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Ochratoxin A induces oxidative DNA damage in liver and kidney after oral dosing to rats

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The nephrotoxic/carcinogenic mycotoxin ochratoxin A (OTA) occurs as a contaminant in food and feed and may be linked to human endemic Balkan nephropathy. The mechanism of OTA-derived carcinogenicity is still under debate, since reactive metabolites of OTA and DNA adducts have not been unambiguously identified. Oxidative DNA damage, however, has been observed *in vitro* after incubation of mammalian cells with OTA. In this study, we investigated whether OTA induces oxidative DNA damage *in vivo* as well. Male F344 rats were dosed with 0, 0.03, 0.1, 0.3 mg/kg bw *per* day OTA for 4 wk (gavage, 7 days/wk, five animals *per* dose group). Subsequently, oxidative DNA damage was determined in liver and kidney by the comet assay (single cell gel electrophoresis) with/without use of the repair enzyme formamido-pyrimidine-DNA-glycosylase (FPG). The administration of OTA had no effect on basic DNA damage (determined without FPG); however, OTA-mediated oxidative damage was detected with FPG treatment in kidney and liver DNA of all dose groups. Since the doses were in a range that had caused kidney tumors in a 2-year carcinogenicity study with rats, the oxidative DNA damage induced by OTA may help to explain its mechanism of carcinogenicity. For the selective induction of tumors in the kidney, increased oxidative stress in connection with severe cytotoxicity and increased cell proliferation might represent driving factors.

Keywords: Comet-assay / *In vivo* / Ochratoxin A / Oxidative DNA damage / Protein oxidation / Rat

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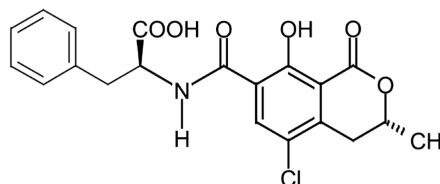
1 Introduction

The mycotoxin ochratoxin A (OTA, molecular mass 403.8 g/mol, Scheme 1), produced by several Aspergillus and Penicillium strains, is a widespread contaminant in food and feed. Human exposure results mainly from plant products such as cereals, grains, coffee, beans, spices, and



Scheme 1. Structure of OTA.

from processed products such as coffee, grape juice, wine, beer, and bread [1]. OTA is detected frequently in human plasma at nanomolar concentrations [1, 2]; the half-life of OTA in humans has been found to exceed 800 h and may be explained by the high affinity of OTA to serum albumin [3].

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Abbreviations: ALT, alanine aminotransferase; **AST**, aspartate aminotransferase; **dG**, deoxyguanosine; **DNPH**, 2,4-dinitrophenylhydrazine; **FPG**, formamido-pyrimidine-DNA-glycosylase; **HBSS**, Hanks buffered salt solution; **HRP**, horseradish peroxidase; **NCTR**, National Center for Toxicology Research, USA; **NOEL**, no observed effect level; **NTA**, nitrilotriacetic acid; **OTA**, ochratoxin A; **PCT**, proximal convoluted tubules

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Although still under debate, there is some evidence that OTA acts as a causative factor in "Balkan endemic nephropathy", a human noninflammatory kidney disease that is also associated with the development of urinary tract tumors. In these patients, plasma levels up to 50 nM (20 µg/L) OTA have been reported [4]. A similar nephropathy has been described for some subjects in regions in France and Northern Africa, suggestive of a correlation with high exposure to OTA [5–7].

