

Original Scientific Paper

THE EFFECT OF OCHRATOXIN A ON THE CONCENTRATION OF PROTEIN CARBONYLS IN RATS

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The mechanism of ochratoxin A (OTA) toxicity has been found to involve the production of free radicals and consequent oxidative stress. Until now, the only studied pathway of OTA-caused oxidative damage was peroxidation of lipids. The aim of this study was to check whether OTA induced the production of protein carbonyls, markers of protein oxidation. Exposed rats (5 per group) were given daily OTA (0.5 mg kg⁻¹ b. w., i. p.) for 7 days, 14 days, or 21 days, and control rats solvent only (TRIS). All animals were killed 24 hours after the last treatment. The levels of OTA in plasma, kidney and liver homogenates increased gradually during the whole length of the experiment. The levels of protein carbonyls in kidney homogenates of OTA-treated rats was significantly higher after days 14 and 21 than in controls ($P < 0.05$). The levels of protein carbonyls in the liver was significantly higher in OTA-treated animals only after day 21 ($P < 0.05$). These results confirm that oxidative stress is involved in the mechanism of OTA toxicity, and that it causes the oxidation of proteins.

KEY WORDS: *free radicals, kidney, lipid peroxidation, liver, mycotoxins, oxidative stress*

Ochratoxin A (OTA) is mycotoxin that naturally contaminates food and feed in all climatic conditions. The main target of OTA toxicity and carcinogenicity in laboratory animals is the kidney. It is supposed to be involved in the aetiology of endemic nephropathy and urothelial tumours that appear in patients with endemic nephropathy with higher incidence and different clinical picture than in other individuals (1).

The mechanism of OTA toxicity is not fully understood, but the proposed mechanism includes inhibition of mitochondrial respiration correlated with a depletion of ATP, inhibition of protein synthesis, and enhanced lipid peroxidation. Furthermore, it is not clear whether OTA carcinogenicity is the result of its direct genotoxic effect or of indirect mechanisms such as induction of cytotoxicity and oxidative damage (2).

Oxidative damage is caused by free radicals, more specifically reactive oxygen species (ROS) that are continuously produced in normal physiological processes (3). Oxidative stress is defined as the imbalance between ROS production and natural antioxidants in biological systems, which leads to the damage of macromolecules such as lipids, proteins, carbohydrates, RNA, and DNA.

The increased OTA-induced ROS production was proven on cultured proximal tubule cells and in bacteria (4, 5). *Rahimtula et al.* (6) reported that OTA enhances lipid peroxidation *in vitro* and *in vivo*. It was also found on Vero cells that OTA induces the formation of malondialdehyde (MDA), one of the final products of lipid peroxidation, in concentration-dependent manner (7). It was reported that a single dose of OTA (0.3 mg kg⁻¹, 1 mg kg⁻¹, or 2 mg kg⁻¹ b. w.) did not increase the concentration of MDA in

plasma, kidney and liver of rats (8). *Meki and Hussein* (9) found that the concentration of MDA increases significantly in the serum, liver and kidney of rats receiving oral OTA doses ($0.25 \text{ mg kg}^{-1} \text{ b. w.}$ per day for four weeks). Our earlier study showed that multiple OTA dosing increased MDA concentrations in urine of rats (10).

Oxidative stress affects side amino acid groups of proteins, forming protein carbonyls that are widely used as markers of protein oxidation (11). Protein carbonyls can be measured using 2,4-dinitrophenylhydrazine that reacts with carbonyls to produce corresponding hydrazone, which can be analysed optically. The possible production of carbonyl derivatives in OTA-treated animals has not been studied by now.

The purpose of this study was to check whether OTA-induced oxidative stress involved the formation of carbonyl proteins and to see the time course of carbonyl protein formation in rat kidney and liver tissue after multiple doses of OTA.

