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Effects of aflatoxin B_1, fumonisin B_1 and their mixture on the aryl hydrocarbon receptor and cytochrome P450 1A induction

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ABSTRACT

Aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) are mycotoxins widely found as cereal contaminants and their co-occurrence in corn has been associated with a high incidence of liver cancer. Both toxins are immunotoxic, with AFB_1 being a procarcinogen, and its bioactivation through specific cytochrome P450 (Cyp) enzymes, such as Cyp1A, being a requirement for hepatocarcinogenic and toxic activities. This study evaluated the effects of these mycotoxins, alone or combined, on activation and expression of Cyp1A and its transcription factor aryl hydrocarbon receptor (Ahr) in hepatoma cell line H4IIE and spleen mononuclear cells of rats. The results demonstrate that in H4IIE cells, AFB_1 induced an increase in Cyp1A activity and *cyp1A* transcription, associated with an enhanced Ahr activity, which suggests that this toxin can act as an Ahr agonist. Moreover, FB_1 caused a small rise in Cyp1A activity and *cyp1A* expression. Similarly in spleen cells, AFB_1 and FB_1 induced overexpression of *cyp1A* and *ahr* genes. This work shows that the response potency was significantly higher for the mixture, indicating the existence of an interaction between both toxins. This study proposes the Ahr pathway activation as a toxicity mechanism of AFB_1 and FB_1, and highlights that FB_1 may increase AFB_1 bioactivation.

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1. Introduction

Aflatoxin B_1 (AFB_1, Fig. 1) is one of the most relevant mycotoxins worldwide due to its widespread occurrence, high toxicity and economic implications. This toxin is mainly synthesized by *Aspergillus flavus* and *Aspergillus parasiticus*, and is commonly found as a contaminant in cereals and oilseeds (Kensler et al., 2011). AFB_1 exposure causes growth stunting, immunosuppression, mutagenicity, genotoxicity, increasing hepatocellular carcinoma (HCC) incidence in animals and humans (Abbès et al., 2010; Sun et al., 2011; Theumer et al., 2010). The toxic and carcinogenic effects of AFB_1 are intimately linked with its biotransformation through the cytochrome P450 (Cyp) to the highly reactive AFB_1-exo-8,9-epoxide (AFBO), which can form adducts with the DNA (Guengerich et al., 2001) and produce reactive oxygen species (ROS) (Mary et al., 2012; Theumer et al., 2010). The predominant site of the AFB_1 metabolism is the liver, with the major human Cyp isoenzymes involved in AFBO formation being Cyp1A2 and Cyp3A4, of which the latter is the most abundant in that organ (Kensler et al., 2011). Different studies carried out with liver microsomes of human, chicken, quail and turkey, and also on human lung cells and lymphoblasts exposed to the concentrations of AFB_1 normally detected in food, have shown that AFBO formation and DNA damage are mostly induced by Cyp1A2 (Diaz et al., 2010a, 2010b; Gallagher et al., 1996; Guo et al., 2006; Klein et al., 2000; Van Vleet et al., 2002). In addition, another major isoenzyme in the AFB_1 metabolism to AFBO formation is Cyp1A1, which is constitutively expressed in most tissues and represents the highest fraction of extrahepatic Cyp (Coutiño Rodríguez et al., 2010). Therefore, variations in Cyp1A expression, due to genetic polymorphism or environmental factors, may be important determinants in the propensity of populations to develop HCC after exposure to AFB_1.

Abbreviations: AFB_1, aflatoxin B_1; Ahr, aryl hydrocarbon receptor; AFBO, AFB_1-exo-8,9-epoxide; βNF, β-naphthoflavone; Cyp, cytochrome P450; DRE, dioxin responsive elements; EROD, 7-ethoxyresorufin-O-deethylase; FB_1, fumonisin B_1; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinases; MIX, AFB_1–FB_1 mixture; ROS, reactive oxygen species; *Sa*, sphinganine; SEM, standard error of the mean; *So*, sphingosine; RT-qPCR, quantitative real time reverse-transcriptase polymerase chain reaction.

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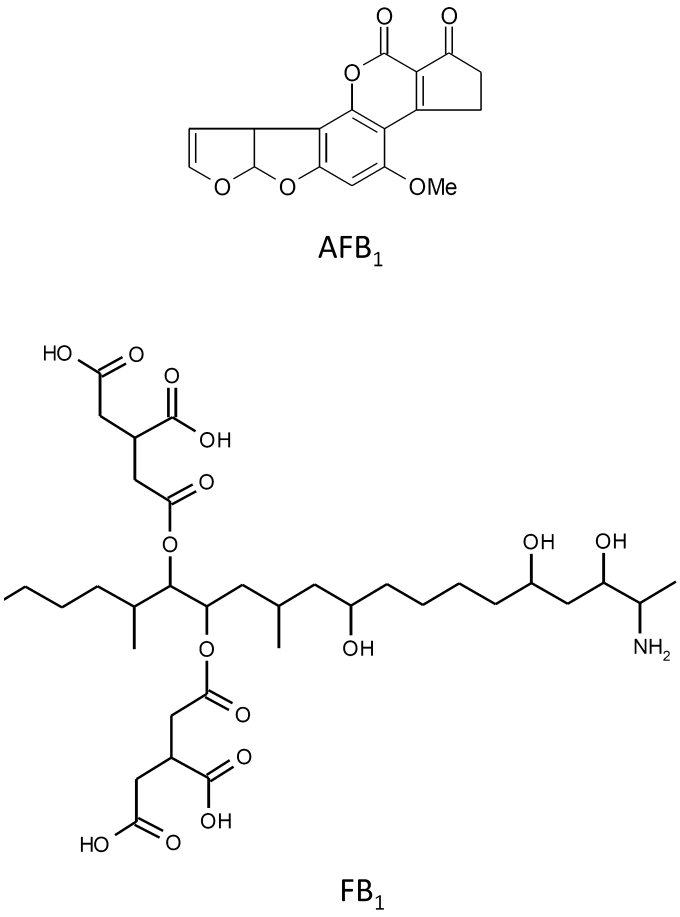

Fig. 1. Chemical structures of AFB_1 and FB_1.