In animal experiments, OTA elicits a number of toxicological effects: it is carcinogenic, nephrotoxic, teratogenic, neurotoxic, and immunotoxic in several species [1]. OTA induces renal tumors in male mice and rats after chronic treatment of both sexes; however, the male gender is considerably more sensitive [8, 9]. In the rat kidney, tumor induction is seen at a very low dose level of 0.07 mg/kg bw *per* day, while tumors were not observed at 0.021 mg/kg bw *per* day. Mammary fibroadenomas were also found in female rats [9]. It is not known yet whether OTA induces tumors by direct adduction to DNA or whether its carcinogenic activity is attributable to indirect mechanisms, such as induction of cytotoxicity, oxidative cell damage, and increased cellular proliferation as a consequence of tissue injury. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Community (EC) Scientific Committee on Food (SCF) have established a provisional tolerable weekly intake (PTWI) of 100 ng/kg bw based on the lowest observed effect level (LOEL) of 8 µg/kg bw for effects on the kidneys in pigs [10], the most sensitive species, applying a safety factor of 500. JECFA concluded in its latest evaluation [1] that in the rat nephrotoxicity preceded carcinogenicity and noted that "the large safety factor applied to the no observed effect level (NOEL) for nephrotoxicity in deriving the PTWI corresponds to a factor of 1500 applied to the NOEL for carcinogenicity in male rats, the most sensitive species and sex for this endpoint". Other risk assessments using nonthreshold models for risk extrapolation have led to provisional tolerable daily intakes of 1.2–5.7 ng/kg bw [11]. Clearly, to resolve the question of threshold *versus* nonthreshold mechanisms of action of OTA, a clearer understanding of the chain of events leading to malignant transformation in the target cell population of the proximal tubule is required.

A genotoxic mechanism of action for OTA has been postulated, based on results of ^{32}P-postlabeling experiments that indicated formation of radio-labeled spots due to DNA lesions induced by OTA treatment [12, 13]. Recently, OTA was reported to undergo photoactivation to form carbon- and oxygen-centered radicals that react with the C8 atom of deoxyguanosine (dG) to form covalent DNA adducts *in vitro* [14]. Moreover, the carbon-bound OTA C8-dG adduct was reported to comigrate with one of the major lesions detected by TLC autoradiography in kidney DNA of OTA-treated rats in ^{32}P-postlabeling experiments [15]. However, the identity of this lesion has not been unequivocally established by confirmatory techniques including MS.

The results of investigations from several other laboratories refute the hypothesis that OTA or a metabolite binds to DNA since OTA-derived adducts have not been identified *in vivo*. In one investigation using tritium-labeled OTA, no radioactivity was detected in DNA of rat kidney with an LOD of 2.7 adducts *per* 10^9 DNA bases, whereas lesions were detected at 3–17-fold higher levels by ^{32}P-postlabeling experiments, indicating that the lesions should not contain an OTA moiety [16]. Moreover, in rats exposed to ^{14}C-labeled OTA, DNA adducts were not detected by accelerator MS (AMS), reported to achieve a detection limit of three adducts *per* 10^9 DNA bases [17]. Furthermore, *in vitro* formation of DNA adducts was not detected after incubation of radio-labeled OTA in various activation systems, using sensitive detection methods including liquid scintillation counting or LC/MS-MS [16–18]. Taken together, the currently available evidence suggests that the lesions observed by the ^{32}P-postlabeling experiments might arise indirectly as a consequence of reactive oxygen species derived from OTA-mediated cytotoxicity rather than by covalent binding of OTA itself or OTA metabolites to DNA.

Indeed, OTA is potently cytotoxic, inducing tubulo-interstitial nephropathy in rats and in other animal species [9, 10]. Likewise, marked cytotoxic and proliferation-stimulating effects have been observed *in vitro* in different cell systems, including cell lines such as Vero, LLC-PK1, V79, CV-1, HEK 293, NRK52E as well as primary cells of the human, rat, and pig renal cortex [19–24]. OTA-dependent induction of apoptosis in kidneys of rats and mice has been reported at rather low dosage and in various cell lines at low micromolar to submicromolar concentrations, including IHKE, CV-1, MDCK-C7, MDCK-C11, OK, HL-60 as well as cultured primary rat proximal tubular cells [21, 22, 25–27]. Free radical formation leading to oxidative cell damage is believed to contribute to both, cytotoxicity and carcinogenicity of OTA. Further evidence for oxidative stress being relevant for the biological effects of OTA is derived from animal studies, describing the attenuation of OTA-dependent toxic effects and reduced formation of DNA lesions in kidney and other organs by coadministration of various antioxidants [*e.g.*, 28, 29]. Changes in renal enzyme activities were also observed in these cotreatment experiments [30, 31]. The hypothesis of oxidative stress being involved in OTA-mediated genetic damage is further strengthened by evidence from studies revealing OTA-dependent lipid peroxidation and free radical formation in mammalian cells and other systems as well as in rats [32–35]. Decreased concentrations of vitamin E in plasma of rats [30], and of glutathione in liver of mice, in primary hepatocytes and in CV-1 cells have also been reported after OTA treatment [22, 36,

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37]. Moreover, OTA has been found to induce DNA strand breaks in different systems and to generate oxidized DNA bases *in vitro* [22, 31, 38, 39].

