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Reactive oxygen species sources and biomolecular oxidative damage induced by aflatoxin B1 and fumonisin B1 in rat spleen mononuclear cells

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ABSTRACT

Aflatoxin B1 (AFB_1) and fumonisin B1 (FB_1) are mycotoxins widely found as cereal contaminants. Their immunotoxicities predispose to infectious diseases and may alter the tumor immunosurveillance of human and animals, but the mechanisms underlying have not been fully elucidated, and the induction of oxidative stress has been proposed as a probable mechanism. This work was aimed at evaluating in spleen mononuclear cells (SMC) from Wistar rats the effects of the exposure, in vitro for up to 48 h, to 20 μM AFB_1, 10 μM FB_1 and AFB_1–FB_1 mixture (MIX), over cellular oxidative status, as well as at elucidating the contribution of different reactive oxygen species (ROS) to biomolecular oxidative damage, the biochemical pathways involved, and the probable interaction of both toxins to induce oxidative stress. All the treatments increased total ROS and oxidation of biomolecules, with MIX having the greatest effects. However, only MIX increased superoxide anion radical. The main ROS involved in oxidation of proteins, lipids and DNA appear to be hydrogen peroxide and hydroxyl radical. The mitochondrial complex I and CYP450 were involved in the ROS generation induced by all treatments. The NADPH oxidase system was induced by FB1 and MIX. The arachidonic acid metabolism contributed to the ROS formation induced by AFB_1 and MIX. These results demonstrate that an interaction between AFB_1 and FB_1 occur in the oxidative stress induction, and show the biochemical pathways involved in ROS generation in SMC. The oxidative stress could mediate the AFB_1 and FB_1 individual and combined immunotoxicities.

1. Introduction

Mycotoxins are fungal metabolites toxic to humans and animals, commonly found as contaminants of food or feed (CAST, 2003).

Aflatoxins are principally produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Among these, aflatoxin B1 (AFB_1) is the predominant form as cereal and oilseed contaminants, and presents the highest toxic potential (CAST, 2003), being hepatotoxic and carcinogenic in human and animals (Nogueira et al., 2009; Pitt, 2000). AFB_1 is classified by the International Agency of Research on Cancer (IARC) as Group 1 carcinogen (IARC, 1993a). This mycotoxin is also mutagenic, teratogenic and immunosuppressive in farm and laboratory animals (IPCS-WHO, 1998). AFB_1 mainly affects the cell-mediated immunity (Williams et al., 2004). This mycotoxin decreased total lymphocytes, T-cells sub-types (CD4(+) and CD8(+)), NK, and TNF-α and IL-1β release in rats (Abbès et al., 2010). Besides, AFB_1 decreased cell proliferation in spleen mononuclear cells (SMC) from rats (Theumer et al., 2003), and inhibited IL-2 mRNA expression in murine thymocytes (Han et al., 1999). The toxicology of AFB_1 involves its biotransformation through the cytochrome P (CYP) 450 to the highly reactive AFB_1-exo-8,9-epoxide, which forms adducts with the DNA (Guengerich et al., 1998). Besides, AFB_1 is able to induce ROS generation (Matur et al., 2011; Adedara et al., 2010), possibly requiring the activation of cytochrome P450. However, the mechanisms of ROS generation induced by AFB_1 have not been completely elucidated.

Fumonisins, mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum*, are mycotoxins commonly found as corn contaminants. The most toxic and abundant of these is FB_1, which

Abbreviations: AFB_1, aflatoxin B1; ArAc, arachidonic acid; DCF, dichlorofluorescein; DCFH-DA, dichlorodihydrofluorescein diacetate; DEX, dexamethasone; dG, deoxyguanosine; DNPH, 2,4-dinitrophenylhydrazine; DPI, diphenyleneiodonium; FB_1, fumonisin B1; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HE, hydroethidine; MAPK, mitogen activated tyrosine kinase; MDA, malondialdehyde; MIX, AFB_1–FB_1 mixture; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PLA2, phospholipase A2; PI, propidium iodide; RET, reverse electron transport; ROS, reactive oxygen species; SMC, spleen mononuclear cells; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; TEP, 1,1,3,3-tetramethoxypropane.