The expression of the Cyp1A subfamily is mainly regulated by the aryl hydrocarbon receptor (Ahr), which is a nuclear transcription factor that also regulates the expression of several genes involved in phases I and II of the metabolism, immune response, cell cycle, differentiation, apoptosis and carcinogenesis (Apetoh et al., 2010; Huang et al., 2012; Ma et al., 2009; Matsumoto et al., 2007; Peng et al., 2009; Stockinger et al., 2011). There are a variety of exogenous and endogenous ligands that can activate Ahr with different affinities, with the dioxin-like compounds being the most potent Ahr ligands due to their polycyclic aromatic structure being easily able to take a planar conformation. However, Ahr activation by a wide variety of non-planar molecules or in a manner independent of the ligand binding has also been reported (Alonso et al., 2008; Barouki et al., 2007; Fernández-Cruz et al., 2011). Once activated, Ahr forms a heterodimer with the Ahr-nuclear translocator protein (ARNT) and is translocated to the nucleus, where it interacts with specific sequences of the DNA, dioxin responsive elements (DRE) and enhances the expression of dependent genes (Rowlands and Gustafsson, 1997).

Fumonisin B_1 (FB_1, Fig. 1) is a mycotoxin mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum*, which disrupts the immune system and causes liver and kidney cancer in rodents and esophageal cancer in humans (Van der Westhuizen et al., 2010). Its most recognized mechanism of action is the disruption of the sphingolipid metabolism, which causes an increase of bioactive sphingoid bases, such as sphinganine (*Sa*) and sphingosine (*So*), and leads to an alteration of the functionality of cell membranes, cell growth, differentiation and cell injury, both *in vitro* and *in vivo* (IPCS-WHO, 2000). Although there is no evidence in the literature indicating that FB_1 is metabolized by Cyp, Martínez-Larrañaga et al. (1996) showed that this toxin can induce Cyp1A and 3A activities in rat liver microsomes. In contrast, Spotti et al. (2000) found an inhibition of Cyp1A activity, while not affecting Cyp3A activity in rat liver microsomes. Therefore, FB_1 may alter the metabolism of AFB_1 and its consequent toxicity.

The co-occurrence of several mycotoxins simultaneously in a single product is a common situation in nature (Rodrigues and Naehrer, 2012a, 2012b), with the coexistence of AFB_1 and FB_1 in cereals, especially in corn, being a worldwide problem (Kimanya et al., 2008; Rodrigues and Naehrer, 2012b; Streit et al., 2012; Wagacha and Muthomi, 2008) that has been associated with a high incidence of human HCC (Klarić, 2012; Li et al., 2001; Sun et al., 2011). Moreover, it is also probable that the immunotoxicity exerted individually by AFB_1 and FB_1 may be raised by co-exposure to both mycotoxins (Mary et al., 2012). However, little is known about the interaction of AFB_1 and FB_1 regarding their toxic and carcinogenic effects and mechanisms.

Scarce or null information is available concerning the effects of AFB_1 and FB_1 on activation of the Cyp 1A and Ahr pathways. In this regard, the aim of the present study was to investigate the effects of AFB_1 and FB_1, alone or combined, on transcription of the *cyp1A* and *ahr* genes and the activity of the corresponding proteins, because this pathway could be involved in the elevated carcinogenicity induced by the mixture of both toxins. We used the rat liver hepatoma cell line H4IIE and the transfected DR-CALUX® cell line as recommended models to study Cyp1A and Ahr induction, respectively. The primary immune cells from rat spleen (spleen mononuclear cells) were also tested, since an increase in ROS production via a Cyp-dependent mechanism has been observed after exposure *in vitro* to the individual or combined mycotoxins (Mary et al., 2012).

2. Materials and methods

2.1. Chemicals

AFB_1 and FB_1 were purchased from Sigma-Aldrich (Buenos Aires, Argentina). 7-ethoxyresorufin, acetic anhydride, acetonitrile, β-naphthoflavone (βNF), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), dicumarol, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), fluorescamine, resorufin, TRI® reagent grade, triethylamine, trypan blue and trypsin were obtained from Sigma-Aldrich (Madrid, Spain). The cell culture media: Eagle Minimum Essential Medium (EMEM), α-Minimum Essential Medium (α-MEM) and Roswell Park Memorial Institute (RPMI)-1640, and also bovine serum albumin (BSA), fetal bovine serum (FBS), L-Glutamine, non-essential amino acids 100x (NEAA), penicillin and streptomycin (P/S, 10,000 U/ml and 10 mg/ml, respectively) were purchased from Lonza (Barcelona, Spain). Stock solutions of AFB_1 and FB_1 were prepared in DMSO and phosphate buffered saline (PBS), respectively, and stored at –20 °C.

2.2. Cell line cultures

The rat hepatoma cell line H4IIE was obtained from the American Type Culture Collection (ATTC) (Manassas, VA, USA), and was cultured in EMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% (v/v) NEAA and 1% (v/v) P/S. The DR-CALUX® (Dioxin Responsive-Chemical Activated Luciferase gene expression) cell line was obtained from Biodetection Systems BV (Amsterdam, The Netherlands) and consisted of the H4IIE cell line stably transfected with luciferase as the reporter gene under direct control of dioxin responsive elements (DRE). The DR-CALUX® cell line was cultured in α-MEM supplemented as described for EMEM. Both cell lines were split weekly with 0.5% (w/v) trypsin/0.02% (w/v) EDTA, and cultured at 37 °C, in a humidified 95% (v/v) air/5% (v/v) CO_2 atmosphere.

