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The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells

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Abstract

Balkan endemic nephropathy (BEN), a disease characterized by progressive renal fibrosis in human patients, has been associated with exposure to ochratoxin A (OTA). This mycotoxin is a frequent contaminant of human and animal food products, and is toxic to all animal species tested. OTA predominantly affects the kidney and is known to accumulate in the proximal tubule (PT). The induction of oxidative stress is implicated in the toxicity of this mycotoxin.

In the present study, primary rat PT cells and LLC-PK_1 cells, which express characteristics of the PT, were used to investigate the OTA-mediated oxidative stress response. OTA exposure of these cells resulted in a concentration-dependent elevation of reactive oxygen species (ROS) levels, depletion of cellular glutathione (GSH) levels and an increase in the formation of 8-oxoguanine.

The OTA-induced ROS response was significantly reduced following treatment with α-tocopherol (TOCO). However, this chain-braking anti-oxidant did not reduce the cytotoxicity of OTA and was unable to prevent the depletion of total GSH levels in OTA-exposed cells. In contrast, pre-incubation of the cell with *N*-acetyl-L-cysteine (NAC) completely prevented the OTA-induced increase in ROS levels as well as the formation of 8-oxoguanine and completely protected against the cytotoxicity of OTA. In addition, NAC treatment also limited the GSH depletion in OTA-exposed PT- and LLC-PK_1 cells.

From these data, we conclude that oxidative stress contributes to the tubular toxicity of OTA. Subsequently, cellular GSH levels play a pivotal role in limiting the short-term toxicity of this mycotoxin in renal tubular cells.
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1. Introduction

Ochratoxin A (OTA) is produced by *Aspergillus* and *Penicillium* strains, and is a frequent contaminant of feed and foodstuffs. Epidemiological studies associate OTA with Balkan endemic nephropathy (BEN), a progressive renal fibrosis, and tumors of the urinary tract. However, the etiology of the disease still remains unclear [1–3]. In animals, OTA was shown to induce a tubulo-interstitial nephropathy similar to BEN [4–6] as well as renal adenoma and carcinoma [7]. Other toxic effects associated with OTA include immunosuppression, hepatotoxicity and teratogenicity (for a review, see Refs. [8–12]).

OTA is known to affect multiple sites of the nephron: acute exposure mainly affects the postproximal parts, while chronic exposure leads predominantly to damage of the proximal tubule (PT) [13]. The exact mechanism of OTA toxicity has not been elucidated as of yet. OTA contains a chlorinated dihydroisocoumarin moiety linked to L-phenylalanine. The observed inhibition of protein synthesis by OTA [14] results from competition with phenylalanine during the aminoacetylation reaction of phenylalanine–tRNA [12]. The inhibition of protein synthesis is thought to underlie most toxic effects of OTA, but protein inhibition alone cannot explain the diverse but apparent site-specific toxic effects. Several studies propose the involvement of oxidative stress to the toxicity of OTA [5,15–17]. Initially,

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Rahimtula et al. [17] have shown that OTA induces lipid peroxidation (LPO) in subcellular fractions. Further evidence showing that OTA is a potent pro-oxidant was provided by Belmadani et al. [15] who reported that OTA induced LPO in primary cultures of brain cells. In addition, the formation of reactive oxygen species (ROS) was significantly increased after exposure of foetal rat telencephalon cells to high OTA concentrations [18]. The kidney is a prominent site for intense oxidative processes in the body and ROS play an important role in the pathogenesis of a variety of renal diseases [19]. Thus, we investigated the relation between the OTA-induced oxidative response and toxicity in its main target cells, renal PT cells, using primary rat proximal tubular cells (PT cells) and continuous proximal tubular cells (LLC-PK_1). Primary cells resemble the in vivo PT cell closely by expressing PT-specific functions including phase I and II biotransformation and organic anion and cation transport [20]. LLC-PK_1 cells are widely used as in vitro model of cultured tubular epithelial cells, as they have retained several characteristics of the PT cell [21]. In contrast to the primary cells, the LLC-PK_1 cells have lost some differentiated functions, including organic anion transport [22]. The OTA-induced stress response was monitored at several levels in these cells, as ROS can attack almost any cellular structure or molecule, including membrane lipids, proteins and DNA [23,24]. The effects of exposure of the cells to OTA on ROS production and cell viability were assessed with respect to concentration- and time-dependency. Furthermore, the effects of the mycotoxin on cellular glutathione (GSH) levels and the formation of 8-oxoguanine were investigated. The established anti-oxidants α-tocopherol (TOCO) and *N*-acetyl-L-cysteine (NAC) were used to determine the contribution of oxidative stress to DNA damage and cytotoxicity induced by OTA.

2. Materials and methods

2.1. Materials

Dimethylthiazol diphenyl tetrazolium bromide (MTT), NAC, OTA and TOCO were obtained from Sigma (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes (Leiden, The Netherlands). DMEM/F12 and medium 199 culture media and supplement G (insulin 10 mg/l, transferrin 5.5 mg/l, sodium-selenite 6.7 μg/l) were obtained from Life Technologies (Breda, The Netherlands). All other chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA). OTA, TOCO and H_2DCF-DA were dissolved in DMSO and diluted in culture medium to the concentrations described in the text. NAC was diluted in ethanol/H_2O (1:1; v/v). Solvent concentrations were 0.1% (v/v) unless specified otherwise. OTA dilutions were prepared in serum-free medium.

