867 μg AFB1/kg feed, EROD activity and P450 1A protein expression were decreased. Testosterone 6β-hydroxylation activity was highly variable, and a significant decrease in testosterone metabolism was observed. Nevertheless, P450 3A protein expression was not significantly reduced compared to the expression in control animals. These results suggest that the impairment of these P450 activities must occur before the decrease in protein expression, and that EROD activity was more strongly affected than testosterone 6β-hydroxylation activity by AFB1. An in vitro study on rabbit hepatocytes suggested that the toxic effects of low doses of AFB1 (0.1 or 1 μM) on P450 might be related to the direct formation of protein adducts with the epoxide metabolite (Guerre et al., 2000). The authors demonstrated that decreases in P450 protein levels did not result from the specific inhibition of P450 mRNA production. The present study is the first to report a toxic impact of AFB1 on hepatic drug-metabolizing enzymes activities in the absence of change in total liver microsomal P540 content, and we found that P450 1A was an early target of the toxin in piglets exposed to 385 μg AFB1/kg of feed. P450 1A and P450 3A have been implicated in the bioactivation of AFB1 in human (Gallagher et al., 1996), but the implication of swine orthologs has never been demonstrated. Additional investigations for the determination of the swine P450 isoforms supporting AFB1 metabolism should interestingly complete our data and would support the hypothesis of protein adduct formation to the epoxide metabolite in pigs.

Benzphetamine and aminopyrine N-demethylation activities were significantly higher in piglets exposed to 867 μg and 1807 μg AFB1/kg of feed than in control piglets. However, we observed no change of the P450 2C protein expression level for these two AFB1 exposure doses. Aniline hydroxylase activity was unaffected by AFB1, regardless of the dose to which the animals were exposed. Our results are in accordance with the study performed on rabbits (Guerre et al., 1996a), since the authors showed no difference in P450 2B, 2C and 2E protein levels between control and treated animals. The increase in N-demethylase activities that we observed may therefore reflect a general imbalance in total liver P450 content following the decrease in the expression of P450 1A and P450 3A.

We also investigated the protein expression and the activity of UGT. These transferase enzymes are largely expressed in liver microsomes (Tukey and Strassburg, 2000). We observed that both protein expression and activity were unaffected for all doses of AFB1 exposure, suggesting that AFB1 did not target this class of microsomal proteins during a subchronic exposure to 385–1807 μg AFB1/kg of feed for 28 days in pigs.

GST are major transferase enzymes involved in AFB1 8,9-epoxide detoxication (Eaton et al., 1994). However in pigs, no publication has ever described AFBO-specific GST activity in liver cytosol. In the present study, CDNB was used as general substrate for all classes of GST (Eaton et al., 1994). More precisely in pigs, CDNB acts as a substrate for GST α and μ classes (Kunze, 1997). We observed no change in GST activity following the addition of CDNB in liver cytosols. Experimental studies in rats have reported the transient induction of GST activity in the liver following acute, but not chronic exposure to AFB1 (Carrillo et al., 1990; Raisuddin et al., 1994). Conversely, in rabbits, AFB1 leads to a decrease in the activity of GST accepting CDNB as a substrate but not in GST activity accepting DCNB as a substrate (substrate of μ class) (Guerre et al., 1996a). Our data for pigs confirm that GST activity is less sensitive to AFB1 toxicity than microsomal P450 enzymes.

In conclusion, this study demonstrates that AFB1 selectively impairs the functioning of P450 enzymes in the absence of associated clinical signs of liver toxicosis. In pigs, P450 1A is the first target of AFB1, and subchronic exposure to AFB1 had no effect on UGT or GST activities. Our results therefore confirm that subchronic dietary exposure to low levels of AFB1 may not be associated with clinical signs. Nevertheless, subtle impairment of certain hepatic drug-metabolizing enzymes might occur in pigs exposed to dietary AFB1 with possible consequences on the normal metabolism of endogenous compounds or associated xenobiotics.

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