2.3. Isolation of the spleen cells

Spleen cells were obtained from male Wistar inbred rats (8 weeks old) that were housed in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Córdoba. The Institutional Experimentation Animal Committee (authorization # 15-09-69934) approved animal handling and experimental procedures. For each experiment, six rats were anesthetized with isoflurane, and spleens were removed aseptically from the animals

and pooled. The splenocyte suspensions were prepared as previously described (Mary et al., 2012), by being seeded at a density of 10^6 cells/ml and cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) glutamine and 1% (v/v) P/S at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

2.4. Cell exposure

The hepatic and spleen cells were incubated with different concentrations of the AFB_1, FB_1 or AFB_1–FB_1 mixtures (MIX) dissolved in culture medium from the stock solutions. The maximal concentrations of DMSO used (0.07% (v/v) in splenocytes and 0.21% (v/v) in the cell lines) were added to the control wells. The treatments with the mycotoxins lasted up to 24 h, depending on the experiment. The range of mycotoxin concentrations were selected on the basis of literature data (maximum permissible levels and intestinal absorption in rats) and our previous studies performed on hepatic cell lines and splenocytes from rat (Abdel Nour et al., 2007; Chuturgoon et al., 2014; Ehrlich et al., 2002; Liu et al., 2012; Lv et al., 2014; Hanioka et al., 2012; Mary et al., 2012; Nucci et al., 2014; Theumer et al., 2010, 2003, 2002). The lower cytotoxicity observed in the CALUX cells allowed the application of higher concentrations in order to observe any possible effects, and as a consequence, the range of concentrations used for the CALUX and for the EROD assays were different.

2.5. Cell viability

The cell viability was studied for 24 h of incubation of spleen and hepatic cells with the mycotoxins or DMSO (0.07–0.21%, v/v), using the trypan blue exclusion test. Briefly, after 24 h of culture, the cells were stained with trypan blue and counted in a Neubauer chamber. These results were expressed as the percentage of viable cells, given by viable cell number/total cell number × 100.

2.6. The 7-ethoxyresorufin-O-deethylase (EROD) activity in H4IIE cells

The EROD assay was used to estimate the activity of Cyp1A, with the Cyp1A dependent EROD activity and the protein content being measured as described previously by Burke and Mayer (1974) and Lubet et al. (1985). H4IIE cells were cultured in 96-well plates (Costar, VWR, Spain) at a density of 2.5×10^4 cells/well. After 24 h, the cells were exposed to different concentrations of AFB_1 (0.31–20 μM), FB_1 (1.56–100 μM) or their mixture (MIX, at the same concentration range as for individual toxins).

Control cells received the maximal DMSO concentration used in the treated cells (0.07%, v/v), and 0.5 μM βNF was used as positive control. After 24 h of treatment, the medium was removed and the cells were washed with PBS (pH 7.5). Then, the plates were frozen in liquid nitrogen, and a reaction mixture comprised of 100 μl/well of PBS (pH 7.5) containing 7-ethoxyresorufin (1.25 μM), dicumarol (20 μM) and NADPH (1.4 μM) was added to each well. The resorufin product fluorescence formation was measured at 532 nm excitation and 590 nm emission wavelengths in a microplate reader Tecan Genios (Maennedorf, Switzerland). Subsequently, 75 μl of fluorescamine (150 mg/l in acetonitrile) were added to each well to measure the protein content (using BSA as a standard) at the excitation and emission wavelengths of 360 nm and 450 nm, respectively. EROD activity was expressed as pmol resorufin/min/mg protein by using a standard curve of resorufin and of BSA.

2.7. DR-CALUX® assay

DR-CALUX® cells were plated in 96-well plates at a density of 2.5×10^4 cells/well. After 24 h, the cells were treated with different concentrations of AFB_1 (0.61–60 μM), FB_1 (3.12–300 μM) or MIX (using the same ranges as applied individually for each mycotoxin) for 24 h, with control cells receiving the maximal DMSO concentration used in the exposed cells (0.21%, v/v) and using βNF (0.5 μM) as positive control. The luminescence emitted by the cells was quantified by means of the SteadyLite Plus Kit (PerkinElmer, Spain) in a liquid scintillation counter (1450 MicroBetaTriLux, PerkinElmer).

2.8. Measurement of cyp1A transcription levels in spleen and H4IIE cells

For the study of *cyp1A* transcription in the H4IIE cell line, cells were exposed to 5 μM AFB_1, 25 μM FB_1 or MIX (5 μM AFB_1 + 25 μM FB_1), and the exposure was stopped after different time periods, up to a maximum incubation time of 16 h. The indicated concentrations of chemicals were chosen from the results obtained in the enzyme measurements, and corresponded to those that would guarantee an induction high enough to be able to observe variations in the transcription levels over time. The mixture obtained using these concentrations induced the greatest increase in EROD activity. In spleen cells, the transcriptional levels of *cyp1A* and *ahr* were determined, with splenocytes being treated with 20 μM AFB_1, 10 μM FB_1 or with MIX (20 μM AFB_1 + 10 μM FB_1), and the doses selected on the basis of previous studies (Mary et al., 2012; Theumer et al., 2010). For all experiments, 0.5 μM βNF was used as positive control, whereas control cells treated with the mycotoxin vehicle were used as negative control.