2.2. Cell culture

2.2.1. Isolation of rat PT cells

Rat PT cells were isolated as described previously [20]. Cell yield was $30-50 \times 10^6$ cells/rat and cell viability was routinely higher than 70% as judged by trypan blue exclusion. Cells were seeded on collagen (rat tail type IV) precoated 60 mm culture dishes (1×10^6 cells/dish) in culture medium containing serum (10% FCS) for 24 h. After 24 h, non-attached cells were removed, pelleted and re-plated (in the same culture dishes) in fresh serum-free culture medium to increase attachment efficiency. Medium was then changed every 3 days with serum-free DMEM/F12 supplemented with 10 mg/l insulin, 5.5 mg/l transferrin, 6.7 μg/ml sodium selenite (supplement G; 1 × concentrated), 10 U/ml penicillin and 10 μg/ml streptomycin. OTA, H_2DCF-DA and TOCO were dissolved in DMSO; NAC was dissolved in H_2O/ethanol (1:1, v/v). The stock solutions were further diluted in culture medium, so that solvent concentrations never exceeded 0.1% (v/v).

2.2.2. LLC-PK_1 cells

LLC-PK_1 cells, obtained from ATCC (number CL-101) were routinely seeded at 4×10^5 cells/75 cm^2 flask and were subcultured every 6–7 days. Two days before experiments, cells were plated at a density of 2.7×10^4 cells/cm^2 culture area in medium 199 supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

2.3. Determination of ROS production

Cells were seeded in 96-well culture plates at 1×10^4 cells/well and allowed to grow for 48 h. In the dose-response experiments, cells were then incubated for 2 h at 37 °C cells in serum-free medium containing 20 μM H_2DCF-DA. After aspiration of H_2DCF-DA, the cells were rinsed once and were subsequently exposed to increasing concentrations of OTA (0–200 μM) in serum- and phenol red-free medium. ROS production was measured after 24 h incubation on a Cytofluor 2300 (Fluorescence Measurement System, Millipore, Bedford, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In the experiments with the anti-oxidants, cells were incubated 48 h after plating with increasing concentrations of TOCO (0–12.5 μM) or NAC (0–4 mM). Following the 24-h pre-incubation period, the cells were treated with H_2DCF-DA as described above. After removal of H_2DCF-DA, cells were exposed to a fixed OTA concentration (100 μM) in the presence of increasing concentrations of TOCO or NAC. The ROS levels were determined 24 h later as described above. The incubation volumes were 50 μl/well unless specified otherwise. Relative ROS production was expressed as the ratio of fluorescence of the treated samples over the response in the appropriate controls [$(\text{fluorescence}_{\text{treatment}}/\text{fluorescence}_{\text{control}})100\%$].

2.4. Determination of cell viability

Cell viability was assessed by measuring the reduction of MTT to formazan by the mitochondrial enzyme succinate dehydrogenase [25]. Cells were seeded on 96-well plates at 1×10^4 cells/well. In the dose-response experiments, cells were exposed to increasing concentrations of OTA (0–200 μM) for 4 and 24 h. Then 0.6 mg/ml MTT solution (final concentration in medium; 3 mg/ml stock dissolved in PBS) was added to the wells. Plates were then incubated for 2 h at 37 °C in a humidified atmosphere. The reaction was terminated by discarding the incubation solutions followed by dissolving the formazan product in 50 μl acidic isopropanol (containing 0.1 M HCl). Absorbance at 595 nm was determined on a Biorad 3550 microplate reader (Biorad, Veenendaal, The Netherlands).

In the experiments with the anti-oxidants, cells were incubated 48 h after plating with increasing concentrations of TOCO (0–12.5 μM) or NAC (0–4 mM). Following the 24-h pre-incubation period, cells were exposed to a fixed OTA concentration (100 μM) in the presence of increasing concentrations of TOCO or NAC. Cell viability was determined 24 h later as described above. The incubation volumes were 50 μl/well unless specified otherwise.

Cell viability was expressed as the relative formazan formation in treated samples as compared to that in solvent-treated controls [(A_{595} treated cells/A_{595} of appropriate control)100%, after correction for background absorbance]. IC_{50} values, defined as the concentrations inducing 50% loss of cell viability, were estimated from the figures. IC_{50} values (24 h OTA incubation) were calculated after fitting the cell viability data with Slide Write plus software, version 4.0 (BIS Netherlands, Ridderkerk, The Netherlands).

