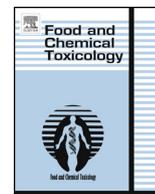




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Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Short communication

Toxicity of aflatoxin B1 towards the vitamin D receptor (VDR)

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ARTICLE INFO

Article history:

Received 8 October 2014

Accepted 27 November 2014

Available online 4 December 2014

Keywords:

Rickets

Aflatoxin B1

Vitamin D

Mycotoxins

ABSTRACT

This research describes an unexpected toxicity of the aflatoxin B1 towards the vitamin D receptors. Rickets is a childhood disease, and calcium deficiency is the aetiological cause in Africa, being primarily associated with nutritional problems; in this research the contribution of aflatoxin B1 exposure during the early months of life is an interesting factor to deepen in order to prevent liver damages or the development of rickets. The results show that the expression of vitamin D receptor in osteosarcoma cell line SAOS-2 is significantly down-modulated by exposure to aflatoxin B1. This seems to suggest that Aflatoxin B1, toxic towards the vitamin D receptor, interferes with the actions of the vitamin D on calcium binding gene expression in the kidney and intestine. Experimental data indicate a 58% and 86% decrease if the cells are exposed to 5 ng/mL and 50 ng/mL of aflatoxin B1, respectively. These results seem to indicate that natural occurrence of the aflatoxin B1 and allelic variant of vitamin D receptor on (F allele) increase the risk of developing rickets of African children.

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1. Introduction

The accumulation of mycotoxins in foodstuff can be observed both on field and during processing or storage of contaminated foodstuff. Mycotoxins are secondary metabolites produced by microfungi and *Aspergillus spp.*, responsible for the production of Aflatoxin B1 (AFB1) is among the most widely diffused ones. The onset of this contaminant can consequently greatly reduce the food safety level, and have strong impact on the entire agriculture–food chain. FAO indicates that about 25% of the entire world harvest is lost for the high level of the mycotoxins in the commodities and to the consequent risk for human health. Worldwide trend is focused on a stricter control of mycotoxins. However, the changing global environment may not be the ideal setting to control and reduce the exposure to these toxins (Marroquín-Cardona et al., 2014).

The most investigated mycotoxin is the aflatoxin B1 (AFB1) (Fig. 1), discovered for the first time in England in 1961 (Armbrecht et al., 1963). This toxin is generally recognized as the molecule with the greatest carcinogenic potential deriving from natural sources, in particular towards the liver and the gastro-intestinal apparatus. AFB1 has been extensively reported as responsible in particular of acute hepatotoxicity in many areas of the world (Cullen and Newberne, 1994; Nault, 2014; Wu and Santella, 2012).

LD₅₀ values observed in animal species tested with single dose of AFB1 show a wide variation with values in a range from 0.5 to 10 mg/kg of body weight.

The AFB1 exposure associated risk through food chain is widely reported in the literature, and its levels in different foodstuffs like peanuts, cereals, fruits or their processed derivatives, are continuously monitored. In many industrialized Countries, AFB1 represents a serious threat, especially related to commodities imported from developing Countries and, for these reason, it is widely regulated.

The many studies on AFB1 have allowed an understanding of the different aspects related to its mode of action, e.g. it interferes with the expression of p53 gene leading to hepatotoxicity, cirrhosis or necrosis. In addition, AFB1 is synergic with other unwanted toxic compounds, and it is capable of increasing the virulence of the hepatitis B virus (Gnonlonfin et al., 2013).

The effects of AFB1 on calcium and vitamin D (Fig. 1) metabolism have been so far poorly investigated (Wild and Gong, 2010) and this correlation has not been exploited by further studies (Sergeev et al., 1988).

Among the main childhood pathologies in Africa and Asia, rickets, a disease caused by a defect in bone formation of newly formed bone matrix, conjugation cartilages, and zones of provisional calcification, plays a relevant role on the early life of infants.

Rickets can affect both human and animals in the first months of life and it is a general realisation that the main causes of this condition are the disorders of both bone metabolism and vitamin D metabolism.

A survey conducted in 59 countries over the past 20 years indicates that the causes of rickets are strictly correlated to the

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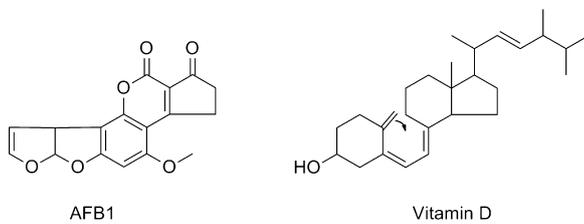


Fig. 1. Aflatoxin B1 and Vitamin D chemical structures.

geographical region and also that rickets is primarily associated with nutritional problems (Pettifor, 2004).

The literature data have shown that the calcium deficiency is the aetiological cause of rickets in Africa and in some Asian subtropical areas, while a deficiency of vitamin D, observed in North America and Europe, is the leading cause of rickets in these countries during the first 18 months of life (Thacher et al., 2006).