The present *in vivo* study was performed to determine whether OTA-induced oxidative DNA damage might be relevant for the nephrotoxicity and carcinogenicity in rodents. Male F344 rats were treated with OTA over a 4-wk period and cells from liver and kidney were isolated for assessment of DNA damage by alkaline single cell gel electrophoresis (comet assay). The OTA dosage covered the range of 0, 0.03, 0.1, and 0.3 mg/kg bw, thus including a nontoxic level close to the NOEL for nephrotoxicity up to a level that would lead to toxicity and tumor formation during long-term treatment [9]. Oxidative DNA damage was measured using additional treatment with formamido-pyrimidine-DNA-glycosylase (FPG). This repair enzyme specifically recognizes oxidized purine and pyrimidine bases, excises the lesions and nicks the DNA at the respective sites resulting in additional DNA damage considered to be oxidation specific [40]. In addition, protein oxidation was used as a secondary biomarker to assess the prooxidant capacities of OTA [41, 42]. The oxidant Fe-nitrilotriacetate (Fe-NTA), a potent nephro-carcinogen in rats [*e.g.*, 43] was included for comparison.

2 Materials and methods

2.1 Chemicals

OTA (*N*-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]carbonyl}-3-phenyl-L-alanine, CAS-no. 303-47-9, purity >99%) was obtained from Dr. Michael Stack, US FDA. 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Aldrich (Milwaukee, WI). BSA, antiDNP antibody was obtained from Sigma (St. Louis, MO). AntiRabbit IgG-horseradish peroxidase (HRP) was bought from Santa Cruz Biotechnology, Santa Cruz, CA, and immuno-star HRP substrate kit was obtained from BioRad, Hercules, CA. B-Per was purchased from Pierce, Milwaukee, WI. Collagenase type IV was provided by Sigma-Aldrich GmbH, Taufkirchen, Germany. Agarose, low melting, and normal melting was obtained from Serva Electrophoresis GmbH, Heidelberg, Germany. All organic solvents and other chemicals were of analytical grade.

2.2 Animal treatment

Groups of five male F344 rats (160–180 g), bred at the National Center for Toxicology Research, USA (NCTR) animal facilities, were gavaged with OTA dissolved in 50 mM $NaHCO_3$ solution (pH 8.2) at the respective dosage (0, 0.03, 0.10, and 0.30 mg/kg bw) for 4 wk at 8.00 a.m. 7 days/wk. Animals were housed in single macrolon cages and had access to water and NIH-31 chow diet *ad libitum*. The animals were sacrificed by decapitation 24 h following the last treatment, and the liver and kidneys were excised. Cells were isolated as described below. In addition, two groups (five animals each) were dosed i.p. with 15 mg/kg bw *per* day Fe-NTA (calculated as Fe, see [30]) and were sacrificed 4 and 24 h after the administration, respectively.

2.2.1 Clinical chemistry

Blood was allowed to clot and then centrifuged at 3000 rpm for 10 min. The serum was then separated and stored at –70°C until analyses. The Cobas Mira Plus (Roche diagnostics, Indianapolis, IN) analyzer was used along with Roche Diagnostics reagents to analyze serum levels of alanine aminotransferase (ALT) (modified IFCC), aspartate aminotransferase (AST) (NADH), creatinine (kinetic Jaffe), and BUN (urease).

Urine samples were collected and stored at –70°C. The Cobas Mira Plus analyzer and Roche Diagnostics reagents were used for creatinine (kinetic Jaffe) and glucose (hexokinase). Sigma reagents were used for microprotein (Brilliant Blue G).

All parameters were calibrated and two levels of controls implemented to ensure accuracy. All maintenance was performed according to the manufacturer.

2.2.2 Histopathology

The formalin-fixed liver and kidney were trimmed, processed, and embedded in Tissue Prep II, sectioned at approximately 5 µm, and for routine histopathology stained with hematoxylin and eosin. When applicable, lesions were graded for severity as one (minimal), two (mild), three (moderate), or four (marked).