2.5. Determination of cellular GSH levels

Total cellular GSH (reduced GSH, GSSG and GSSR) measurements were based on the method described by Griffith [26]. Cells were seeded at 5×10^4 cells/well in 24-well plates (for rat PT cells, the plates were coated with collagen type I as previously described [20]). The (pre-) incubation volumes were 500 μl. Cells were either pre-incubated for 24 h with solvent (0.1% DMSO or ethanol), 5 μM TOCO, 2 mM NAC (PT cells) or 4 mM NAC (LLC-PK_1). Initially, for measurements of solvent controls, cells were treated with either 0.1% (v/v) DMSO (vehicle for TOCO) or 0.1% (v/v) ethanol/ddH_2O [1:1] (vehicle for NAC) and results interpreted independently. However, replicate measurements (two groups of triplicate measurements) showed that these solvent control groups did not differ significantly and are thus presented as one group (solvent in Table 1). Following the pre-incubation period, cells were exposed to OTA (0, 6.25 or 100 μM). After 24 h, the incubation medium was aspirated and cells were harvested in 100 μl cold harvest buffer [Triton X-100 0.05% (w/v), EDTA 0.05 mM in PBS]. The amount of protein was determined in 10 μl of this suspension, while 10 μl sulfosalicylic acid 25% (w/v) was added to the remaining 90 μl sample. After centrifugation of the samples (12,000 × *g*, 5 min 4 °C), 20 μl triethanolamine (T-1377, Sigma) was added. GSH levels were determined in a mixture containing 5.3 mM NADPH and 0.6 mM 5-5′ dithio-bis (2-nitrobenzoic acid) [DTNB]. After incubation for 5 min at 30 °C, 25 μl sample or GSH standard (0, 0.5, 1.0, 2.0 and 3.0 mg/ml) was added. The reaction was initiated with glutathione reductase (0.5 U/sample) and the increase in absorption at 412 nm and 30 °C was determined. Specific activity was calculated by comparison to a standard curve. Activity was expressed as nanomoles per milligram total cellular protein.

Table 1
The effect of anti-oxidant treatment on GSH levels (nmol/mg protein) in rat PT- and LLC-PK_1 cells exposed to OTA for 4 and 24 h

OTA	Anti-oxidant	PT cells		LLC-PK_1	
		4 h OTA	24 h OTA	4 h OTA	24 h OTA
OTA 0 μM					
	Solvent[a]	3.2 ± 1.3	1.5 ± 0.4	22.9 ± 3.3	15.8 ± 2.1
	TOCO	4.1 ± 0.7	2.0 ± 0.9	26.6 ± 2.4	23.8 ± 2.1 *
	NAC	28.2 ± 4.4 *	21.1 ± 2.0 *	26.8 ± 2.5	16.5 ± 2.3
OTA 6.25 μM					
	Solvent[a]	3.7 ± 1.2	2.0 ± 2.3	20.4 ± 4.0	12.2 ± 6.8
	TOCO	4.9 ± 1.4	1.9 ± 1.3	23.5 ± 3.7	9.0 ± 3.8
	NAC	38.2 ± 4.5 *,#	15.5 ± 3.9 *,#	31.1 ± 4.0#	24.1 ± 6.5
OTA 100 μM					
	Solvent[a]	5.5 ± 1.5	ND	18.2 ± 2.0	3.4 ± 2.3 *,#
	TOCO	5.2 ± 1.2	ND	21.6 ± 1.7	5.9 ± 1.2 *
	NAC	30.4 ± 4.1 *,§	3.8 ± 0.9 *,§	28.4 ± 2.5§	8.3 ± 8.8 *

Cells were pre-incubated for 24 h with 5 μM TOCO, 2 mM NAC (PT cells) or 4 mM (LLC-PK_1) NAC. Following pre-incubation with the anti-oxidant, OTA was added and GSH levels were determined 4 and 24 h later. The data represent means ± S.D. from triplicate measurements from at least two independent experiments. Symbols indicate significant difference ($P < 0.05$). ND: below limit of detection (< 30 pmol/mg protein).

[a] Solvent-treated cells refer to cells treated with 0.1% (v/v) DMSO (vehicle for TOCO) or 0.1% (v/v) ethanol/ddH_2O [1:1] (vehicle for NAC).

* Significantly different from GSH levels in non-exposed (0 μM OTA) solvent-treated cells.

[#] Significantly different GSH levels in solvent-treated cells exposed to 6.25 μM OTA.

[§] Significantly different GSH levels in solvent-treated cells exposed to 100 μM OTA.

2.6. Detection of OTA-induced oxidative DNA damage

LLC-PK_1 cells were seeded at 5×10^4 cells/chamber on LabTek 8-chamber slides (Nalgene, cat. no. 177445, Life Technologies, Breda, The Netherlands) 2 days before the start of the experiment. As reference, cells were exposed to methylene blue (2 mM; MB) for 1 h followed by 30 min exposure to ambient light, a treatment known to generate primarily 8-oxoguanine adducts [27]. For experiments

involving OTA, LLC-PK$_1$ cells were either pre-incubated with solvent (0.1% EtOH) or with 4 mM NAC for 24 h. Cells were then exposed to 15 μM OTA for 4 and 24 h. The cells were fixed using cold 4% paraformaldehyde for 30 min. The Biotrin OxyDNA assay (Biotrin, cat. no. BIO81-DNA, Dublin, Ireland) was used to detect the formation of 8-oxyguanine adducts. The Biotrin OxyDNA assay was performed according to the manufacturers' instructions. Micrographs were taken immediately following the experiments using a fluorescence microscope (Olympus BX60, Hamburg, Germany; with a U-MNB fluorescence filter wheel, excitation wavelength between 470 and 490 nm, emission wavelength 520 and 550 nm, magnification × 20).

2.7. Protein determination

Total cellular protein was determined according to the method described by Lowry et al. [28].

2.8. Statistical analysis

Significance of differences between different data groups was first determined using a one-way ANOVA analysis within a study and then by *t*-test pairwise comparison (Bonferroni) of the groups of data using SYSTAT 8.0. Differences were considered significant if $P < 0.05$.

