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Autor: Cavin, Christophe / Marin-Kuan, Maricel / Scholz, Gabriele
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Mechanism of action: a key element for ochratoxin A risk assessment*

Christophe Cavin, Maricel Marin-Kuan, Gabriele Scholz, Benoît Schilter, Thierry Delatour
Nestlé Research Center, Quality and Safety Department, PO Box 44,
Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

Introduction

Ochratoxin A is a naturally occurring mycotoxin produced by several species of *Aspergillus* and *Penicillium* fungi. As a consequence of its widespread occurrence in a variety of food commodities such as cereals, coffee, cocoa, wine, and dried fruits products, the human population is continuously exposed to OTA (1, 2). In animal models, OTA was shown to produce a wide array of toxicological effects, including nephrotoxicity, neurotoxicity, immunotoxicity and carcinogenicity (1). In an NTP-carcinogenicity bioassay, OTA administration was associated with an increased incidence of renal tumours in male rats while females appeared much less susceptible (1). Similar effects were observed in mice, although at much higher doses (1, 3). The actual effect of dietary exposure to OTA in human remains unclear. Data suggest a possible role for OTA in the development of specific kidney diseases (e.g. the Balkan endemic nephropathy (BEN) and urinary tract tumours, however such a direct correlation has not been demonstrated yet. Up to now epidemiological studies have not allowed the assessment of the actual health impact of OTA exposure in humans (4, 5). In this context, risk evaluation relies mainly on the use of toxicological data obtained through animal experimentation.

Mechanisms of OTA toxicity and carcinogenicity

Renal carcinogenicity is considered the highest health concern raised by OTA exposure and has been therefore assumed has the pivotal effect (1). The mechanism of OTA carcinogenicity in animal models has not been fully characterized. Its elucidation would allow the most appropriate risk evaluation procedure to be applied to address the safety significance of OTA contamination of foods. Direct acting genotoxin (which binds directly to DNA) are assumed to act through a non-threshold

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mechanism triggering the application of a low-dose modeling for quantitative risk assessment. Epigenetic effects are normally assumed to act through a threshold mechanism indicating the use of an approach based on the application of uncertainty factors to the pivotal animal No Observed Adverse Effect Level (NOAEL) for safety assessment. There is currently significant scientific debate on the mechanism involved in OTA carcinogenesis.

A) Advance on the potential direct genotoxicity of OTA

Evidence based on various experimental approaches such as comet assay, micronucleus assay, abasic sites, and ^{32}P -postlabelling indicates that OTA produces DNA damage (1, 3). Mutagenicity studies appeared inconsistent since they led to either negative or positive responses to OTA. Some authors have argued that OTA acts through a direct genotoxic mechanism. This was based on the use of the ^{32}P -postlabelling method, showing that OTA treatment resulted in the formation of spots in mice, rats and monkeys, which were interpreted as OTA derived DNA-adducts (6, 7). It was found that the level of spots in male rats was higher than in females, suggesting a correlation between the incidence of DNA adducts and the well known sex-dependent carcinogenicity of OTA in rats. This approach was also applied in the mouse. Formation of spots, rationalized in terms of OTA-guanine adducts, was observed. However, it was shown that the incidence and number of spots was much higher than that previously reported with rats, contrasting with the known relative *in vivo* sensitivities of mice and rats for OTA-mediated carcinogenicity. Using radiolabelled OTA, no direct DNA-binding of OTA moieties have been found (8–10). The administration of rats with [^3H]-OTA (1000 mCi/mmol, 210 $\mu\text{g}/\text{kg}$ b.w.) did not reveal any covalent binding with DNA when the treatment was performed over 24 h, with detection limits in assay of <1.3 adducts per 10^{10} nucleotides in kidney and <5.6 adducts per 10^{11} nucleotides in the liver. Similarly, the incubation of rat kidney S-9 with NADPH in the presence of OTA did not reveal any detectable DNA-binding (detection limit at 0.2 adduct per 10^7 nucleotides). These data further confirmed results from studies showing that rat microsomes (liver and kidney) or human cytochromes P450 exhibited either no or low activity toward OTA. Overall the available data strongly indicate that OTA is poorly metabolized and is unlikely to involve electrophilic intermediates (8–9, 11). To further investigate the potential binding of OTA to DNA, the radiocarbon content of DNA isolated from liver and kidney male rats treated with a single dose of [^{14}C]-OTA (0.25 mCi/mmol, 500 $\mu\text{g}/\text{kg}$ b.w.) was measured by accelerator mass spectrometry (detection limit at 3 adducts per 10^9 nucleotides), and no difference was observed between control and treated animals. However, the photoirradiation of a mixture OTA-2'-deoxyguanosine in aqueous solution was reported recently to give rise to two guanine-OTA adducts (12, 13). One of them, comprehensively characterised by NMR and MS, exhibited a covalent bond between the carbon C-8 of the guanine and the carbon C-5 of the coumarin moiety of OTA. This adduct was

