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ScienceDirect
Toxicology and Applied Pharmacology 224 (2007) 174–181
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

MAPK-ERK activation in kidney of male rats chronically fed ochratoxin A at a dose causing a significant incidence of renal carcinoma

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Received 21 November 2006; revised 16 May 2007; accepted 26 June 2007
Available online 3 July 2007

Abstract

Kidney samples of male Fischer 344 (F-344) rats fed a carcinogenic dose of OTA over 7 days, 21 days and 12 months were analysed for various cell signalling proteins known to be potentially involved in chemical carcinogenicity. OTA was found to increase the phosphorylation of atypical-PKC. This was correlated with a selective downstream activation of the MAP-kinase extracellular regulated kinases isoforms 1 and 2 (ERK1/2) and of their substrates ELK1/2 and p90RSK. Moreover, analysis of effectors acting upstream of PKC indicated a possible mobilisation of the insulin-like growth factor-1 receptor (lGFr) and phosphoinositide-dependent kinase-1 (PDK1) system. An increased histone deacetylase (HDAC) enzymatic activity associated with enhanced HDAC3 protein expression was also observed. These findings are potentially relevant with respect to the understanding of OTA nephrocarcinogenicity. HDAC-induced gene silencing has previously been shown to play a role in tumour development. Furthermore, PKC and the MEK-ERK MAP-kinase pathways are known to play important roles in cell proliferation, cell survival, anti-apoptotic activity and renal cancer development.

Keywords: Ochratoxin A; PKC-ζ; MAP kinases; Cell signalling; Nephrocarcinogenicity

Introduction

The mycotoxin ochratoxin A (OTA) is a naturally occurring fungal metabolite produced by several *Aspergillus* and *Penicillium* species. Consequent on its widespread occurrence in food, continuous exposure to measurable levels has been observed in humans and some data suggests a possible role for dietary OTA in the development of specific kidney diseases and urinary tract tumours (Fink-Gremmels, 2005). However, the available epidemiology is insufficient for assessing the significance of OTA in food as a potential risk to human health (Fink-Gremmels, 2005). Risk evaluation therefore relies mainly on the use of toxicological data obtained through animal experimentation, in which OTA has been shown to produce a wide array of toxicological effects, including nephrotoxicity, nephrocarcinogenicity, neurotoxicity and immunotoxicity (WHO, 2001; O'Brien and Dietrich, 2005; EFSA, 2006).

It is widely acknowledged that the risk assessment of dietary OTA based on available animal data would significantly benefit from the elucidation of the mechanisms of carcinogenicity (Fink-Gremmels, 2005). Although genotoxicity is likely to play a role in OTA carcinogenicity, the actual molecular mechanism of action, either directly through the formation of covalent DNA adducts or indirectly through epigenetic pathways is still unknown and highly debated (Turesky, 2005; Manderville, 2005; Fink-Gremmels, 2005). Several modes of action relevant as potential epigenetic mechanisms of OTA carcinogenicity have been reported and include protein synthesis inhibition, oxidative stress and alterations of cell signalling (Schilter et al., 2005; EFSA, 2006). It is generally recognised that some

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0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2007.06.014

toxicities may induce cell regeneration and proliferation, which may subsequently lead to cell transformation and tumour development.

In a recent study, OTA was administered to male rats for up to 2 years. Renal tumours, mainly carcinomas, were discovered during the last 6 months (Mantle et al., 2005). Gene expression profile was studied in groups of animals from this study, taken at intervals from 7 days to 12 months, and a disruption of pathways regulated by the transcription factors hepatocyte nuclear factor 4 alpha (HNF4α) and nuclear factor-erythroid 2-related factor 2 (Nrf2) was the most striking effect observed in the kidney (Marin-Kuan et al., 2006). The disruption of the Nrf2 pathway was characterised by an inhibition of Nrf2 binding to the antioxidant responsive element (ARE) promoter, resulting in a reduction in the expression of downstream genes. Since many Nrf2-regulated genes are involved in antioxidant defence, the final consequence of this effect was an increase in oxidative DNA damage (Schilter et al., 2005; Cavin et al., 2007).

Only limited published data was available on mechanisms potentially responsible for the inhibition of Nrf2- and HNF4α-regulated pathways. Some evidence indicated a role for atypical protein kinase C zeta (PKC-ζ) (Hashimoto et al., 2005; Bloom and Jaiswal, 2003; Numazawa et al., 2003; Roy et al., 2001). In addition, histone deacetylases (HDACs), a group of enzymes involved in gene silencing, were shown to inhibit the expression of genes modulated by HNF4α (Hirai and Chida, 2003). In the present study, the potential effects of OTA on PKC-dependent pathways and on the activity of HDAC enzymes were investigated.

Materials and methods

Ochratoxins. As previously described (Mantle et al., 2005), standardised OTA production was performed by growing *A. ochraceus* isolate D2306 (Harris and Mantle, 2001) in shaken solid substrate fermentations at 28 °C for 2 weeks to yield a product containing 5–6 mg OTA/g. More specifically, 40 g of sterilised shredded wheat (Cereal Partners UK, Welwyn, UK) in 500 ml Erlenmeyer flasks was inoculated with a concentrated spore suspension in water (16 ml) and the flasks shaken at 200 rpm and 10 cm eccentric throw. An aliquot of each fermentation was assayed for OTA concentration by HPLC with diode array detection (Harris and Mantle, 2001). Batches of fermentation product contained ochratoxin B (OTB) equivalent to 5–10% of the amount of OTA. No other mycotoxins (e.g. penicillic acid, citrinin) were biosynthesised in this fermentation (Mantle et al., 2005).