3. Results

3.1. OTA-induced production of ROS

Fig. 1 shows that OTA concentration-dependently induced the formation of ROS in both cell types. ROS levels of primary rat PT cells were already significantly elevated above the levels in solvent-treated (DMSO) controls after 4 h of incubation with 12.5 μM OTA (Fig. 1A).

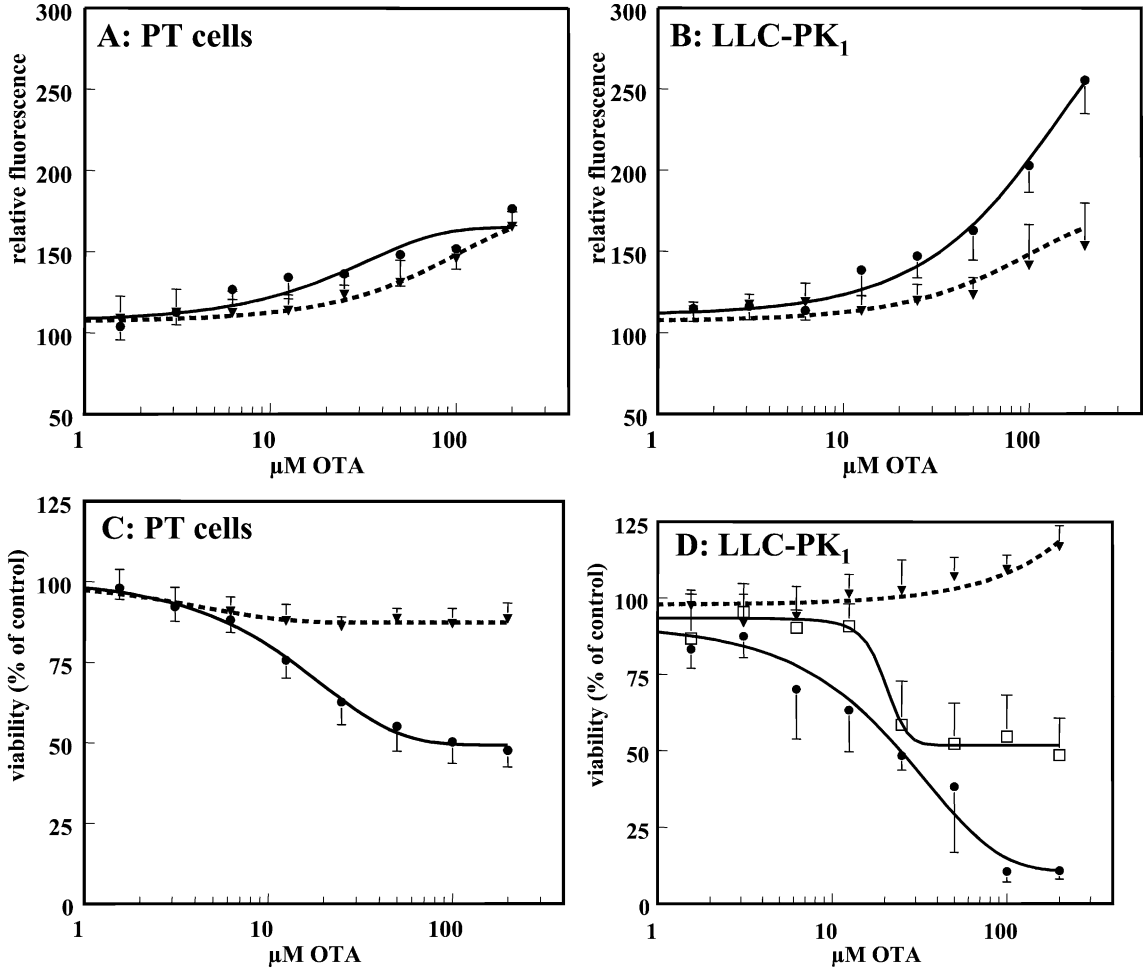


Fig. 1. OTA-induced ROS formation (A and B) and loss of cell viability (C and D) in primary PT cells and LLC-PK$_1$. Primary PT (A and C) and LLC-PK$_1$ cells (B and D) were exposed to increasing concentrations of OTA and the ROS production (A and B) or cell viability (C and D) after 4 h (- -▼- -) and 24 h (—●—) of incubation with OTA was measured as described in the Materials and Methods section. The effect of serum (10% FCS) (—□—) on cell viability after exposure of LLC-PK$_1$ cells for 24 h to increasing concentrations of OTA was also determined (D). The data are expressed as relative response as compared to DMSO-treated controls (100%) and represent means ± S.D. from quadruplicate measurements from at least two independent experiments. The results of statistical analysis are delineated in the text.

After 24 h, the ROS production was significantly elevated by OTA concentrations as low as 3.1 μM. In contrast, a significant increase of ROS levels was observed following exposure of continuous LLC-PK$_1$ cells to a minimal effective concentration of 25 μM OTA after 4 h and 12.5 μM OTA after 24 h, respectively (Fig. 1B). These data indicate that this mycotoxin induces a ROS response in a concentration- and time-dependent manner. The time-dependent increase in ROS levels was more pronounced in LLC-PK$_1$ cells as compared to the primary PT cells. In LLC-PK$_1$ cells, the formation of ROS induced by OTA concentrations >50 μM after 24 h incubation was significantly increased as compared to the ROS response evoked by equimolar concentrations OTA after 4 h. In contrast, the ROS response to OTA in rat PT cells after 24 h exposure did not differ statistically from that after 4 h.