The total RNA was extracted from cultured H4IIE cell lines and spleen cells using TRI® reagent, according to the manufacturer's instructions. Also, single-step reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis was carried out with 100 ng of RNA for each sample, using iScript RT-PCR kit with SYBR Green (BIO-RAD, Madrid, Spain), according to the manufacturer's instructions. The thermal cycling conditions were 10 min at 50 °C for the reverse transcription, 5 min at 95 °C to inactivate the reverse transcriptase, followed by 35 cycles at 97 °C for 15 s, 48 °C for 30 s and 72 °C for 1 min. All PCR reactions were performed in a Line-gene K system BIOER Technology (Hangzhou, China) with the specific primers (see below), and the mRNA relative quantification was normalized with the corresponding levels of *β-actin* mRNA. Results were represented as Ct values, where Ct was defined as the threshold cycle number at which the product was first detected by fluorescence. The fold change in relative expression was then determined using the comparative Ct method, also referred to as $2^{-\Delta\Delta Ct}$, an amount of target gene normalized to an endogenous control (β-actin) and relative to the base control, where $\Delta\Delta Ct = (Ct_{target} - Ct_{\beta\text{-actin}})_{sample} - (Ct_{target} - Ct_{\beta\text{-actin}})_{control}$ (Livak and Schmittgen, 2001). The sequences of the forward and reverse primers used to amplify the different genes in H4IIE and spleen cells were 5′-GTCATCTGTGCCATATGCTTTG-3′ and 5′-GCTTAGA TTGACTATGCTGAGCAG-3′, respectively, for *cyp1A* (primers allowing a region common to *cyp1A1* and *cyp1A2* to be amplified) with a product size of 74 bp (Nishimura et al., 2007). For *ahr* cDNA, these primers were 5′-AAACCAAAGACACGGGAT-3′ and 5′-TCGGACTCTGAAACTTGCTTAGG-3′, respectively, with the product size being 179 bp (Sonneveld et al., 2007). For *β-actin* cDNA amplification, the sequences of the forward and reverse primers used were 5′-CATCACCATCGGC AACGA-3′ and 5′-GATGTCCACGTCACACTTCATGA-3′, respectively, and the product size was 137 bp.

2.9. Statistical analysis

Results from these studies are expressed as the mean ± standard error of the mean (SEM) from a minimum of three independent experiments. For each experiment, all treatments were performed in triplicate for the EROD and DR-CALUX® assays and in duplicate for the RT-qPCR. Statistical analyses of the data were performed using the one-way ANOVA followed by Bonferroni's *post hoc* test, utilizing GraphPad InStat software version 3.01 (La Jolla, CA, USA). Multiple comparisons were only carried out when the ANOVA p value was lower than 0.05, using an alpha value of 0.05.

The effective concentration for a 50% maximal response (EC_{50}) was calculated using SigmaPlot version 12.0 (Systat Software Inc, USA). The estimation of the concentration–response function and the calculation of the EC_{50} were carried out by fitting the EROD and DR-CALUX® assay results to the following regression model equation for the sigmoid curve:

$y = \max/[1 + (x/EC_{50})b11] + \text{minwhere max is the maximal response observed, b is the slope of the curve and min is the minimal response.}$

3. Results

3.1. Cytotoxicity

The potential cytotoxic effects of AFB_1, FB_1 and MIX were evaluated on cell lines and splenocytes exposed for 24 h to the mycotoxins. The cell viability was not significantly affected ($p < 0.05$) except in the cell lines exposed to the highest concentration of AFB_1 (20 μM), where a decrease in viability to 80% was observed. No toxicity was observed for FB_1 at any of the tested concentrations (data not shown).

3.2. EROD activity assay

Exposure of H4IIE cells to different doses of AFB_1 for 24 h resulted in a concentration-dependent significant increase of EROD activity from 0.31 μM ($p < 0.01$), reaching a maximum at the 10 μM concentration ($p < 0.001$, Fig. 2). The decrease in EROD activity produced by the highest concentration of AFB_1 tested may have been related to the significant decrease in cell viability induced by treatments containing 20 μM of this toxin. No changes were observed in the EROD activity in cells exposed to different concentrations of FB_1, except for the 50 μM concentration ($p < 0.05$). When H4IIE cells were cultured with mixtures of both mycotoxins, there were significant dose-dependent increases in EROD activity with respect to control from 0.31 μM AFB_1 and 1.56 μM FB_1 ($p < 0.01$) to a maximum response at 5 μM AFB_1 and 25 μM FB_1 ($p < 0.001$). Moreover, these inductions were significantly higher than those found for AFB_1 alone. The EC_{50} value for AFB_1 was 3.16 μM, but was above the highest concentration tested for FB_1. The EC_{50} derived from the MIX curve corresponded to a 2.33 μM concentration of AFB_1 and 11.66 μM of