2.3 Isolation of rat liver and kidney cells

2.3.1 Liver cells

After exsanguination and preparation of the liver, a small piece of the left liver lobe was placed in 1 mL of ice-cold Hanks buffered salt solution (HBSS, see [44]) (pH 7.4, containing 20 mM EDTA and 10% DMSO). The tissue was minced into fine pieces over ice with a pair of scissors according to Tice *et al.* [45]. After settling of remaining tissue pieces, aliquots of the supernatant containing hepatocytes in suspension were used for the comet assay and for determination of viability (trypan blue exclusion assay, see [22]).

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2.3.2 Kidney cells

Cortices of the kidneys were excised and placed in 1 mL of ice-cold HBSS (pH 7.4, containing 20 mM EDTA and 10% DMSO). The cortex tissue was minced into small pieces using scalpels, incubated with collagenase (30 min, Type IV, 5 U/mL) and thereafter with trypsin (30 min) as described earlier [22] with slight modifications. Cells were prepared by mechanical disintegration using a microliter pipet and washed with HBSS containing fetal calf serum (FCS). Aliquots were taken for the comet assay and for determination of viability (trypan blue exclusion assay, see [22]).

2.4 (Oxidative) DNA damage

Alkaline single cell gel electrophoresis was performed as described elsewhere [40, 46, 47] with slight modifications [48]. Briefly, aliquots of cell suspensions were centrifuged (400*g*), cells resuspended in low-melting agarose (65 µL), spread onto a frosted glass microscope slide, precoated with a layer of normal melting agarose (two gels *per* slide), coverslipped and kept at 4°C for solidification. After removing the cover glass, slides were immersed in a lysis solution for 1 h at 4°C. After cell lysis, slides were washed three times in enzyme buffer, drained, covered with 50 µL of either enzyme buffer (one slide) or FPG enzyme, kindly provided by Dr. Collins (one slide), sealed with a cover glass, and incubated for 30 min at 37°C.

After DNA unwinding (pH 13.5, 20 min, 4°C), horizontal gel electrophoresis (BioRad Sub Cell GT) was carried out at 4°C for 20 min (BioRad 300 power supply, 25 V, 300 mA, 0.89 V/cm). Slides were washed thereafter three times with Tris-buffer (0.4 mol/L, pH 7.5) and kept at 4°C until further processing. The slides were stained with ethidium bromide (40 µL, 10 µg/mL) and viewed microscopically with a Zeiss Axioskop 20, equipped with filter set 15 (excitation: BP 546/12; emission: LP 590). Slides were analyzed by computerized image analysis (Perceptive Instruments, Suffolk, Great Britain), scoring 2×50 images *per* slide (two gels *per* slide). DNA migration was expressed as mean fluorescence intensity of comet tail in percent of total comet fluorescence (TI%). Basic DNA damage (without FPG treatment) and total DNA damage (including oxidative DNA damage as detected by additional FPG treatment) were determined. Oxidation-specific DNA damage (ΔTI%) was calculated as difference between total and basic DNA damage.

2.5 Isolation of kidney and liver subcellular fractions

Kidney and cytosolic fractions were obtained by differential centrifugation. Organ tissues (1 g) were pottered on ice with four parts of buffer w/v containing 5 mM HEPES, 210 mM manitol, 70 mM sucrose, 1 mM EDTA, 0.3 mM defferoxamine mesylate, 0.25 mM PMSF, and protease inhibitor cocktail (1 mL/100 mL buffer, Sigma, P8340) at pH 7.4. The homogenate was centrifuged for 30 min at $12\,000 \times g$ at 4°C. The supernatant was subjected to centrifugation at $105\,000 \times g$ for 1 h at 4°C, and the cytosolic fraction was snap frozen in liquid nitrogen and then stored at –80°C until use.