MATERIAL AND METHODS

Chemicals

OTA (99 % purity), EDTA, and guanidine HCl were purchased from Sigma Chemicals (St. Louis, MO, USA). Ethanol, ethyl acetate, trichloroacetic acid (TCA), hydrochloric acid (HCl), NaCl, KH_2PO_4 , K_2HPO_4 , TRIS, KCl, and 2,4-dinitrophenylhydrazine (2,4-DNPH) were from Kemika (Zagreb, Croatia).

Water (Merck, Darmstadt, Germany) and methanol (Kemika, Zagreb) used for HPLC mobile phase were of HPLC grade, and all other chemicals were of *pro analysis* grade.

Animal assay

Adult female Wistar rats (190 g of weight) were kept in macrolone cages. Animals were fed on a standard diet for laboratory rodents (Pliva, Zagreb) and had free access to water.

The rats were randomised in six groups, each of five animals. The animals were receiving daily intraperitoneal (i. p.) doses of either with OTA dissolved in TRIS buffer ($0.5 \text{ mg kg}^{-1} \text{ b. w.}$) or the solvent only (control animals) for up to three weeks.

Twenty-four hours after the days 7, 14, and 21 of treatment, the animals were killed with natural gas. Blood samples were obtained directly from the heart in heparinised test tubes. Plasma was separated by

centrifugation and stored at -20°C until analysis. Liver and kidneys were collected, washed in physiological solution, and weighed. Prior to analysis kidney and liver samples were kept frozen at -20°C .

OTA assay

The concentration of OTA in plasma, kidney, and liver homogenate (10 % in saline) was determined by high performance liquid chromatography (HPLC) according to the methods of *Peraica et al.* (12) and *Bauer and Gareis* (13).

Protein carbonyl assay

Protein carbonyl content was determined in kidney and liver homogenates by measuring the reactivity of carbonyl groups with 2,4-DNPH, as described elsewhere (14). Tissue homogenates (10 %) were prepared using phosphate buffer (pH 7.4) containing 1.15 % KCl and 3 mmol L^{-1} EDTA. EDTA was added to prevent further formation of free radicals. All analyses were performed in duplicate. Each sample of homogenate was divided in three aliquots of 0.5 mL. Proteins were precipitated with 10 % TCA and centrifuged at 2000 g for 10 minutes. One pellet was re-suspended with 1 mL of 2 mol L^{-1} HCl (blank), and other two pellets (duplicates) with an equal volume of 0.2 % 2,4-DNPH in 2 mol L^{-1} HCl. All samples were incubated at room temperature for one hour. The samples were precipitated with 10 % TCA and centrifuged as before. The pellets were then washed twice with 1.0 mL of ethanol:ethyl acetate (1:1) to eliminate traces of 2,4-DNPH. Proteins were finally dissolved in 2.0 mL of 6 mol L^{-1} guanidine HCl, and centrifuged as before. Protein concentration was calculated at 280 nm in the HCl blank using BSA in 6 mol L^{-1} guanidine HCl as standard. Carbonyl concentration was determined from the absorbance at 370 nm with the use of a molar absorption coefficient of $22.0 \text{ L mmol}^{-1} \text{ cm}^{-1}$. The results were expressed as nmol of 2,4-DNPH bound on mg of protein.

Statistics

Differences between treated and control groups were determined using the two-tailed Student's *t*-test for independent samples. Probability values $P < 0.05$ were considered statistically significant. A computer program Statistica 5.5 was used for statistical analysis.

RESULTS

During the experiment, treated animals showed no change in body weight in comparison to control animals.

The OTA levels in plasma ($\mu\text{g mL}^{-1}$) and kidney and liver homogenates ($\mu\text{g g}^{-1}$ of tissue) of treated animals are presented as mean values \pm standard deviation (Figure 1). OTA levels in plasma, kidney and liver tissue homogenates increased gradually from day 7 to 21.