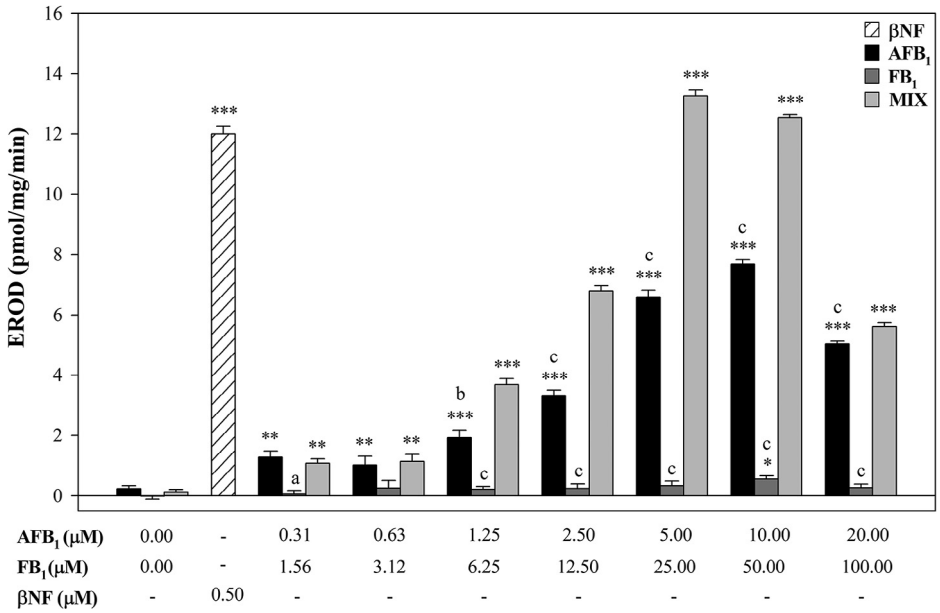

Fig. 2. Individual and combined effects of AFB_1 and FB_1 on EROD activity. Cyp1A activity was measured using an EROD assay in H4IIE cells exposed to different concentrations of AFB_1, FB_1 or MIX ($AFB_1 + FB_1$) for 24 h. βNF (0.5 μM) was used as positive control. Each bar represents the mean ± SEM (n = 4). Asterisks indicate differences relative to the control ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Letters indicate differences between MIX and the individual mycotoxins ($^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$).

FB_1. Although the AFB_1 EC_{50} was similar for both AFB_1 and MIX curves, the potency of the response was significantly higher for the MIX exposure.

3.3. DR-CALUX® assay

The dose–response curves shown in Fig. 3 were obtained after exposure of DR-CALUX® cells for 24 h to increasing concentrations of the individual and combined mycotoxins. Cells cultured with FB_1 did not reveal significant differences in luminescence relative to the control, although there was a trend toward higher values. In contrast, when *ahr* activity was determined in cells exposed to different doses of AFB_1 and in mixtures, significant increases were recorded for all treatments ($p < 0.01$). The EC_{50} values for AFB_1 and FB_1 were 4.61 and 208.28 μM, respectively, while the EC_{50} from the MIX curve corresponded to a concentration of 4.42 μM for AFB_1 and 22.10 μM for FB_1. Although AFB_1 EC_{50} values for AFB_1 and MIX curves were similar, the relative luminescence levels stimulated by the AFB_1–FB_1 mixtures were significantly greater than those produced by the corresponding concentrations of individual mycotoxins.

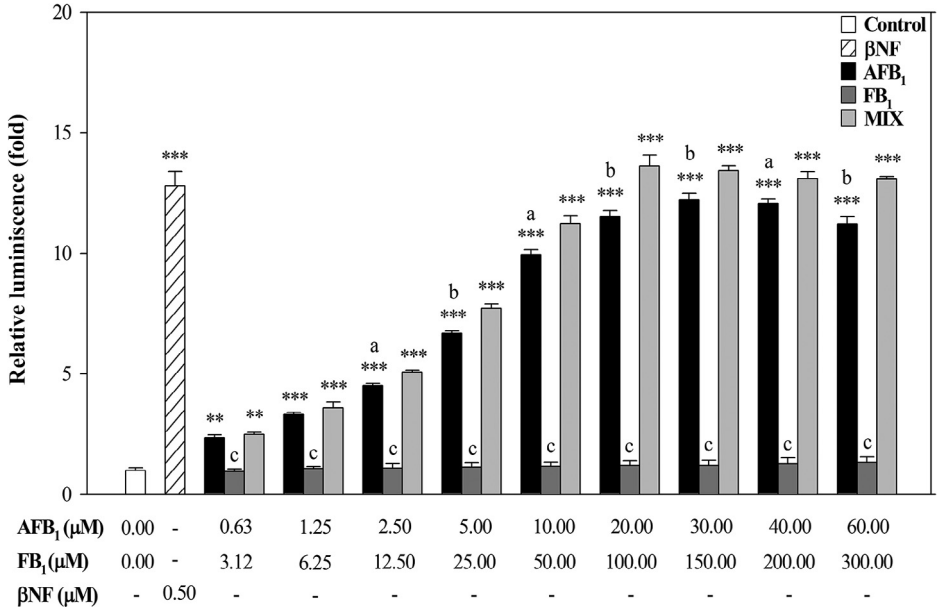

Fig. 3. Individual and combined effects of AFB_1 and FB_1 on Ahr activity, determined by bioassay DR-CALUX® after 24 h of treatment with different concentrations of AFB_1, FB_1 or MIX ($AFB_1 + FB_1$). βNF (0.5 μM) was used as positive control. Each bar represents the mean ± SEM (n = 4). Asterisks indicate differences relative to the control ($^{**}p < 0.01$; $^{***}p < 0.001$). Letters indicate differences between MIX and the individual mycotoxins ($^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$).

3.4. Effects of AFB_1, FB_1 and MIX on cyp1A mRNA levels in H4IIE cells

In an attempt to elucidate whether the increase of Cyp1A activity observed with the EROD assay by AFB_1 and FB_1, alone or combined, was accompanied by an enhancement in the expression of the *cyp1A* gene, RT-qPCR was performed on the H4IIE cell line. These cells were stimulated with 5 μM AFB_1, 25 μM FB_1 or MIX (5 μM AFB_1 + 25 μM FB_1), and studied at increasing time periods from 0.5 to 16 h.