3.2. Cytotoxicity of OTA

The cell viability of primary rat PT cells incubated for 4 h with increasing concentrations of OTA in serum-free medium decreased slightly, but this decrease was not significant. However, 24 h OTA exposure in serum-free medium clearly decreased cell viability in a concentration-dependent manner (Fig. 1C). Loss of cell viability was also time-dependent as viability after 24 h exposure to OTA was significantly lower in PT cells than after 4 h incubation for OTA concentrations of 12.5 μM and higher.

This concentration- and time-dependent loss of cell viability following OTA exposure was also observed in LLC-PK$_1$ cells. Interestingly, OTA-induced ROS production clearly preceded the loss of cell viability in LLC-PK$_1$ cells as cell viability was unaffected after 4 h incubation with OTA, while a distinct ROS response was observed at OTA concentrations exceeding 50 μM (Fig. 1D). In contrast, cell viability was even slightly increased after 4 h exposure to high OTA concentrations (100 and 200 μM) in serum-free medium ($P<0.05$ as compared to vehicle-treated cells). However, exposure to increasing concentrations of OTA for 24 h in serum-free medium resulted in a loss of cell viability in LLC-PK$_1$ cells (Fig. 1D). Loss of cell viability after 24 h was significantly different from that after 4 h OTA incubation at 12.5 μM and higher. However, the presence of serum in the incubation medium attenuated the cytotoxicity of OTA (data not shown).

In serum-free medium, the calculated IC$_{50}$ values following 24 h exposure to OTA were approximately 83 and 25 μM for PT cells and LLC-PK$_1$ cells, respectively, whereas in the presence of 10% serum, the IC$_{50}$ was approximately 50 μM. A concentration close to the IC$_{50}$ values for both cell types (100 μM for rat PT cells; 25 μM for LLC-PK$_1$) were chosen for successive experiments investigating the mechanism of OTA-mediated toxicity. The subsequent experiments were performed in serum-free medium.

3.3. Influence of anti-oxidants on the OTA-induced ROS response

Cells were pre-incubated for 24 h with the selected anti-oxidative agent and subsequently challenged with OTA still in the presence of the anti-oxidant. Treatment of rat PT cells with several concentrations (1.56–12.5 μM) of TOCO significantly decreased the OTA-induced ROS response (Fig. 2A; hatched bars). Treatment with 6.25 and 12.5 μM TOCO completely prevented the OTA-induced ROS response. Similarly, the ROS production in rat PT cells treated with 1, 2 and 4 mM NAC following exposure to OTA was not statistically different from the basal ROS levels in solvent-treated control cells (Fig. 2B; hatched bars).

In LLC-PK$_1$ cells treated with increasing concentrations (1.56–12.5 μM) TOCO and exposed to OTA, the formation of ROS was not different from that in solvent-treated cells, but was significantly in comparison with the response in cells exposed to OTA alone (Fig. 2C; hatched bars). Treatment of LLC-PK$_1$ cells with 0.5–4 mM NAC significantly decreased the OTA-mediated elevation of ROS levels at all NAC concentrations tested, in a dose-dependent manner. Treatment with 4 mM NAC appeared to completely prevent loss of cell viability in cells exposed to 25 μM OTA.

3.4. Influence of anti-oxidants on the OTA-induced loss of cell viability

In addition to monitoring the effects of anti-oxidants on the OTA-induced ROS response, the effects of TOCO and NAC on the cell viability were determined. As depicted in Fig. 2A (black bars), the treatment of the cells with TOCO up to 12.5 μM could not prevent the OTA-induced loss of cell viability in rat PT cells. In contrast, NAC treatment showed a clear dose-dependent protective effect in rat PT cells and at the tested concentrations between 0.5 and 4 mM NAC (Fig. 2B; black bars).

Similarly as observed in PT cells, TOCO up to 12.5 μM was unable to protect LLC-PK$_1$ cells against the cytotoxicity of OTA (Fig. 2C; black bars). In contrast, NAC treatment provided a dose-dependent protection of LLC-PK$_1$ cells against the cytotoxicity of OTA (Fig. 2D; black bars). In fact, the viability of the cells treated with 4 mM NAC followed by exposure to OTA was not statistically different from that of solvent-treated controls.

3.5. The role of GSH

Incubation with NAC strongly elevated GSH levels in control PT cells, while TOCO incubation did not affect basal GSH levels (Table 1). Exposure for 4 h to OTA did not statistically affect GSH levels. The GSH levels of NAC-treated PT cells were significantly higher after exposure to 6.25 and 100 μM OTA as compared to solvent- and TOCO-