2.6 Oxidative protein damage

Protein oxidation: BSA was oxidized with Fe/ascorbate, followed by derivatization with DNPH as previously described [42, 49]. Briefly, one volume of BSA (5 mg/mL in 50 mM HEPES/PBS, pH 7.4) was mixed with one volume Fe/ascorbate mix (200 µM $FeCl_3$ in 50 mM ascorbate) and the mixture incubated at 37°C overnight. After incubation, the sample was precipitated with TCA and the pellet was solubilized in B-Per. Concentrated HCl (5 N) was added to achieve a final concentration of 1 N HCl, and the oxidized protein was derivatized with DNPH (0.2 mL of 10 mM DNPH in 2 N HCl *per* 1 mL sample) for 30 min at room temperature. Thereafter, the sample was precipitated with TCA (10% v/v), the pellet was washed three times with ethanol/ethyl acetate (1/1) and dried under a stream of nitrogen. The pellet was dissolved in 6 M urea and the absorbance read at 375 nm to determine the extent of protein oxidation as pmol of carbonyl sites *per* mg of protein for calibration curves. To establish background levels of carbonyl sites for the immunoblot assay, BSA was pretreated with $NaBH_4$ to reduce preexisting carbonyl sites (Section 2.7).

2.7 Immunoquantitation of oxidized protein

Liver and kidney cytosolic protein samples under study were treated with DNPH as described above for oxidized BSA. Protein samples and DNPH-modified BSA standards were blotted onto NC membranes (5 µg protein in 0.3 mL TBS buffer (0.2 M Tris, 1.5 M NaCl, pH 7.4) using a Minifold II (Schleicher & Schuell). The wells are washed with 0.5 mL of TBS and protein binding was verified by staining the membrane with Ponceau S. After blocking active sites with 5% nonfat milk (1 h at RT), the membrane was washed 3× in TBST (TBS buffer containing 0.05% Tween 20) and probed with antiDNP antibody diluted 1:20 000 in 0.5% milk for 1 h at room temperature. The membrane was washed three times with TBST buffer, followed by incubation for 1 h with antiRabbit IgG-HRP, diluted 1:20 000. The membrane was washed again 3× with TBST buffer and subjected to enhanced chemiluminescent reaction using the immuno-star HRP substrate kit. Oxidized protein was

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visualized with an Alpha Innotech Imager (San Leandro, CA) using FluorChem 8000 software. The integrated density values (IDV) were used to quantitate the DNPH-modified proteins.

2.8 Statistics

For determination of statistical significance, the Student's test (unpaired) was performed between the corresponding groups. Before conducting the Student's test, individual values were logarithmically transformed to ensure a standardized normal distribution, an operation entailing a slight loss of information but increasing statistical power. Significance levels were marked as follows: *: $p < 0.05$; **: $p < 0.01$.

3 Results and discussion

OTA treatment did not affect food or water consumption neither did it change animal growth and final body weights: 244 ± 18 for control animals *versus* 245 ± 27 g for the upper dosage level group (mean ± SD), respectively. OTA treatment induced histopathological changes in the kidney at the top dosage (0.3 mg/kg). In the epithelium of the proximal convoluted tubules (PCTs) morphological changes were observed predominantly in the region of the corticomedullary junction. The lesions included apoptosis and karyomegaly. To a lesser degree, acute cellular swelling and cytoplasmic vacuolization were also observed. Urine analysis revealed proteinuria, glucosuria, and polyuria at the top dosage (0.3 mg/kg) further supporting OTA-induced damage to the PCT. No significant differences were detected between the lower dosage groups and control animals. Liver morphology and function of OTA-treated animals were unchanged, based upon histopathologic examination and assay of serum ALT and AST, respectively.

The Fe-NTA-treated positive control kidneys also had treatment-related changes, as expected, which consisted of acute tubular nephrosis. Livers of Fe-NTA-treated animals showed centrilobular hepatocellular hypertrophy.

Dose-dependent basic DNA damage (without FPG treatment) and oxidative DNA damage (with FPG treatment) in liver and kidney of OTA-treated male F344 rats are shown in Fig. 1. Control animals exhibited higher basic and oxidative DNA damage in the kidneys than in the liver. Administration of OTA did not result in a significant increase of basic DNA damage (Fig. 1a and b, open bars), as compared to the control group. However, oxidative DNA damage was increased in liver cells of animals exposed to 0.03 mg/kg bw *per* day OTA ($p = 0.001$, 1% ΔTI) and to 0.30 mg/kg bw *per* day OTA ($p = 0.022$, 3% ΔTI). Likewise, in kidney cells, a significant increase of oxidative DNA damage