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causes esophageal and hepatic cancer in humans, and liver and kidney cancer in rodents (van der Westhuizen et al., 2010; IPCS-WHO, 2000). The IARC evaluated FB_1, as probably carcinogenic to humans (Group 2B) (IARC, 1993b). Besides, FB_1 modulates the immunity in animals. In broilers, this fumonisin decreased the viability of lymphocytes (Dombrink-Kurtzman et al., 1993), and inhibited the expression of IL-1beta, IL-2, IFN-alpha and IFN-gamma (Cheng et al., 2006). Furthermore, FB_1 modified the excretion of IL-4 and IL-10 in SMC, and decreased H_2O_2 released by adherent peritoneal cells (APC) from rats (Theumer et al., 2002). FB_1 inhibits the ceramide synthetase activity and disrupts the sphingolipid metabolism (IPCS-WHO, 2000). In addition, FB_1 can produce oxidative stress and/or apoptosis, depending on the species and the cell types (Stockmann-Juvala and Savolainen, 2008). The mechanisms used by FB_1 to induce ROS have begun to be evaluated in recent years (Domijan and Abramov, 2011), but have not been clearly understood yet.

The natural co-occurrence of AFB_1 and FB_1, especially in corn, is a worldwide problem and has been associated with a high incidence of human hepatocellular carcinoma (CAST, 2003). Moreover, it is also likely that the immunomodulation exerted individually by AFB_1 and FB_1 may be enhanced by co-exposure to both these mycotoxins. AFB_1–FB_1 mixture reduced cell proliferation, and IL-2 and IL-10 release from SMC, and decreased H_2O_2 release by APC from rats. These immunosuppressive effects induced by the AFB_1–FB_1 mixture were higher than those induced by individual mycotoxins (Theumer et al., 2003). However, the biochemical events leading to immune system impairment by this toxin mixture are still unknown.

The immunotoxicity of the mycotoxins may predispose to the infectious diseases (Grenier et al., 2011); and might alter the tumor immunosurveillance. Moreover, recent studies suggest that such an immunotoxic effect elicited by the mycotoxins could be closely related to ROS generation (Kotan et al., 2011; Theumer et al., 2010).

Oxidative stress arises when ROS synthesis exceeds the ability of the antioxidant defenses to eliminate them, leading to oxidation of proteins, lipids and DNA (Hwang and Kim, 2007). The ROS, including the superoxide anion radical ($O_2^{\bullet -}$), H_2O_2 and the hydroxyl radical ($^{\bullet}OH$), may be generated in vivo by: (a) the mitochondria at the electron transport complexes I–III, being the predominant site of high ROS production the complex I (Murphy, 2009); (b) the NADPH oxidase system, which can be activated by the tyrosine kinase, the mitogen activated tyrosine kinase (MAPK) and the PKC biochemical pathways (Curnutte et al., 1994; Dewas et al., 2000); (c) the CYP450 monooxygenases; and (d) the metabolism of arachidonic acid (ArAc), which is released from membrane glycerophospholipids by phospholipase A2 (PLA2) and is subsequently transformed by cyclooxygenases or lipoxygenases into bioactive eicosanoids (Circu and Aw, 2010).

This work was aimed at evaluating the ROS generation elicited by AFB_1 and FB_1, alone or in combination, in SMC from Wistar rats, as well as at elucidating the probable interaction of both toxins to induce oxidative stress. Furthermore, the metabolic pathways involved in the ROS generation and the contribution of different ROS to induce biomolecular oxidative damage were also assessed, by the specific inhibition of the ROS-generating pathways and by incorporating ROS scavengers into the SMC cultures, respectively. In the present study it was demonstrated that these mycotoxins, alone or as a mixture, affect the oxidative status in SMC, by increasing ROS levels and biomolecular oxidative damage. The action of AFB_1–FB_1 mixture promoted a stronger pro-oxidant activity by the AFB_1 and FB_1 interaction.

2. Materials and methods

2.1. Chemicals

RPMI-1640 medium and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Laboratories. 1,1,3,3-Tetramethoxypropane (TEP), 2-thiobarbituric acid (TBA), 2,4-dinitrophenylhydrazine (DNPH), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 8-hydroxy-2′-deoxyguanosine (8-OHdG), AFB_1, catalase, deoxyguanosine (dG), dexamethasone (DEX), diphenyleneiodonium (DPI), FB_1, genistein, guanidine hydrochloride, hydroethidine (HE), indomethacin, propidium iodide (PI), proteinase K, RNAse A, rotenone, superoxide dismutase (SOD) and trichloroacetic acid (TCA) were all purchased from Sigma–Aldrich, Buenos Aires, Argentina. UO126 and the Bradford reagent were obtained from Cell Signaling Technology (Beverly, MA, USA) and Bio-Rad Laboratories (Buenos Aires, Argentina), respectively. Nuclease S1 and alkaline phosphatase were supplied by Promega (Madison, WI, USA). All other chemicals were provided by Sigma–Aldrich (Buenos Aires, Argentina), and Sintorgan (Buenos Aires, Argentina) was obtained at the highest analytical grade available.