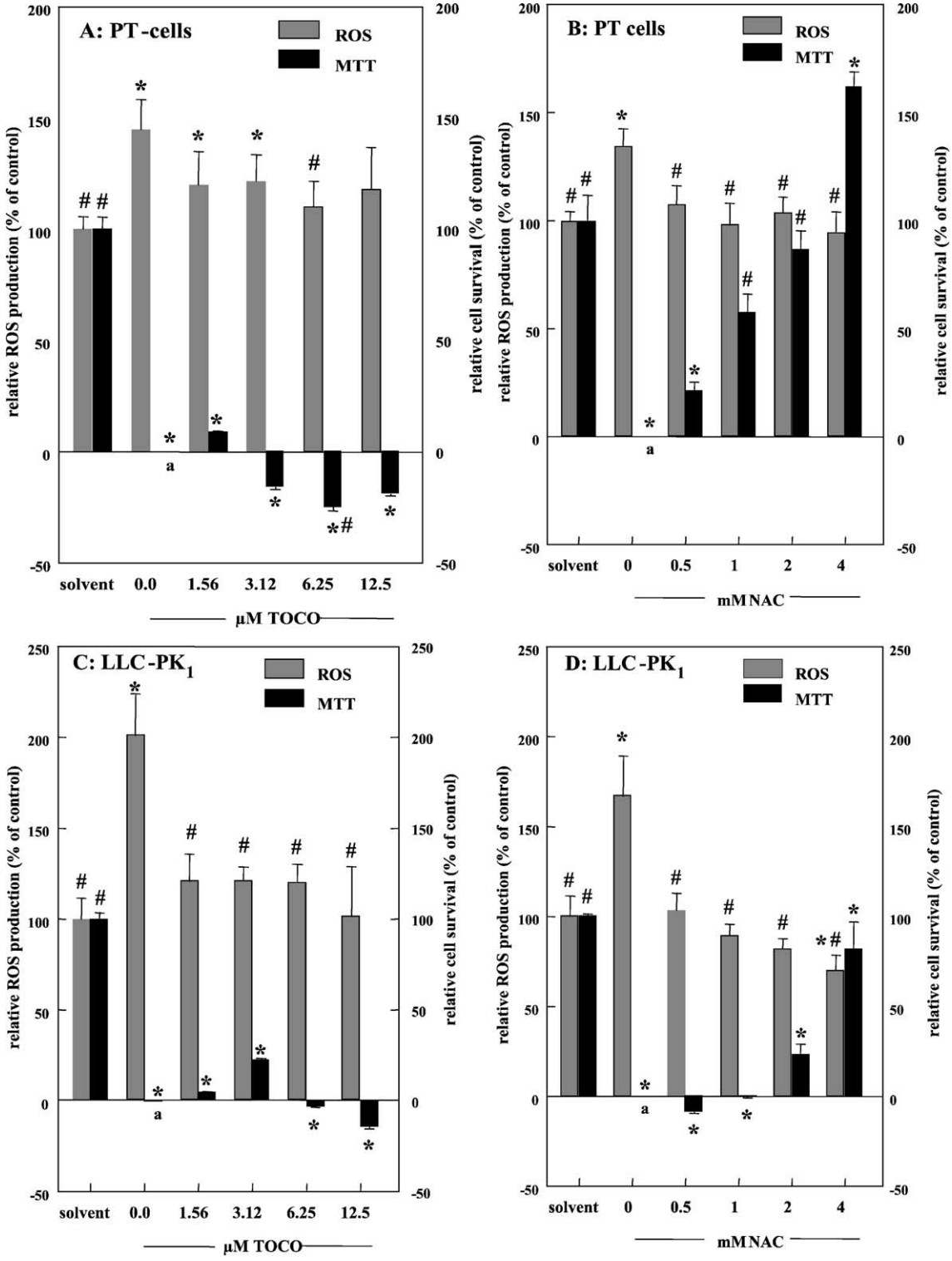


Fig. 2. The effect of TOCO and NAC on OTA-induced ROS response and loss of cell viability in renal tubular cells. Primary PT cells and LLC-PK_1 cells were pre-incubated for 24 h with indicated concentration of TOCO (A and C) or NAC (B and D). Following pre-incubation, OTA was added and the production of ROS (hatched bars) or the cell viability (MTT assay; black bars) was measured 24 h later. Cells were exposed to 100 μM OTA for ROS determinations (A–D). For determination of the effect on cell viability (MTT assay), PT cells were exposed to 100 μM OTA (A and B), while LLC-PK_1 cells were incubated with 25 μM OTA for 24 h (C and D). The OTA concentrations were chosen on the basis of the IC_{50} values in the respective cell types. The data represent the relative response in treated cells as compared to the response in solvent-treated cells (100%). The results of the MTT assay are expressed as protection against the OTA-induced loss of viability; therefore, the viability of the cells exposed to OTA alone (0 μM TOCO or NAC) was set at 0% (indicated in the figure as "a"). The data represent means ± S.D. of quadruplicate measurements from at least two independent experiments. * Significantly different from response in solvent-treated cells ($P<0.05$). # Significantly different from the response in OTA-alone treated cells ($P<0.05$).

treated cells (Table 1). TOCO treatment did affect the OTA-induced GSH depletion.

A similar pattern was observed after 24 h exposure to OTA, although the GSH levels were below the limit of detection (< 30 pmol/mg protein) after exposure to 100 μM OTA, indicating significant depletion of GSH (Table 1). Moreover, an evident decrease of GSH levels following 24 h OTA exposure was observed in NAC-treated PT cells. TOCO treatment did not result in preservation of the GSH levels in OTA-exposed cells.