On the other hand, everywhere, calcium deficiency always represents a severe threat also for children aged over two years. Literature data suggest the vitamin D diet supplementation during pregnancy can reduce the frequency of rickets among infants and children as shown by follow up monitoring.

The African continent is a geographical and socio-economic area that experiences a relevant risk for the onset of rickets. The climatic conditions are in fact ideal for the colonization and the growth of *Aspergillus spp.* and for the production of AFB1. Moreover, the poor diet and the low dietary origin vitamin D availability makes the onset of rickets more frequent and severe (Li et al., 2001; Xi et al., 2011; Zheng et al., 2004). Low calcium intake surely plays an important role in the development of rickets among African children, even if some scientific evidence suggests that additional nutritional, genetic or hormonal factors which reduce calcium absorption may contribute to the genesis of the disease in susceptible children. Taking into account these observations, our target has been to show that AFB1 ingested with the food diet, interferes with the vitamin D hormonal system and, consequently, interferes with the calcium metabolism. We demonstrate that the exposure to AFB1 affects the VDR expression; this may prevent the receptor from mediating the actions of Vitamin D on calcium binding genes expression in the kidney and intestine (Christakos et al., 1989, 2010).

2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell line SAOS-2 was purchased from ATCC (USA) and was grown, according to recommendation of the supplier, in Dulbecco's modified Eagle's medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% foetal calf serum, 1% streptomycin–penicillin mix (Bio-Whittaker) in 5% CO₂ atmosphere at 37 °C. The medium was changed every 48 h.

2.2. AFB1 treatment

AFB1 was re-suspended with 100% dimethyl sulphoxide (DMSO) and the concentration was adjusted to 10 mg/mL. SAOS-2 cells were plated at a density of 3×10^5 cells/well in 6-well plates and were exposed to different concentrations of AFB1 (5 and 50 ng/mL) for 1 hour. The final concentration of DMSO (v/v), used as solvent of AFB1, was maintained under 5% also in the control (cells treated only with DMSO). Results are representative of two independent experiments.

2.3. RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. One microgram of each RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. Real-time PCR was carried out in a iCycler Thermal Cycler (Bio-Rad, Berkeley, CA, USA) using the SYBR Green I Master Mix (Bio-Rad) and specific primers for: VDR (Fw 5'-TCCAGTTCGTGTAATGATG-3', Rev 5'-GAAGGTCATCTGAATCTTC-3'); β -actin (Fw 5'-cgacaggatgcagaaggaga-3', Rev 5'-

cgtcatactctgcttctgctg-3'). β -actin house-keeping gene was used as a reference gene for relative quantification. All real-time PCR reactions were performed in duplicate.

The relative quantification in gene expression was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Normalization: $\Delta C_T = C_T(\text{sample}) - C_T(\text{endogenous control})$; $\Delta\Delta C_T = \Delta C_T(\text{sample 1}) - \Delta C_T(\text{sample 2})$. Relative quantification = $2^{-\Delta\Delta C_T}$. Efficacy of the PCR amplification of controls and test was identical; parallelism of standard curves of the control and test was confirmed.

2.4. Statistical analysis

The Student's test was used to evaluate the statistical significance of differences using the non parametric Mann–Whitney test. P values were considered significant when $P < 0.05$.

2.5. Western blot analysis

Total protein extract was obtained as follows: cells were washed twice with cold PBS, then were lysed in lysis buffer (10 mM Hepes pH 7.8, 250 mM NaCl, 5 mM EDTA pH 8.0, 1% Nonidet P-40, 1 mM sodium orthovanadate and 50 mM sodium fluoride) and protease inhibitors (1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μ g/mL aprotinin and 1 μ g/mL pepstatin A) for 30 min on ice. The extract was centrifuged in a microfuge at maximum speed for 10 min and the supernatant was quantified using the Bio-Rad protein assay reagent, resolved on SDS–PAGE and then transferred to Hybond membranes (Amersham Biosciences, NJ, USA). Non-specific binding sites were blocked for 2 h with 5% milk in Tris–Tween buffered saline (tTBS) (5 mM Tris pH 7.5, 15 mM NaCl, 0.1% Tween-20), washed three times with tTBS and incubated with the following primary antibodies: anti-VDR (Santa Cruz, Biotechnology), 1:500 and anti-GAPDH (Origene), 1:2000. The secondary antibodies were IgG anti-mouse and IgG anti-rabbit (Amersham), respectively, conjugated to horseradish peroxidase 1:5000 and detected with ECL western blot detection system (Amersham). Results are representative of two independent experiments.

3. Results and discussion

The human osteosarcoma cell line SAOS-2, represents a useful in vitro model to verify the behaviour of VDR when it is exposed to compounds different from the Vitamin D.