shown to co-migrate on TLC plates with a spot observed in DNA of OTA-administered rats and pigs by ^{32}P -postlabelling. Additional analytical work is still necessary to definitely demonstrate the actual formation of this adduct *in vivo* and establish its biological significance.

B) Putative epigenetic mechanisms of OTA

Protein synthesis inhibition is thought to be a major mode of OTA toxicity (14). The exact molecular mechanism involved is not known. It is currently thought that the mechanism may involve constraint of peptide elongation through competition with phenylalanine in the reaction catalysed by phenylalanyl-tRNA synthetase (14). This hypothesis was supported by *in vivo* and *in vitro* data showing that co-administration of phenylalanine with OTA prevented the OTA-mediated protein synthesis inhibition (14). Many studies have indicated that inhibition of protein synthesis is likely to be involved in most of the acute toxic effects of OTA. When injected simultaneously, phenylalanine prevented OTA lethality (15), immunotoxicity (16) and teratogenicity (17). The role of protein synthesis inhibition in effects observed at lower doses is less obvious. There is currently no *in vivo* study available which directly addresses protein synthesis inhibition at chronic doses producing kidney tumours.

The nephrotoxic potential of OTA may be related to mitochondrial dysfunction leading to energy shortage and production of oxygen reactive species. The treatment of isolated renal proximal tubules with large concentrations of OTA resulted in an inhibition of mitochondrial respiration (18). Other *in vitro* studies applying direct treatments of mitochondrial preparations found that OTA affected respiration and oxidative phosphorylation through an impairment of the mitochondrial membrane and an inhibition of the succinate-supported electron transfer activities of the respiratory chain (19). In addition, ATP synthesis in mitochondria isolated from renal cortex was significantly inhibited by micromolar concentrations of OTA (20). In contrast to the data outlined above, nanomolar concentrations of OTA produced a stimulation of mitochondrial activity in cell cultures (21). Based on the data available, a role for mitochondrial dysfunctions in OTA toxicity and carcinogenicity is possible although more research will be necessary to clarify its relevance at chronic, low dose exposure *in vivo*.

Several reports have suggested a potential role for oxidative stress in OTA toxicity and carcinogenicity (22–24). Several *in vivo* and *in vitro* studies have shown that OTA induced oxidative damage. For example, an increased formation of the lipid peroxidation product malondialdehyde (MDA) was observed in rats treated orally with 120 $\mu\text{g}/\text{kg}$ bw/day of OTA over 60 days (24). Moreover, a 24 h treatment of male rats at an OTA dose of 1 mg/kg led to a 22% decrease in the plasma level of α -tocopherol (8). Moreover, antioxidants were shown to prevent OTA-mediated increase in MDA production *in vitro* (25) and *in vivo* (26) and the injection of superoxide dismutase and catalase provided protection against OTA-induced

