Food and Chemical Toxicology 46 (2008) 2665–2671

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Effect of ochratoxin A on redox-regulated transcription factors, antioxidant enzymes and glutathione-S-transferase in cultured kidney tubulus cells

C. Boesch-Saadatmandi [a], A. Loboda [b], A. Jozkowicz [b], P. Huebbe [a], R. Blank [c], S. Wolffram [c], J. Dulak [b], G. Rimbach [a,*]

[a] Institute of Human Nutrition and Food Science, Christian-Albrechts-University Kiel, Herrman-Rodewald-Strasse 6, 24118 Kiel, Germany
[b] Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
[c] Institute of Animal Nutrition and Physiology, Christian-Albrechts-University Kiel, Germany

ARTICLE INFO

Article history:
Received 5 November 2007
Accepted 21 April 2008

Keywords:
Ochratoxin A
Glutathione-S-transferase
Nrf2
AP-1
Kidney tubulus cells
Oxidative stress
Redox signalling
Nephrotoxicity

ABSTRACT

Ochratoxin A (OTA), a mycotoxin mostly produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, is a worldwide contaminant of food and feedstuff. OTA is nephrotoxic and a renal carcinogen in rodents. The underlying molecular and cellular mechanisms by which OTA exhibits its toxicity have yet not been fully clarified. In the present study the effects of ochratoxin A on the activity of redox-regulated transcription factors, antioxidant enzymes, as well as glutathione-S-transferase (GST) have been studied in cultured kidney tubulus cells (LLC-PK1). Confluent LLC-PK1 cells were incubated with increasing concentrations of OTA for 24 h. OTA decreased SOD activity and enhanced intracellular levels of reactive oxygen species (ROS) as measured by flow cytometry. Furthermore OTA resulted in a down-regulation of GST mRNA and activity levels. Lower GST levels were accompanied by a decreased transactivation of activator protein-1 (AP-1) and NF-E2-related factor-2 (Nrf2), which mediate GST gene transcription. Present data indicate that enhanced ROS production and an impairment of GST activity, possibly due to an AP-1 and Nrf2 dependent signal transduction pathway, may be centrally involved in OTA induced nephrotoxicity.

1. Introduction

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by several species of the fungal genera *Penicillium* and *Aspergillus*. Due to their widespread occurrence, OTA is routinely detected in a wide range of food and feedstuff. Cytotoxic and carcinogenic properties of OTA towards the kidney, its main target tissue, have been consistently described in numerous studies in rodents (Bendele et al., 1985; Boorman et al., 1992; Castegnaro et al., 1998). Based on this evidence, OTA has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC) (see Fig. 1).

Furthermore, OTA has been associated with the Balkan endemic nephropathy (BEN) and is possibly implicated in the development of urinary tract tumours (Pfohl-Leszkowicz and Manderville, 2007; Pfohl-Leszkowicz et al., 2002), although substantiating data demonstrating a clear involvement of OTA in the aetiology of BEN are still lacking (Stefanovic et al., 2006). However, porcine nephropathy, a tubulointerstitial disease similar to BEN, which occurring in Northern Europe, is caused by OTA (Krogh, 1992).

Despite intensive investigative effort, the molecular and cellular mechanism of OTA-induced nephrotoxicity and carcinogenicity remain unclear. OTA toxicity has been associated with inhibition of protein synthesis, mitochondrial dysfunction, formation of DNA adducts, disruption of calcium homeostasis, and the generation of reactive oxygen species (Baudrimont et al., 1997; Gekle et al., 2005; Kamp et al., 2005a; Ringot et al., 2006; Schaaf et al., 2002).

Taking into account that OTA enhances the production of free radicals, the activity of redox-regulated transcription factors and antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) may be affected by OTA. It has been recently suggested that a disruption of Nrf2 related signal transduction pathways is involved in OTA induced impairment of antioxidant defence and cellular detoxification (Cavin et al., 2007; Marin-Kuan et al., 2006). The transcription factor Nrf2, a member of the basic leucine zipper (bZIP) transcription factor family is a potent activator of antioxidant response element (ARE)-mediated gene expression, moderating the transcriptional induction of a battery of genes encoding for phase II enzymes including glutathione-S-transferase (GST) and known to be involved in cellular protection against xenobiotics and oxidative

Abbreviations: AP-1, activator protein-1; ARE, antioxidant responsive element; BEN, Balkan endemic nephropathy; bzip, basic leucine zipper; CAT, catalase; FCS, fetal calf serum; GPx, glutathione peroxidase; H_2DCF-DA, dihydrodichlorofluorescein A; IARC, International Agency for Research on Cancer; Nrf2, NF-E2-related factor-2; OTA, ochratoxin A; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author. Tel.: +49 431 8802583; fax: +49 431 8802628.
E-mail address: rimbach@foodsci.uni-kiel.de (G. Rimbach).