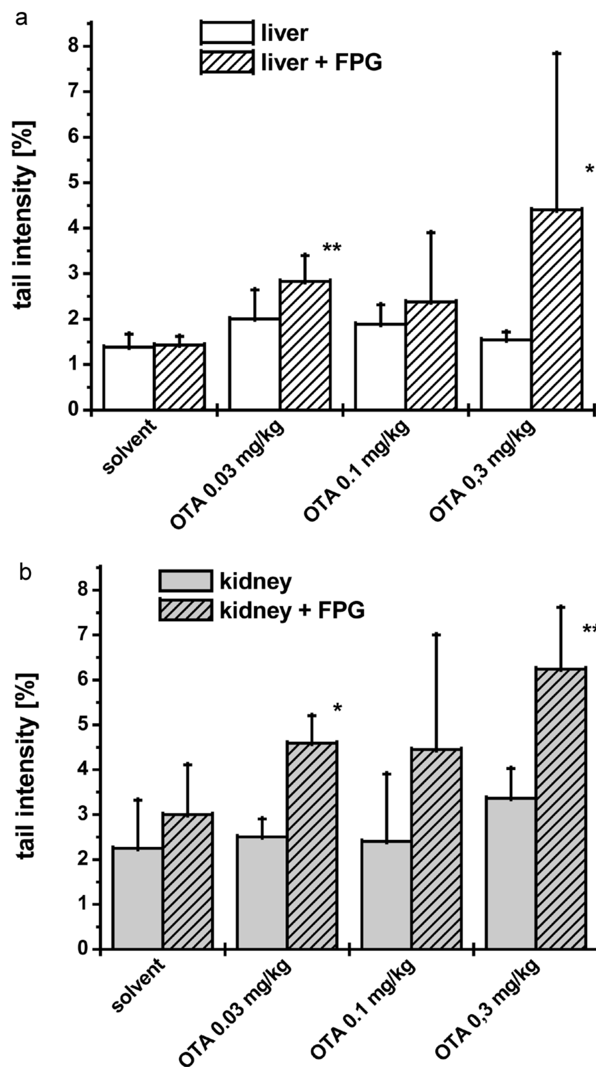

Figure 1. Basic and total DNA damage (TI%), including FPG sensitive sites (hatched bars) (a) in rat liver cells and (b) in rat kidney cells after oral treatment with OTA. Mean values ± SD of five animals. *, $p < 0.05$; **, $p < 0.01$.

occurred in the low and highest dose group (0.03 mg/kg bw *per* day OTA: $p = 0.020$ and 0.30 mg/kg bw *per* day OTA: $p = 0.004$). Animals treated with 0.10 mg/kg bw *per* day OTA also exhibited an increase of oxidative DNA damage in liver and kidney. However, this was not statistically significant due to the high variation of the TI% in both organs within this dose group (Fig. 1a and b, hatched bars).

These results agree well with results of our previous studies *in vitro*, which showed oxidative DNA damage in cell lines and primary rat kidney cells, already at submicromolar, noncytotoxic concentrations [22, 50].

Oxidative DNA damage induced by OTA in a dose-dependent manner was detected at a dosage level as low as 0.03 mg/kg bw *per* day after 4 wk of treatment, very close

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to the NOEL for nephrotoxicity in long-term carcinogenicity bioassays. The induction of oxidative DNA damage is considered to be a premutagenic genotoxic event [51]. In a 2-year carcinogenicity study in F344 rats, oral administration of 0.07 mg/kg bw *per* day OTA (5 days/wk) led to an increase in tumor incidence in the kidneys of male animals, whereas tumor formation was not observed in animals treated with 0.021 mg/kg bw *per* day OTA [9]. The results of the present study show for the first time increased oxidative DNA damage in liver and kidney of rats after 4 wk of OTA treatment already at a dosage as low as 0.03 mg/kg bw. It might be speculated that accompanying cytotoxic and other effects of OTA, such as the triggering of proliferative signaling cascades as described by Gekle *et al.* [21], might not suffice yet at dosages as low as 0.021 mg/kg bw/day to propagate tumor formation within the life span of the rats. Likewise, such accompanying OTA effects might largely be absent in liver even at dosages that already drive tumor formation in the kidney. Although this is still highly speculative and needs further confirmation, it is noteworthy that Schilter *et al.* [52] have presented evidence that OTA given chronically to rats mediates totally different responses in kidney and liver with respect to altered gene expression profiles. Disruption of pathways regulated by the transcription factors hepatocyte factor 4 alpha (HNF 4 alpha) and nuclear factor-erythroid 2-related factor (Nrf2) was observed in kidney but not in liver. Inhibition of Nrf2 binding to the antioxidant response element (ARE) promoter leads to reduced expression of the corresponding gene batteries, involving predominantly detoxification and antioxidative defense proteins. This supports the premise that oxidative stress is likely to play an important role in OTA toxicity and carcinogenicity. Of note, increased cell proliferation and karyomegaly have been reported in the kidney tubular epithelium but not in the liver of OTA-treated animals at a long-term dosage of 0.07 and 0.21 mg/kg bw *per* day [9]. In the present study, similar lesions were observed at 0.3 mg/kg bw *per* day in the kidney, but no indication for toxicity was seen in the liver. A combination of cell proliferation and increased oxidative stress has been found to induce tumors in the kidney of rats and mice treated with the oxidants Fe-NTA and potassium bromate [43, 53, 54].