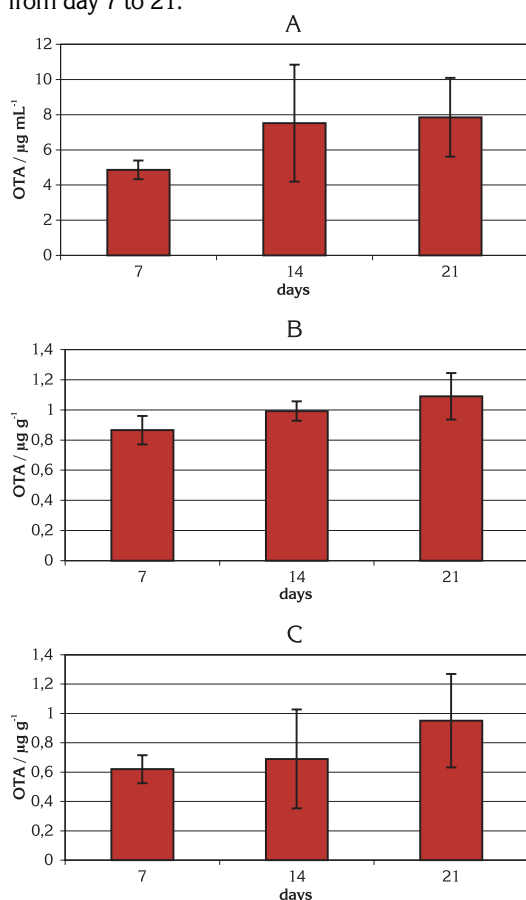
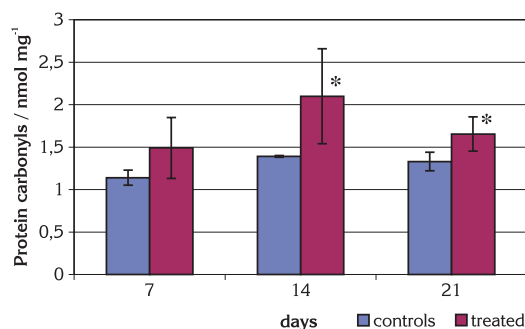


Figure 1 Levels of ochratoxin A in the plasma (A), kidney (B) and liver (C) of rats given OTA intraperitoneally in the dose of $0.5 \text{ mg kg}^{-1} \text{ b. w. per day}$ for 7, 14 and 21 days ($N=5$ per group)

Figure 2 shows the levels of protein carbonyls (nmol mg^{-1}) in the kidney homogenates of OTA-treated animals and control animals. The levels of protein carbonyls was higher ($P<0.05$) in the kidney homogenates of animals given 14 and 21 doses of OTA than in control animals. The highest level of protein carbonyls in the kidney was found after day

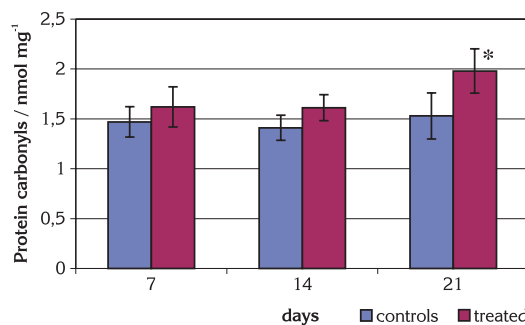
14. In the liver homogenates of OTA treated animals, the level of protein carbonyls increased steadily from the beginning of treatment, but the difference between the treated and control groups was significantly higher only after day 21 ($P<0.05$) (Figure 3).