0278-6915/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2008.04.023

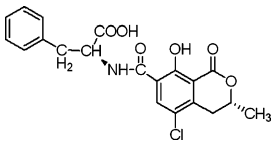

Fig. 1. Structure of ochratoxin A.

stress (Tanigawa et al., 2007; Wasserman and Fahl, 1997). GST is regulated by inducible activator protein-1 (AP-1) binding, however, little is known on the effect of OTA on AP-1 activity.

Although there is preliminary evidence indicating that OTA may affect Nrf2 activity (Cavin et al., 2007), systematic studies on the role of OTA and its metabolites on Nrf2 dependent signal transduction pathways are missing. Therefore, the objective of the present study was to further expand knowledge of OTA impact on Nrf2 as well as AP-1 activity, antioxidant enzymes and GST in cultured kidney tubulus cells.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture medium and supplements were purchased from PAA (Coelbe, Germany). Other chemicals and reagents were obtained from Sigma (Deisenhofen, Germany) if not specified otherwise in the following sections. Ochratoxin A (CAS no. 303-47-9) and B (CAS no. 4825-86-9) were produced by inoculation of wheat with *Aspergillus ochraceus* (NRRL 3174). Crystalline OTA and OTB were obtained in a purity of greater than 99 % by isolation and purification from contaminated wheat as described earlier (Xiao et al., 1996). Ochratoxin α was synthesized by hydrolysis of ochratoxin A with hydrochloric acid according to the procedure reported by Xiao et al. (1995).

2.2. Cell culture and treatments

Porcine kidney tubuli cells (LLC-PK1), were obtained from the Institute for Applied Cell Culture (IAZ, Munich, Germany). They were routinely grown in high glucose DMEM medium, supplemented with 10% fetal calf serum (FCS), streptomycin (100 U/ml) and penicillin (100 μg/ml), and kept under standard conditions (37 °C, 5% CO_2). For experiments, kidney cells were subcultured for 48 h starting with an initial cell density of 2.5×10^4 cells per cm^2. OTA was dissolved in methanol as a 100 mmol/l stock solution, stored at −20 °C, and further diluted in DMEM medium for individual experiments.

2.3. Cytotoxicity and ROS production

To determine OTA-induced cytotoxicity, LLC-PK1 cells (in 24-well plates) were incubated for 24 h with increasing concentrations of OTA ranging from 1 to 100 μmol/l in medium with or without FCS. The viability was measured via neutral red assay. In this assay, the ability of viable cells to incorporate neutral red into lysosomes is photometrically measured at 540 nm (Borenfreund and Puerner, 1985; Valacchi et al., 2001). Viability is expressed as percent of control cells; solvent controls were treated with 0.1% methanol only.

The OTA-induced generation of reactive oxygen species (ROS) was measured by using the fluorescent probe dihydrodichlorofluorescein (H_2DCF-DA). Confluent LLC-PK1 cells (12-well plates) were pre-treated for 30 min at 37 °C with 20 μmol/l H_2DCF-DA, which is intracellularly hydrolysed by esterases to form non-fluorescent H_2DCF and then rapidly oxidised to highly fluorescent DCF in the presence of ROS. Cells were then washed with phosphate buffered saline (PBS) and incubated with increasing concentrations of OTA (0, 0.5–2.5 μmol/l) for 1.5 h in FCS-free medium. Medium was discarded, the cells resuspended in PBS and fluorescence intensity measured in 10,000 cells of each sample via flow cytometry (FACS Calibur, Beckton Dickinson). In preliminary experiments flow cytometry was found superior compared to the fluorescent plate reader protocol since the latter often produces unspecific and high background signals. Additional incubations of OTA for 24 h and in concentrations higher than 2.5 μmol/l were performed but they did not result in further enhancement of ROS levels as compared to control cells (data not shown). The amount of intracellular ROS generation, paralleled by an increase in fluorescence intensity, was calculated as % of control cells.