The RT-qPCR results (Fig. 4) showed that AFB_1 significantly enhanced *cyp1A* expression at all times studied, with a maximal effect of 160-fold with respect to the control after 4 h of treatment ($p < 0.001$). In contrast, FB_1 exposure did not show a significant effect upon the *cyp1A* expression for any of the times tested, except after 8 h of exposure, a time at which the mRNA level of this gene was increased 8-fold relative to control ($p < 0.05$). The toxin mixture also significantly enhanced the *cyp1A* expression in H4IIE cells at all times tested, with a maximum effect of 280-fold compared to the control after 4 h of treatment ($p < 0.001$). Moreover, the MIX effect on mRNA *cyp1A* was significantly stronger than the effects induced by AFB_1 treatment at short times (up to 4 h), and by FB_1 treatment at all times, suggesting an additive or synergistic interaction between the two mycotoxins at 0.5, 2 and 4 h of culture exposure. After 8 h of treatment, no statistically significant differences were observed between AFB_1 and MIX.

3.5. Effects of AFB_1, FB_1 and MIX on cyp1A and ahr mRNA levels in spleen cells

RT-qPCR was performed in order to investigate whether the *cyp1A* expression dependent on Ahr was also affected in splenocytes by the mycotoxins. Immune cells were treated with 20 μM AFB_1, 10 μM FB_1, or MIX (20 μM AFB_1 + 10 μM FB_1), and the expression of *cyp1A* and *ahr* mRNA was studied at increasing time periods from 2 to 24 h. When spleen cells were incubated with AFB_1, the *cyp1A* gene transcription was significantly increased compared to control after 2, 4 and 8 h of culture exposure, with 4 h being the time at which the largest increase was registered (8-fold, $p < 0.001$) (Fig. 5). In contrast, in immune cells exposed to FB_1, a significant rise in the expression of that gene relative to control was only observed after 8 h of treatment (6-fold, $p < 0.001$) (Fig. 5). Furthermore, in splenocytes incubated with MIX, a significant increase in *cyp1A* mRNA levels were observed from 2 to 16 h, reaching a maximal expression of 13-fold after 4 h of treatment ($p < 0.001$), with respect to the control. In addition, these mRNA levels were significantly higher than those induced in cells treated with the individual mycotoxins from 4 to 16 h (Fig. 5). On the other hand, the effect of the mycotoxins on *ahr* mRNA expression revealed that all treatments significantly enhanced the expression of this gene at all tested times, except after 24 h of culture (Fig. 6). In this case, the exposure to AFB_1 produced a maximal increase of about 60-fold observed after 2 h of treatment ($p < 0.001$), while the incubation with FB_1 provoked a maximum rise of about 61-fold after 4 h of treatment ($p < 0.001$), with respect to the control. The study of *ahr* gene transcription upon MIX treatment revealed similar results to those observed in spleen cells exposed to AFB_1, resulting in the highest increase of about 64-fold occurring after 2 h of treatment ($p < 0.001$). However, this significant rise was more sustained by the combined action of both mycotoxins.

4. Discussion

The present work provides new data related to the induction of Cyp1A and Ahr after AFB_1 and FB_1 exposure *in vitro*, which contribute to a better understanding of the mechanisms of action underlying their reported toxic and carcinogenic effects. Additionally, due to their frequent co-occurrence, effects resulting from their mixture were also studied.

Although Cyp activation by AFB_1 has been previously reported, its effect on the regulation of *cyp1A* gene expression has not yet been clarified. In this study, it was shown that AFB_1 induced *cyp1A* transcription and Cyp1A activation, which was accompanied by the enhancement of Ahr activity in the hepatocyte cell lines. These findings strongly suggest that the increase in *cyp1A* transcription was due to Ahr activation induced by AFB_1, presumably via the classical pathway (Delescluse et al., 2000). This toxin has a planar polycyclic aromatic structure similar to that of the known ligands,

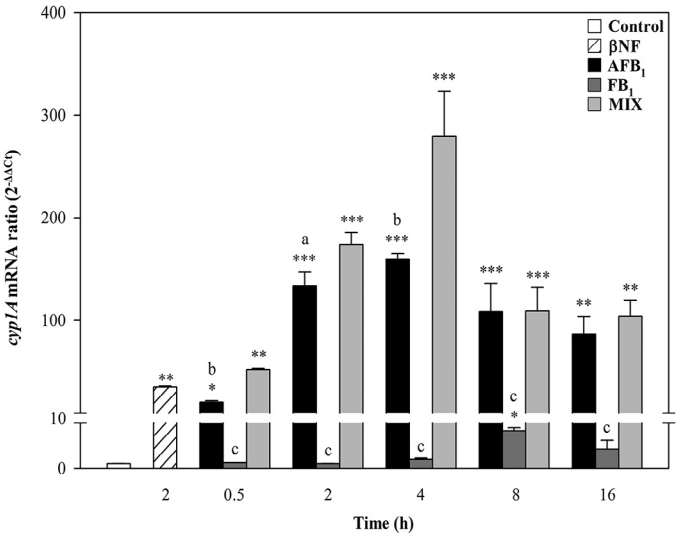

Fig. 4. Individual and combined effects of AFB_1 and FB_1 on *cyp1A* mRNA expression in H4IIE cell line determined by RT-qPCR. These cells were incubated with 5 μM AFB_1, 25 μM FB_1 or MIX (5 μM AFB_1 + 25 μM FB_1) for 0.5, 2, 4, 8 and 16 h. Cells exposed to βNF (0.5 μM) for 2 h were used as positive control. Bars represent the mean ± SEM (n = 3). Asterisks indicate differences with respect to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Letters indicate differences between MIX and the individual mycotoxins ([a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$).