Animal treatment. Male Fischer 344 (F-344) rats (B. & K. Universal Ltd., Hull, UK) were administered OTA in diet given daily over 2 years (Mantle et al., 2005; Marin-Kuan et al., 2006). As from their weight of ~175 g, daily dietary intake of OTA was 300 μg/kg bw but was held at 100 μg/rat after animals reached 333 g. Throughout the study, animals were housed in cages on absorbent paper under tightly controlled conditions (21±1 °C, 55±10% relative humidity, air-exchange, 12 h light–dark cycle). Animal growth and welfare were monitored by regular weighing and daily surveillance. For purpose of the present study, time points were selected to represent early (7 and 21 days) and later (12 months) response. At each time point, 4 control and 4 treated animals were randomly chosen for tissue harvest. Kidneys from these rats were immediately snap-frozen in liquid nitrogen. All handling and procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Tissue homogenates. Briefly, for phosphorylation analysis, tissues were homogenised using the FastPrep system (Q.BIOgene, Germany) with RIPA buffer consisting of 150 mM Tris–HCl, pH 8.0 containing 1% (vol/vol) Igepal CA630, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS (Sigma). Protease inhibitor cocktail and phosphatase inhibitor cocktail I and II were added at the manufacturer's recommended concentrations (Sigma). The homogenates were incubated on ice for 5 min followed by centrifugation at 8000×*g* for 10 min at 4 °C. Supernatant containing the soluble protein was recovered for further analysis. For protein expression analysis, homogenates were prepared by sonication of the tissues for 5 sec in 10 mM Tris–HCl lysis buffer, pH 7.4 (Merck), followed by centrifugation at 10,000×*g* for 5 min. Protein concentration was determined with a BioRad Protein assay kit (Bio-Rad, Richmond, CA).

Cell signalling analysis and protein expression by Western blotting. Cell signalling response was evaluated following carefully the Western blot instructions from the antibodies manufacturer (Cell Signalling Technology, Inc.). Using the MiniCell XCellSureLock system (Invitrogene Ltd., Paisley, UK), 15 μg of protein, mixed with a pre-stained standard marker (SeaBlue® Plus 2), was loaded in the NuPAGE® NOVEX Bis–Tris Pre-Cast Gel 4–12% System (Invitrogene). Protein transfer onto a PVDF transfer membrane (Invitrogene) was carried out at 30 V for 1 h. Transferred membranes were first probed with antibodies at the manufacturer recommended dilution in 1× TBS containing 5% BSA, 0.1% Tween-20 at 4 °C under gentle shaking, overnight. Antibodies directed against phosphorylated and total phosphoinositide-dependent kinase-1 (PDK1), phospho-PKCs (Phospho-PKC sampler kit), total atypical protein kinase C (PKC-ζ) , phosphorylated and total p38 mitogen-activated protein kinase (p38), phosphorylated and total SAP/JNK NH2-terminal kinase (SAP/JNK), phosphorylated and total ETS-domain protein Elk1 (Elk-1), phosphorylated and total ribosomal-S6 primary antibodies, insulin growth factor receptor (IGFr), epidermal growth factor receptor (EGFr) and histone deacetyltransferases (HDAC 1,3,4,5,6 and 7) were applied. All antibodies were purchased from Cell Signalling Technology, Inc. except the ones against phosphorylated and total extracellular signal-regulated kinase 1 and 2 (ERK1/2), which were obtained from Abcam (Abcam, UK). Secondary probing was performed with the horseradish peroxidase-linked HRP-conjugated goat anti-rabbit IgG antibody in 1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk with gentle agitation for 1 h at room temperature. Final washing was repeated 3 times for 5 min each with TBS 0.1% Tween-20. Chemiluminescence of the immunoblots was detected using ECL solution (Amersham). Membranes were wrapped in polythene film and exposed to Kodak films. Chemiluminescence of specific protein bands was quantified using the Advance Image Data Analyser (AIDA-raytest, Germany). Phosphorylation is presented as ratio of quantified phosphoprotein and total protein. Statistical analysis of phosphoprotein was performed by application of Student's *t* test.

Histone deacetylase activity (HDAC). Homogenates from kidney tissue (50 mg) were prepared using the FastPrep system (Q.BIOgene, Germany). Nuclear proteins were extracted using the compartmental protein extraction kit (BioChain Institute, Inc., CA) following the manufacturer's instructions. Deacetylase activity was determined in kidney extracts (10 mM Tris–HCl buffer, pH 7.4 (Merck)) using a fluorometric detection kit in accordance with manufacturer's instructions (Upstate). Statistical analysis of measured HDAC activities was performed by application of Student's *t* test.

Results

OTA was administered to male rats for 2 years as described. The effect of OTA treatment on phosphorylation mediated cell signalling was investigated at different time points by measuring both the expression and activation (phosphorylated) status of relevant proteins.

As shown in Fig. 1, at all time points the level of phospho-PKC-ζ (atypical-PKC) was increased in OTA-treated animals as compared to controls. Baseline expression of total PKC-ζ