2.4. Relative expression of mRNA by real-time PCR

We studied the effect of OTA on the transcription level of selected genes encoding for proteins involved in antioxidant defence and detoxification. Cells (in 6-well plates) were incubated with OTA at different concentrations (0, 2.5, 10 and 25 μmol/l) for 24 h. Cells were then washed with PBS, scraped and centrifuged after transfer into a falcon tube. The supernatant was discarded and the resulting pellet resuspended in RLT lysis buffer including ß-mercaptoethanol. The samples were further processed for RNA-isolation (RNeasy kit, Qiagen, Hilden, Germany). Total RNA was quantified photometrically at 260 nm and quality confirmed by gel electrophoresis. Primers for porcine genes were designed by standard procedures (Primer3, NCBI BLAST) and purchased from MWG (Ebersberg, Germany). Primer sequence information is given in Table 1. Real-time PCR was performed as a one-step method using QuantiTect SybrGreen Kit from Qiagen (Rotorgene RG-3000) and gene expression standardized to the expression level of the housekeeping gene, 18S RNA. Results are calculated as % of controls.

Table 1
Primer sequences and conditions for real-time PCR experiments

Primer	Forward primer	Reversed primer	Annealing temp. (°C)
18S RNA	AGTCGGCATCGTTTATGGTC	CCGCAGCTAGGAATAATGGA	58
GST	CCTCCTATGGATGCGAAAAA	ATGATATTGCGTGCGAACAA	58
GPX	GAACTGAATGCACTGCAGGA	GTTCACGTCCCCTTTCTCAA	58
CAT	CAGCTTTAGTGCTCCCGAAC	AGATGACCCGCAATGTTCTC	58
SOD	AAAGGACTGGCTGAAGGTGA	CCAATGATGGAATGGTCTCC	58

2.5. Enzyme activity measurements

LLC-PK1 cells, grown in 10 cm plates, were treated with OTA for 24 h (0, 2.5, 10, 25 μmol/l). They were washed with PBS, detached by scraping, centrifuged, and the resulting cell pellet stored at −80 °C. Cell pellets were thawed, resuspended in 300 μl lysis buffer (PBS, 1 mmol/l EDTA), homogenized and centrifuged (12,000*g*, 4 °C, 10 min). The resulting supernatants were used for activity measurements. Activity of SOD was determined by inhibition of pyrogallol autoxidation according to Marklund and Marklund (1974). GPx, which catalyses the reduction of hydrogen peroxide to water, was measured in an indirect assay based on the method of Lawrence and Burk (1976). CAT activity measurement was performed according to Johansson and Borg (1988), a method which determines the production of formaldehyde (from methanol as a hydrogen donor) with the chromogen Purpald. The method of Habig and Jakoby (1981) was applied to measure GST in cell supernatants by means of glutathione conjugation rate with the substrate CDNB (1-chloro-2,4-dinitrobenzene). The protein content was determined by BCA Protein Kit (Pierce, Illinois, USA).

2.6. Reporter gene studies

To evaluate the OTA effect on the activity of ARE, a plasmid ARE-luc, containing the ARE sequences driving the expression of luciferase was used (it was a kind gift from J.A. Johnson, University of Wisconsin, Madison, Wisconsin, USA). For measuring activity of AP-1, a construct containing four tandem copies of the AP-1 enhancer driving the secreted alkaline phosphatase gene (pAP1-SEAP) was purchased from Clontech. Cells growing in 24-well plates were transiently transfected with 0.5 μg of the vector by SuperFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Twenty-four hours later, cells were stimulated with varying concentrations of OTA (0, 2.5, 10 and 25 μmol/l). The activity of luciferase was determined in cell lysates and SEAP in cell culture media at 24 h after stimulation. Values of chemiluminescent luciferase measurement were normalised for total cell protein. Chemiluminescent SEAP assay was done according to the manufacturer's protocol (Clontech) with a modification. The samples of cell culture media (7.5 μl) were combined with dilution buffer (12 μl) in microtiter plates for chemiluminescent assays and heated at 65 °C for 30 min. After this time assay buffer (15 μl) and substrate (15 μl) were added and SEAP activity was detected by plate chemiluminometer (EG&G Berthold, Bad Wildbad, Germany). In addition, ARE-luc transfected cells were incubated for 24 h with 25 μmol/l ochratoxin B (OTB), the dechlorinated OTA analogue as well as with 25 μmol/l ochratoxin α (OTα). The concentration of OTB and OTα used were not cytotoxic.

2.7. Statistical analysis

Statistical calculations were performed with SPSS. The effect of different concentrations of OTA on individual parameters was analysed by analysis of variance (ANOVA) and Dunnett's post hoc test. Comparison of two groups (of means) was done with Student's *t*-test. Data represent means with SEM of at least three experiments and subsequent cell passages. Within each experiment, two to three wells were treated identically. The exact number of replicates is given in the respective figure legends.