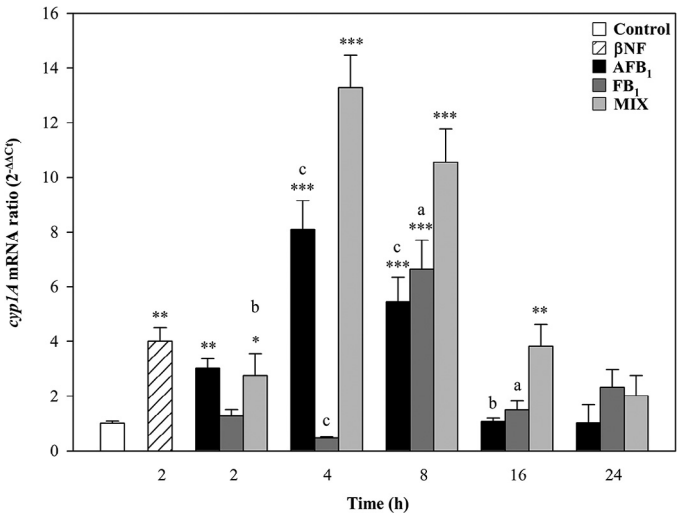

Fig. 5. Individual and combined effects of AFB_1 and FB_1 on *cyp1A* mRNA expression in spleen cells determined by RT-qPCR. These cells were incubated with 20 μM AFB_1, 10 μM FB_1 or MIX (20 μM AFB_1 + 10 μM FB_1) for 2, 4, 8, 16 and 24 h. Cells exposed to βNF (0.5 μM) for 2 h were used as positive control. Bars represent the mean ± SEM (n = 3). Asterisks indicate differences with respect to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Letters indicate differences between MIX and the individual mycotoxins ([a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$).

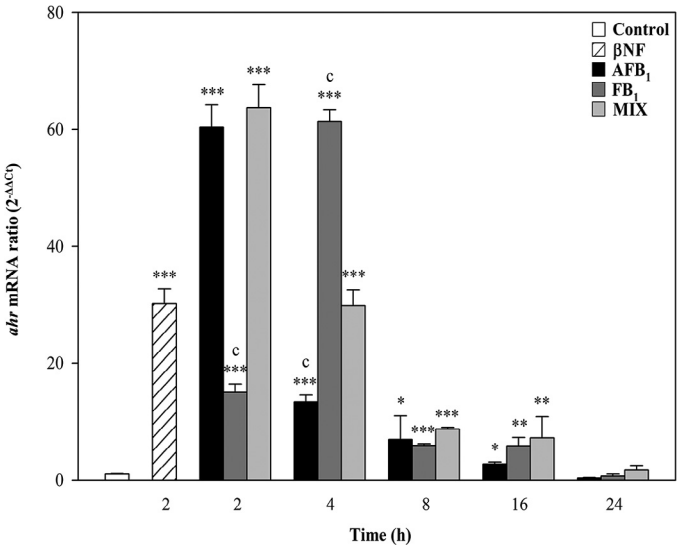

Fig. 6. Individual and combined effects of AFB_1 and FB_1 on *ahr* mRNA expression in spleen cells determined by RT-qPCR. These cells were incubated with 20 μM AFB_1, 10 μM FB_1 or MIX (20 μM AFB_1 + 10 μM FB_1) for 2, 4, 8, 16 and 24 h. Cells exposed to βNF (0.5 μM) for 2 h were used as positive control. Bars represent the mean ± SEM (n = 3). Asterisks indicate differences with respect to control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Letters indicate differences between MIX and the individual mycotoxins ($^{c}p < 0.001$).

which allows it to bind to Ahr and induces receptor translocation to the nucleus, with subsequent transcription of its target genes such as *cyp1A1/2*.

The present results are in agreement with those obtained in spleen cells, with AFB_1 producing an increase in *cyp1A* mRNA levels. However, marked differences were found in the degree of induction of these mRNA levels in hepatic and immune cells, which may have been related to the differential regulation of expression and activity of existing isoforms in these cells, since Cyp1A2 is mainly present in liver whereas Cyp1A1 is primarily found at the extra-hepatic level (Coutiño Rodríguez et al., 2010). In addition, this disparity may be associated with differential regulation of Ahr activity (Tijet et al., 2006). The findings of the present investigation also concur with those obtained by Chirulli et al. (2007), who showed that the Ahr ligand β-naphthoflavone differentially increased the expression and activity of Cyp1A1/2 in different tissues, with the liver being the organ where most Cyp1A induction was recorded.

In the literature, there are only a few conflicting reports that have studied the effects of FB_1 on Cyp activity (Martínez-Larrañaga et al., 1996; Spotti et al., 2000). Of these, our results are similar to those obtained by Martínez-Larrañaga et al. (1996), who reported an increase of Cyp1A activity in the liver of Wistar rats exposed to FB_1. In addition, the findings of the present work demonstrated that FB_1 was able to enhance *cyp1A* gene transcription in H4IIE and spleen cells, but to a lesser extent and duration than AFB_1. This effect was faster for the AFB_1 treatment, with a maximal effect occurring at 4 h, whereas FB_1 treatment revealed a maximum effect at 8 h. The differences found between both mycotoxins with respect to time, duration and amplitude of the observed induction may have been related to the more effective activation of Ahr by AFB_1 than FB_1, as observed in hepatic cells.

Although Ahr plays a central role in *cyp1A* gene regulation, the activation of this transcription factor could not explain the *cyp1A* increase induced by FB_1, since by using the DRE-luciferase construct in DR-CALUX cells, it was observed that FB_1 failed to activate the DRE site, thus suggesting that *cyp1A* gene expression by this mycotoxin did not involve Ahr activation. In fact, other compounds have also been reported to induce *cyp1A* by Ahr-independent mechanisms, through up-regulation or activation of certain transcription factors such as hepatic nuclear factor 4-α (HNF4-α), retinoid X receptor, peroxisome proliferator activated receptor-γ coactivator (PGC-1) and peroxisome proliferator-activated receptor-α (PPAR-α) (Delescluse et al., 2000; Hu et al., 2007;Kruber et al., 2011; Martínez-Jiménez et al., 2006; Sérée et al., 2004). Although the mechanisms by which FB_1 induces *cyp1A* expression have not yet been elucidated, we propose PPAR-α activation to be a possible mechanism, since Martínez-Larrañaga et al. (1996) showed that this mycotoxin induced peroxisomal proliferation in rats, with some other studies also indicating that sphingoid bases, including *Sa* and *So*, can bind and activate PPAR-α (Tsuji et al., 2009; Van Veldhoven et al., 2000).