In LLC-PK_1 cells, GSH levels were not statistically affected after 4 h incubation with OTA, similarly as observed in PT cells (Table 1). Table 1 shows that NAC treatment resulted in significantly higher GSH levels after 4 h exposure to 6.25 and 100 μM OTA, as compared to solvent-treated cells. TOCO treatment had no effect on the GSH levels in OTA exposed LLC-PK_1 cells.

The decrease in GSH levels in LLC-PK_1 cells following 24 h OTA exposure was more pronounced at 100 μM OTA than that at 6.25 μM OTA suggesting a dose-dependent trend (Table 1). TOCO treatment appeared to have slightly increased the basal GSH levels, but was unable to preserve GSH levels after 24 h exposure to OTA. The GSH levels of TOCO-treated cells decreased to the same extent as in solvent-treated cells after exposure to OTA. Table 1 shows that NAC treatment resulted in significantly higher GSH levels after 24 h incubation with 6.25 μM OTA as compared to solvent- and TOCO-treated cells, although this effect was only significant as compared to TOCO-treated cells (Table 1). Taken together, these results illustrate that NAC treatment preserved GSH levels in OTA-exposed PT- and LLC-PK_1 cells. This protective effect by NAC treatment was more pronounced in PT cells as compared to NAC-treated LLC-PK_1 cells.

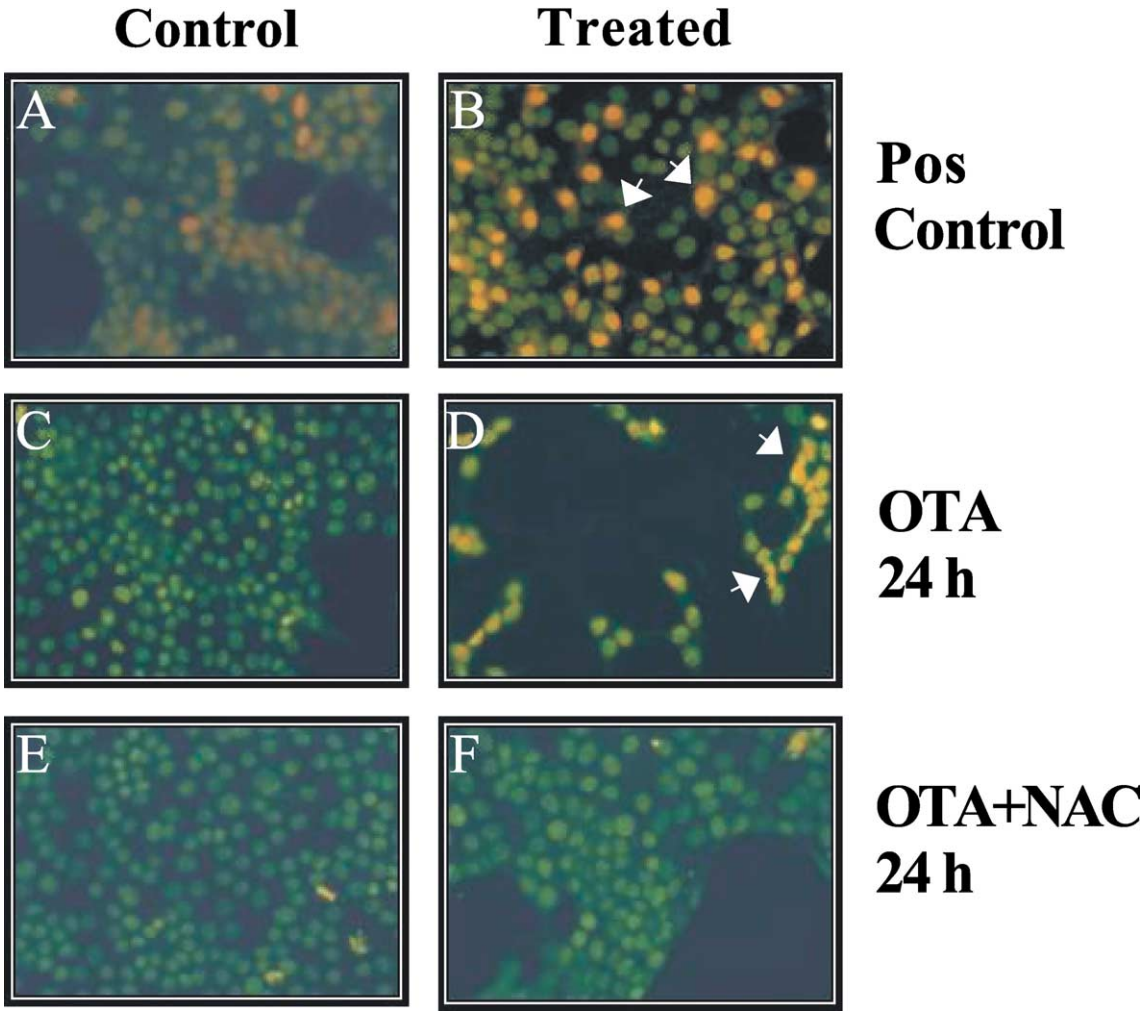


Fig. 3. Formation of 8-oxoguanine adducts in OTA-exposed LLC-PK_1 cells. (A and B) LLC-PK_1 cells were exposed for 1 h to solvent (panel A) or 2 mM methylene blue (Pos Control) (panel B) followed by 30 min exposure to ambient light. Formation of 8-oxoguanine was assessed using the Biotrin oxyDNA assay kit as described in the Materials and Methods section. (C and D) LLC-PK_1 cells were exposed for 24 h to solvent (panel C) or 15 μM OTA (panel D) for 24 h and assessed for 8-oxoguanine formation. (E and F) Cells were treated with 4 mM NAC for 24 h before 24 h exposure to solvent (panel E) or 15 μM OTA (panel F). The fixed cultures were then assayed for the formation of 8-oxoguanine adducts. The arrows indicate representative 8-oxoguanine positive cells. The presented micrographs were taken from representative areas from one of two independent experiments. Original magnification: × 20.