Figure 2 Levels of protein carbonyls in the kidney of rats given either OTA intraperitoneally in the dose of $0.5 \text{ mg kg}^{-1} \text{ b. w. per day}$ (for 7, 14 and 21 days) or solvent alone (controls) ($N=5$ per group)



* significantly different $P<0.05$

Figure 3 Levels of protein carbonyls in the liver of rats given either OTA intraperitoneally in the dose of $0.5 \text{ mg kg}^{-1} \text{ b. w. per day}$ (for 7, 14 and 21 days) or solvent alone (controls) ($N=5$)



* significantly different $P<0.05$

DISCUSSION

According to the available literature, there is no doubt that OTA induces free-radical production and the consequent oxidative stress in bacteria and in cultured cells (Vero cells, proximal tubular kidney cells) (4, 5, 7). Although there are several reports regarding lipid peroxidation, there are no data on the effect of OTA on the oxidation of proteins neither in cultured cells nor in experimental animals.

In our experiment, the concentration of OTA increased steadily in plasma, kidney and liver homogenates of animals killed 24 h after 7, 14 and 21 daily i. p. doses of OTA (0.5 mg kg⁻¹ b. w.). Protein carbonyl concentration was measured in the kidney and liver homogenates at the same time points.

The concentration of protein carbonyls increased in the kidney tissue of OTA-treated rats, but the effect did not correspond to the increase of OTA concentration in plasma, kidney, and liver. The concentration of protein carbonyls was significantly higher in the kidney tissue of rats treated for 14 and 21 days. However, the highest concentration was observed after the 14th treatment, and after this time point, the concentration of protein carbonyl decreased even though the OTA treatment continued. This finding is in accordance with our earlier study in which multiple OTA treatment increased the release of kidney enzymes into urine until two weeks of treatment, and decreased afterwards, despite continued OTA treatment (10). This effect was probably caused by lipid peroxidation of the kidney cells, because the changes in MDA concentration had the same pattern. The effects of OTA on the activity of the kidney enzymes and the concentration of MDA in urine in our previous study as well as the effect on protein carbonyls in the present study indicate that some compensatory mechanism may be involved in the kidney. This mechanism obviously does not exist in the liver, because the concentration of protein carbonyls increased continuously during the whole length of the experiment and reached its peak after the last (21st) treatment.

In this study the increased protein carbonyls in the kidney and liver confirm that oxidative stress is involved in the mechanism of OTA toxicity. Not only does oxidative stress increase lipid peroxidation, but it also affects proteins.

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Sažetak

DJELOVANJE OKRATOKSINA A NA PROTEINSKE KARBONILE U BUBREGU I JETRI ŠTAKORA

Poznato je da je jedan od mehanizama toksičnog djelovanja mikotoksina okratoksina A (OTA) stvaranje slobodnih radikala i posljedični oksidativni stres. Oksidativni stres uzrokuje oštećenje makromolekula kao što su DNA, proteini, lipidi i ugljikohidrati. Jedini do sada proučavani učinak oksidacijskog stresa što ga uzrokuje OTA jest peroksidacija lipida. Cilj ovog istraživanja bio je utvrditi da li OTA uzrokuje stvaranje proteinskih karbonila, markera oksidacije proteina. Štakori (5 u grupi) dobivali su svakodnevno intraperitonealno OTA (0,5 mg kg⁻¹ tj. t.) ili otapalo (kontrola). Životinje su žrtvovane 24 sata nakon 7., 14., odnosno 21. tretmana. Razina OTA u plazmi, bubregu i jetri povećavala se postepeno tijekom pokusa. Razina proteinskih karbonila u bubregu tretiranih životinja bila je značajno veća ($P < 0,05$) nakon 14. odnosno 21. tretmana negoli u kontrolnih životinja. U jetri je razina proteinskih karbonila postepeno rasla tijekom pokusa, a razlika u tretiranih i kontrolnih životinja bila je značajna samo nakon 21. tretmana ($P < 0,05$). Ovi rezultati potvrđuju da OTA uzrokuje nastanak oksidativnog stresa zbog čega dolazi do oksidacije proteina.

KLJUČNE RIJEČI: *lipidna peroksidacija, mikotoksin, oksidativni stres, slobodni radikali*

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