The present investigation demonstrated that FB_1 and AFB_1 were able to increase the *ahr* mRNA levels in primary spleen cells, indicating that these mycotoxins can stimulate transcription of this gene. However, although the expression control of Ahr target genes has been extensively studied, little is known about the molecular events that regulate *ahr* gene expression (Shimba et al., 2003; Shin et al., 2007). Related to this, some studies have indicated that known Ahr agonists may induce mitogen-activated protein kinase (MAPK) activation, such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), which have been shown to stimulate *ahr* expression (Tan et al., 2004; Yim et al., 2004). These types of mechanisms may have been present in these mycotoxins, since previous results have indicated that FB_1 activates the MAPK/ERK pathway in spleen primary cells (Mary et al., 2012) and in the liver (Rumora et al., 2007) of rat, with Chen et al. (2009) suggesting that AFB_1 can activate the MAPK/JNK pathway in hepatic cells. Furthermore, several authors have shown that oxidative stress can induce Ahr activation (Elbekai and El-Kadi, 2005; Kalthoff et al., 2010; Ramadass et al., 2003; Wu et al., 2008), thereby providing evidence for a cross-talk between oxidative stress and the Ahr pathway. Thus, taking into account that previous studies have demonstrated the induction of oxidative stress *in vivo* and *in vitro* by the mycotoxins used in the present work (Lin et al., 2006; Mary et al., 2012; Theumer et al., 2010), we cannot discard that this mechanism may have contributed to Ahr activation by AFB_1 and FB_1.

The findings of this investigation have indicated that the effects of AFB_1–FB_1 mixtures are stronger than those found for the mycotoxins alone. In H4IIE cells, Cyp1A activity and Ahr transduction were only induced by AFB_1 and MIX, but with the latter showing an enhanced response that was greater than the sum of the individual actions of these mycotoxins for most of the tested concentrations. In this cell line, the *cyp1A* transcription was observed for AFB_1, FB_1 and the MIX, but again the activation of the expression of this gene was significantly higher for MIX compared to AFB_1 and FB_1. It should be noted that although no EROD and Ahr inductions were observed after exposure to FB_1, this mycotoxin might still induce *cyp1A* gene expression, albeit at a much lower potency than AFB_1, which may explain the greater responses found with MIX. In spleen cells, a similar effect was observed on *cyp1A* transcription, with the results revealing that the maximum effects of individual and combined toxins on the *ahr* gene expression in spleen cells occurred before the largest increases observed in the *cyp1A* transcription, suggesting that the expression of the latter gene was, at least in part, a consequence of an increased *ahr* mRNA level (Tijet et al., 2006).

The potential interaction between the mycotoxins to induce *cyp1A* up-regulation may have been the result of: the combination of the Ahr activation by AFB_1, the increased expression of *ahr* by both toxins, and the possible activation of intracellular signal transduction systems that involved protein tyrosine kinases (TK), ERK or protein

kinase C (PKC) by FB_1 (Gopee and Sharma, 2004; Mary et al., 2012; Rumora et al., 2007), since these kinases facilitate and/or amplify the functionality of Ahr, thus favoring the binding of the receptor to its target genes (Fang et al., 2013; Tan et al., 2004; Yim et al., 2004).

Our results suggest that the large increase in *cyp1A* mRNA levels induced by the mycotoxin mixture in H4IIE cells might have favored AFB_1 biotransformation to the highly carcinogenic metabolite AFBO and the malignant transformation of hepatic cells. Additionally, Ahr activation may also promote the carcinogenicity of AFB_1 and FB_1, because some previous evidence has indicated that this receptor appears to be important in carcinogenesis and the progression of several carcinomas (Koliopanos et al., 2002; Lin et al., 2003; Peng et al., 2009). In fact, Ahr has recently emerged as a critical regulator of immune responses that affects both the innate and adaptive systems and plays an important role in regulatory T cells, which are regarded as inhibitors of anti-tumor immunity (Apetoh et al., 2010; Pot, 2012). Therefore, the activation of the Ahr signaling pathway by AFB_1 and FB_1 might alter the tumor immunosurveillance and thereby contribute to the carcinogenicity of both mycotoxins.

In summary, the results of the present work demonstrate that AFB_1 and FB_1, either alone or in combination, increase *cyp*1A transcription and Cyp1A activity, as well as up-regulate Ahr. The AFB_1–FB_1 mixture induces enhanced effects compared with those produced by the mycotoxins alone; indicating that the presence of FB_1 may increase the bioactivation and subsequent carcinogenicity of AFB_1. Although the rat liver hepatoma cell line H4IIE and the transfected DR-CALUX cell line are widely recommended models to study Cyp1A and Ahr induction, respectively, and although some information on the hepatotoxic effects of AFB_1 and FB_1 and carcinogenic effects of AFB_1 in rats is available, further studies *in vivo* should still be conducted in order to confirm the findings obtained in this work.

Conflict of interest

Dr. Fernández-Cruz, Dr. Mary and Dr. Rubinstein report grants from Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo and Dr. Mary and Dr. Rubinstein report grants from Agencia Nacional de Promoción Científica y Tecnológica, during the conduct of the study.

Transparency document

The Transparency document associated with this article can be found in the online version.

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