2.2. Cells and treatments

Cells were obtained from male Wistar inbred rats (6–8 weeks old) kept in environmentally controlled rooms with a 12-h light–dark cycle. For each experiment, 6–8 rats were anesthetized with isoflurane and spleens were removed aseptically from the animals and pooled. SMC suspensions were prepared as previously described (Theumer et al., 2010), being seeded at a density of 10^6 cells/ml and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere with 5% CO_2.

For all studies, SMC were treated with 20 μM AFB_1 dissolved in DMSO (final concentration: 8 mM), 10 μM FB_1 dissolved in PBS or with the two-toxin mixture (20 μM AFB_1 + 10 μM FB_1, MIX). The doses were selected on the basis of the literature data and previous studies (Theumer et al., 2002, 2003). The treatments with the mycotoxins lasted up to 48 h, depending on the experiment.

The Institutional Experimentation Animal Committee (authorization #15-09-69934) approved the animal handling and experimental procedures.

The cell viability and death were studied up to 48 h of incubation of SMC with the mycotoxins or the AFB_1 vehicle (DMSO), using trypan blue exclusion test, MTT test and the flow cytometric detection of hypodiploid DNA.

2.2.1. MTT test

After 44 h of incubation in the presence or absence of different concentrations of mycotoxins and the AFB_1 vehicle, the SMC were incubated for 4 h with 0.45 mg/ml MTT. The medium was then removed, and formazan crystals were solubilized in DMSO and assessed spectrophotometrically (Benchmark Microplate Reader, Bio-Rad) at 570 nm (reference wavelength of 630 nm). Wells with 5 μM As_2O_3, and wells without the addition of MTT, were used as positive and negative controls, respectively. Wells containing culture medium and MTT but no cells acted as blank. The results are expressed as percentage of viability = [(MTT OD treated cells-blank)/(MTT OD untreated cells-blank)] × 100.

2.2.2. Flow cytometric detection of hypodiploid DNA

After 48 h of incubation with or without the mycotoxins and the AFB_1 vehicle, the SMC were fixed in 70% ice-cold ethanol overnight at 4 °C, and then incubated with 50 μg/ml PI and 100 μg/ml RNase A for 0.5 h at 37 °C (Nicoletti et al., 1991). The cells were analyzed using a flow cytometer (FACSCantoII, Becton Dickinson) and 30,000 events were acquired for each sample (excitation at 488 nm, emission at 600 nm, and recollection filter from 564 to 606 nm). The decrease in DNA content was used as cell death marker.

2.3. Analysis of splenocyte subpopulations

Splenocyte subpopulations were stained with the following conjugated monoclonal antibodies (mAb, BD Biosciences) against surface markers: CD3-APC, CD4-PE Cy5, CD8-FITC, NK-FITC, B220-PE, CD11b/c-FITC, either alone or in combination. Isotype staining was performed with IgG-conjugated antibodies. After preincubation of cells for 30 min on ice with anti-CD32, cells were incubated with mAb for 30 min on ice. Following this, cells were analysed by flow cytometry, with a minimum of 100,000 events.

2.4. Detection of intracellular ROS generation

Intracellular production of ROS was determined using DCFH-DA (Kang et al., 2003). Briefly, the SMC were pre-incubated for 0.5 h with or without 2 μM DPI (NADPH oxidase inhibitor), 10 μM UO126 (ERK/MAPK pathway inhibitor), 10 μM genistein (tyrosine kinase inhibitor), 1 μM rotenone (complex I mitochondrial electron-transport-chain inhibitor), 0.01 μM DEX (PLA2 inhibitor), 1 μM indomethacin (cyclooxygenases 1 and 2 inhibitor) or 50 μM ciprofloxacin (CYP450 1A and 3A isoenzyme inhibitor). Then, the mycotoxins were added to the medium,