To understand if AFB1 could affect the VDR expression, SAOS-2 cells were treated with increasing concentrations of AFB1 (5 and 50 ng/mL) for 1 hour, after which VDR mRNA and protein expression was measured by real-time quantitative PCR (Rt-qPCR) and western blot, respectively. Interestingly, we observed that the exposure of SAOS-2 cells to the AFB1 resulted in a dose dependent down-regulation of VDR mRNA expression measured by RT-qPCR expression. Treatment with 5 ng/mL of AFB1 significantly decreased VDR mRNA expression (58%, $P < 0.0001$) and cells exposition to 50 ng/mL of AFB1 further reduced the expression of VDR mRNA (86%; $P < 0.0001$), as shown in Fig. 2A. Western blot analysis also confirmed that the protein expression of VDR was down-modulated by AFB1 as shown in Fig. 2B.

These results indicate that the exposure to AFB1 affects the vitamin D receptor expression and, consequently, can interfere with vitamin D metabolism and, in cascade, increases the risk for rickets onset in the children population.

To support this experimental strategy, it can be noted that recent studies outlined the presence of polymorphisms in the VDR gene. Nevertheless, the influence of these allelic variants on VDR protein function and signalling is still largely unknown.

Specific variations in the allelic frequencies of VDR polymorphisms among Europeans, Africans, Amerindians, and Asians, have been described (Uitterlinden et al., 2004). A study conducted on Nigerian children suggested that a VDR variant (F allele) is associated with increased risk of developing rickets, raising the possibility that VDR gene polymorphism may be an important factor in determining an individual susceptibility to developing rickets when faced with dietary calcium deficiency (Nejentsev et al., 2004). Other genetic association studies could be needed to assess completely the VDR polymorphism role in the risk of rickets onset (Fischer et al., 2000).

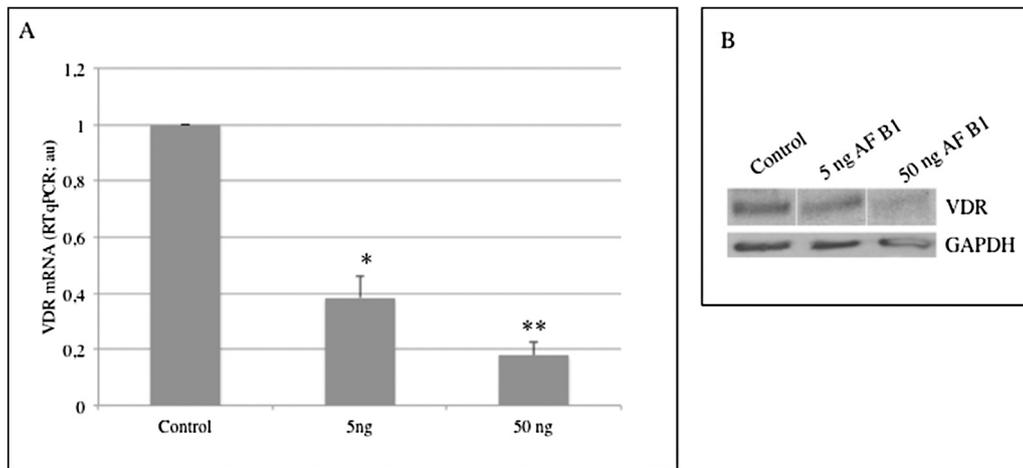


Fig. 2. VDR mRNA and protein expression after treatment of SAOS-2 cells with AFB1. SAOS-2 cells were exposed to increasing concentration of AFB1 (5 ng, 50 ng) or DMSO (control) and analyzed after 1 h. (A) VDR mRNA levels evaluated by quantitative Real Time PCR (RT-qPCR) analysis. * $P < 0.0001$ vs the corresponding DMSO-treated cells (control). ** $P < 0.00001$ vs the corresponding DMSO-treated cells (control). Error bars represent standard deviations of two independent experiments. (B) VDR protein expression levels evaluated by western blot analysis. GAPDH was used as loading control. One representative blot out of two performed is presented.

Our data support that the individual polymorphisms in the VDR gene may show an increased susceptibility to AFB1, resulting in an increased formation of AFB1-DNA adducts that block DNA replication and transcription of VDR gene, leading to the decreased expression of the receptor.

The chemical structures of vitamin D and AFB1 are partially overlapping because, as shown in Fig. 1, similarities between the two poly-aromatic systems of the vitamin D and the AFB1 are evident, and this could explain the partial interferences of mycotoxin towards the physiological activity of the VDR.

In addition, the VDR polymorphism in the African population and the high exposure to AFB1 present endemically in the food chain, suggests the increased susceptibility of African children to develop rickets.

The results of our experiment on SAOS-2 cell line exposed to AFB1 concentrations simulating those found in foodstuff available in Africa open a new perspective and suggest considering AFB1 as an important aetiological cofactor that increases the risk of developing rickets in the first part of life of children with severe problems on their lease of life.

Conflict of interest statement

Authors do not have any conflict of interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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