nephrotoxicity in rats. In a few studies, oxidative damage by OTA were linked to DNA damage. In cell culture, an OTA-dependent increase in DNA damage (such as formation of 8-oxoguanine) was correlated with a production of reactive oxygen species (23, 27) and in mice, antioxidant vitamins reduced OTA-mediated increase in chromosomal aberrations (28) and the formation of ^{32}P -postlabelling spots (29). However, OTA-mediated oxidative damage and DNA damage were not always corroborated. In an other study, treatment of male rats with OTA (up to 2 mg/kg bw administered by gavage) did not increase the formation of biomarkers of oxidative damage such as the lipid peroxidation marker malondialdehyde (MDA) in plasma, kidney and liver, or the DNA damage marker 8-oxo-7,8-dihydro-2'-deoxyguanosine in kidney DNA (8). In contrast, in this last study a significant increase in the expression of the marker of oxidative stress response HSP 32 was observed specifically in the kidney and not in the liver. Altogether these data suggest that production of oxygen radicals from reactions involving directly OTA is quite weak, generating only low level of oxidative stress under chronic exposure. This may explain some inconsistencies found in the different *in vitro* and *in vivo* studies.

C) New plausible mechanism of OTA carcinogenicity from toxicogenomic data

In a recent study, OTA was administered to male rats for 2 years (at 300 $\mu\text{g}/\text{kg}$ bw up to a weight of 333 g, and then 100 $\mu\text{g}/\text{rat}$). The dose administered produced a renal tumor incidence of 25 % after 2-years (30). Gene expression profile was studied in groups of animals from this study, taken in intervals from 7 days to 12 months (Marin-Kuan). Surprisingly, in kidney samples, many genes expected to be induced by oxidative stress were actually significantly down regulated by OTA in the kidney but not in the liver. Importantly many of these genes were found to contain an Antioxidant Regulatory Element (ARE) in their promoter region. The ARE-motif is recognized by the Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a member of the "Cap-n-Collar" family of basic-region leucine zipper transcription factors (31, 32). Nrf2 is involved in both the basal expression and the induction of many genes including genes encoding for detoxification, cytoprotective and antioxidant enzymes (31, 33). Taken together, these data suggested the hypothesis that OTA might inhibit the Nrf2-mediated gene expression pathway. Considerable biological consequence would be anticipated from such an effect. Inhibition of Nrf2 might be expected to impair the antioxidant defense of the target kidney cells making them more susceptible to chronic low level of oxidative stress and damage.

In the same study, gene expression profile showed a specific disruption in kidney cells of an other pathway regulated by the transcription factor hepatocyte nuclear factor 4 alpha (HNF4 α). Previous data have suggested that a reduction of HNF4 α pathway may be associated with kidney carcinogenicity (34). Moreover, recent data suggest that repression of HNF4 α may be indirectly linked to a specific signaling cascade of mitogen-activated protein kinases (MAPKs), involving the

extracellular regulated kinases 1 and 2 (ERK1/2). ERK1/2 is generally thought to regulate cell proliferation and it has been implicated in cancer development including renal carcinoma (35). Interestingly, OTA was found to increase the phosphorylation and activity of ERK1/2 in Madin-Darby canine kidney-C7 cells (MDCK-C7, from collecting duct) and in renal proximal tubule cell lines (OK cells, NRK-52E) (36, 37). To date, no study has addressed the effects of OTA on MAPKs *in vivo*. Therefore, some more work is required to clarify the exact role of these pathways on the carcinogenicity of OTA.

Conclusion

There is significant debate within the scientific community on the use of the rat carcinogenicity data to assess the potential carcinogenic risk associated with human exposure. The question of a direct genotoxicity of OTA which would trigger the use of a conservative linear dose response modelling approach for risk assessment is still open. However up to now, the actual formation of a specific OTA DNA adduct has not been demonstrated *in vivo*. The observed OTA-mediated DNA damage (e.g. ³²P-postlabelling lesions, clastogenicity) may not necessarily require direct OTA-DNA binding, but they may also be related to other, indirect mechanisms involving possible epigenetic modes of actions such as oxidative stress, inhibition of protein synthesis and cytotoxicity. Although data reported no drastic oxidative stress response following OTA treatment, low level of chronic oxidative radicals has already been associated with carcinogenesis (38). Recent data suggest that oxygen radicals may also result from normal oxidative metabolism due to an OTA-mediated reduction of the antioxidant cellular defense in the target kidney cells. This may be a plausible mechanism of OTA nephrocarcinogenicity. This mechanism does not involve direct binding of OTA to DNA and is therefore thresholded. Of relevance for risk assessment, it would trigger the use of a health based guidance value established by the application of uncertainty factors to the NOAEL obtained in animal studies (1).