Taken together, the *in vitro* and *in vivo* results support the hypothesis that OTA-dependent oxidative DNA damage occurs at low exposure concentrations and therefore can be considered a likely causative factor contributing to the induction of kidney tumors.

Fe-NTA acts as a potent oxidant in kidney, resulting in tumorigenesis [*e.g.*, 43]. Fe-NTA was used as a prooxidant and positive control in our study. We observed differences in oxidation markers induced by treatment with OTA 24 h after the last application as compared to Fe-NTA. The treatment of rats with Fe-NTA resulted in significantly increased

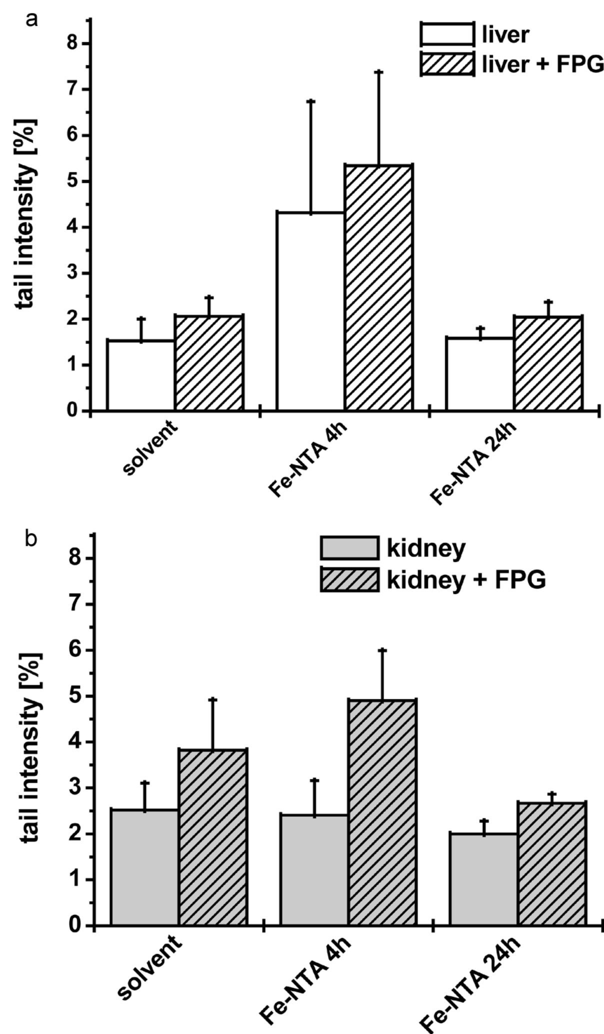

Figure 2. Basic and total DNA damage (TI%), including FPG sensitive sites (hatched bars) (a) in rat liver cells and (b) in rat kidney cells after i.p. treatment with Fe-NTA. Mean values ± SD of five animals. *, $p < 0.05$.

basic ($p = 0.011$) and oxidative ($p = 0.024$) DNA damage in liver cells already 4 h after administration. However, the DNA damage was rapidly repaired and the level of DNA oxidation returned to basal values within 24 h after injection of Fe-NTA (Fig. 2a). Fe-NTA treatment resulted in a 25% increase in oxidative DNA damage of kidney cells after 4 h which however did not reach statistical significance (Fig. 2b). Tail intensities were even lower in treated animals than in the control animals 24 h after injection, even though the dose of Fe-NTA used in this study resulted in renal toxicity and treated animals displayed acute tubular nephrosis (necrosis) in the PCT of the cortex. These animals also clearly showed an induction of apoptosis (data not shown). However, the DNA damage caused during apoptosis would not be detected with the comet assay under our experimental conditions [46].

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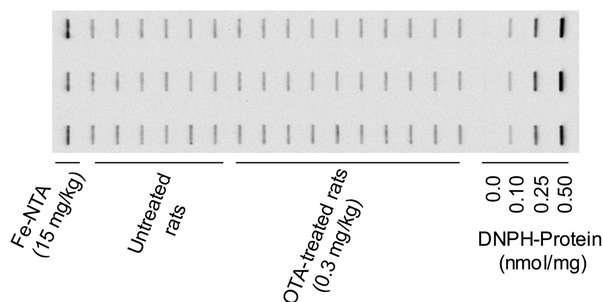

Figure 3. Immunslot blot analysis of protein oxidation in rat kidney through measurement of protein carbonyl formation, following derivatization with DNPH and immunodetection. Kidney of a rat dosed i.p. with 15 mg/kg bw Fe-NTA was used as a positive control.

We used protein oxidation as another indication of an oxidant effect of OTA and Fe-NTA *in vivo*. Formation of protein carbonyl groups is a major pathway of protein oxidation that is mediated by transition metals and other prooxidants [41, 42]. Oxidized proteins are readily detected by immunodetection methods, following derivatization of the carbonyl sites with DNPH, which serves as the antigenic marker [54]. Protein oxidation was detected in kidney of Fe-NTA-treated rats 4 h posttreatment, with a 3.5-fold increase in protein carbonyls in the cytosolic fraction as compared to untreated animals (126 ± 30 *vs*. 36 ± 10 pmol/mg protein; mean ± SD, $N = 3$; $p < 0.01$) (Fig. 3). The level of protein carbonyls returned to basal levels at 24 h posttreatment, obviously as a consequence of rapid protein degradation. There was, however, no increase in protein oxidation in liver cytosols of Fe-NTA-treated animals despite the observation of minimal to mild centrilobular hepatocellular hypertrophy (data not shown). In contrast to Fe-NTA, even the highest dose treatment of OTA (0.3 mg/kg bw *per* day) did not increase the level of protein oxidation in kidney (34 ± 1 pmol/mg protein; mean ± SD, $N = 10$; $p > 0.05$) (Fig. 3) and liver (data not shown).

Fe-NTA treatment induces various types of oxidative cell damage in rodent kidney. However, the reports in the literature are inconsistent concerning the kinetics, *i.e.*, the rate of formation *versus* elimination of oxidative damage [56–60]. Our findings argue for multiple biomarkers and measuring time points being required to obtain reliable and relevant information on the type and extend of cellular/tissue damage induced by such prooxidants.

4 Concluding remarks

One plausible mechanism of OTA-induced carcinogenesis that can be envisaged to be operative is DNA damage resulting from oxidative stress [*e.g.*, 22, 30, 31, 34–37, 61]. Several *in vivo* studies have shown that repeated dosing of rats with OTA (0.25 mg/kg bw *per* day for 4 wk, or 0.12 mg/kg bw *per* day for 8 wk) leads to increased levels of reactive oxygen species in liver, kidney, and plasma. As a result, decreased hepatic GSH and plasma vitamin E levels have been observed [30, 62, 63]. Moreover, OTA was reported to increase 8-oxodG formation and oxidative DNA strand breaks in various cell lines [22, 31] and to promote oxidative damage and strand breakage of supercoiled DNA *in vitro* in the presence of transition metals [32].

In the present study using the highly sensitive comet assay, we have found that OTA induces oxidative DNA damage in liver and kidney of rats already when applied at a dosage as low as 0.03 mg/kg bw. The selective induction of tumors in the rat kidney by OTA may be attributable to oxidative DNA damage in combination with cell-specific cytotoxic and proliferation-stimulating effects including activation of various signaling cascades and other cellular effects induced by this mycotoxin. Thus, oxidative DNA damage as an initiating step in combination with proliferative response might lead to cell-specific malignant transformation within the kidney [21, 22, 64].

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