Summary

Ochratoxin A (OTA) is a mycotoxin occurring naturally in a wide range of food commodities resulting into continuous exposure of the human population. The health significance of this exposure is highly debated. It depends upon the mechanism of OTA-toxic action, which has not been elucidated yet. One of the key features of OTA is its strong selectivity for the kidney. In rodents, OTA has been shown to be nephrotoxic and nephrocarcinogenic. Risk evaluation for humans is dependent on the use of the rat carcinogenicity data and upon the mechanisms of action involved. The observed OTA DNA-damage could have been generated via direct OTA-DNA binding however, they may also be related to indirect mechanisms involving epigenetic modes of actions. In this short review, the putative mechanisms of OTA carcinogenicity has been briefly discussed and the implication for OTA risk assessment has been addressed.

Zusammenfassung

Ochratoxin A (OTA) ist ein Mycotoxin, das in den verschiedensten Lebensmitteln natürlicherweise vorkommt, und dem die Bevölkerung kontinuierlich ausgesetzt ist. Die Bedeutung dieser Exposition für die Gesundheit ist Gegenstand intensiver Diskussionen. Sie ist abhängig vom Mechanismus, welcher der Toxizität zugrunde liegt, und der bislang nicht vollständig geklärt ist. Eines der Hauptmerkmale der OTA-Toxizität ist ihre Selektivität für die Niere. In Nagern erwies sich OTA als nierentoxisch und nierenkarzinogen. Die Abschätzung des gesundheitlichen Risikos für den Menschen basiert auf den Ergebnissen von Langzeit-Karzinogenesestudien in Ratten und dem der Karzinogenität zugrundeliegenden Mechanismus. Die beobachtete Schädigung der DNA durch OTA könnte auf einer direkten DNA-Interaktion basieren, sie könnte jedoch auch mit einem indirekten Mechanismus verbunden sein, der auf einer epigenetischen Wirkungsweise beruht. Dieser Übersichtsartikel beschreibt die möglichen Mechanismen der OTA-Karzinogenität und diskutiert deren Bedeutung für die Abschätzung des gesundheitlichen Risikos für den Menschen.

Résumé

L'ochratoxine A (OTA) est une mycotoxine naturellement présente dans une large gamme de denrées alimentaires qui conduit la population humaine à une exposition permanente. L'impact de cette exposition sur la santé est vivement débattu car il repose sur la connaissance du mode d'action de la toxicité de l'OTA qui, à ce jour, n'a toujours pas été élucidée. Toutefois, l'accumulation d'OTA dans le rein a été clairement établie et, chez certains rongeurs, son action néphrotoxique et néphrocancérogène a été démontrée. Chez l'Homme, l'évaluation du risque repose sur les données de cancérogénèse chez le rat ainsi que sur les mécanismes d'action potentiels de l'OTA. Certains travaux suggèrent que les dommages infligés à l'ADN par l'OTA proviendraient de la formation d'adduits entre l'OTA et des nucléobases mais, dans l'état actuel des connaissances, de tels dommages sont également attribuables à une action indirecte mettant en jeu des mécanismes épigénétiques. Dans cette brève revue des mécanismes de l'OTA susceptibles de provoquer une cancérogénèse, l'évaluation du risque afférent à la consommation d'OTA dans les aliments est abordée.

Key words

Mycotoxin, ochratoxin A, oxidative stress, carcinogenicity, genotoxicity, nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)

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Corresponding address: Christophe Cavin PhD., Nestle Research Center,
P.O. Box 44, Vers-chez-les Blanc, CH-1000 Lausanne 26, Switzerland,
Phone +41 21 785 80 99, Fax +41 21 785 85 53, E-mail: christophe.cavin@rdls.nestle.com