3.6. OTA-induced oxidative DNA damage

Fig. 3 shows the formation of 8-oxoguanine in LLC-PK_1 cells after treatment with OTA, as assessed with the Biotrin OxyDNA assay. The 8-oxoguanine adduct is a sensitive marker of DNA base damage caused by oxygen free radicals [24,29] and was detected in oxidant-treated cell cultures using the same assay [30,31].

Panel A and B show the effect of methylene blue (MB) treatment in combination with ambient light, which was used as positive control. The combination of visible light and MB is known to generate primarily 8-oxoguanine adducts [27]. Panel A shows background staining, while after 30 min, treatment with 2 mM MB clearly increased the number of positively stained cells. This indicates that LLC-PK_1 cells respond to MB treatment by an increase in 8-oxoguanine formation.

In contrast, exposure of LLC-PK_1 cells for 4 h to 15 μM OTA did not result in the formation of 8-oxoguanine adducts (data not shown), which is in agreement with the absence of ROS response below 25 μM OTA after 4 h incubation (Fig. 1B).

Exposure of LLC-PK_1 cells to 15 μM OTA (panel D) for 24 h resulted in a clear increase in 8-oxoguanine adduct formation as compared to the solvent-treated cells (panel C). This concentration of OTA found to induce oxidative DNA damage was comparable to those elevating ROS levels significantly in 24 h (Fig. 1B; 12.5 μM).

Pre-treatment of LLC-PK_1 cells with NAC resulted in a slight reduction in the level of background staining as compared to solvent-treated controls (panel E vs. C). However, treatment of the cells with 4 mM NAC to 15 μM OTA prevented the formation of 8-oxoguanine adducts (panel F), suggesting that the observed OTA-induced 8-oxoguanine formation was ROS-dependent.

4. Discussion

Previous studies implicate oxidative pathways in the toxicity of OTA [5,15,16,18,32,33]. The present study was conducted to investigate the role of oxidative stress in the diverse toxic responses of renal tubular cells following exposure to this mycotoxin.

Exposure of primary PT- and LLC-PK_1 cells to OTA increased the ROS levels in a concentration- and time-dependent manner. Interestingly, OTA-induced ROS production preceded the loss of cell viability particularly in LLC-PK_1 cells, indicating that the ROS response may contribute to the OTA-induced cytotoxicity and is not a consequence of it. This assumption is supported by the observation that NAC treatment completely protected cells against the OTA-induced loss of viability. Previous studies in rodents exposed to OTA show that several anti-oxidants (e.g. SOD and catalase) also provide protection in vivo [5,34].

The ROS-detecting probe, H_2DCF-DA, detects a wide range of ROS species including $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$ [35]. Lipid peroxides, although at a slow rate and in the presence of lipoxygenase or Fe^{2+}, were also shown to oxidize this probe [35]. The effects of different anti-oxidants, including TOCO and NAC in the present study, superoxide dismutase and catalase in previous studies [5], indicate that more than one ROS might be involved in the observed oxidative stress following OTA exposure.

TOCO is a chain-braking anti-oxidant, particularly effective in scavenging peroxyl and alkoxyl radicals, thus preventing the propagation of LPO [23,36]. The lack of cytoprotection by TOCO observed in PT and LLC-PK_1 cells suggests that LPO contributes only marginally to the cytotoxicity of OTA. This seems to be in contrast to previous studies suggesting LPO as possible mechanism of OTA toxicity [16,17]. However, our findings are in agreement with the conclusion of Aleo et al. [37] that despite the fact that OTA stimulates LPO apparently preceding cell death, this does not appear to contribute to OTA-induced proximal tubular cell death. Interestingly, the latter study also showed that OTA inhibits site I and II mitochondrial respiration and the authors concluded that OTA toxicity in renal PTs was related to mitochondrial dysfunctioning [37]. It remains to be established, however, if oxidative stress, in particular LPO, is involved in the reported loss of mitochondrial function.

Another indication of OTA-related oxidative stress is provided by the observed depletion of the cellular GSH pool in the tubular cells following 24 h exposure to OTA. TOCO treatment did not preserve GSH levels, while pre-incubation with NAC apparently prevented GSH levels to decrease below critical levels following exposure to OTA. Prevention of OTA-induced loss of GSH by NAC was most pronounced in PT cells, as compared to the modest protection observed in LLC-PK_1 cells. This also corresponds to the relatively greater protective effect of NAC in PT cells on the OTA-mediated ROS response and loss of viability. The quantitative differences of NAC treatment in the two renal cell types may be explained by a decreased ability of the LLC-PK_1 cells to transport and accumulate NAC. For instance, LLC-PK_1 are shown to lack the organic anion transport system [22], while we have shown previously that rat PT cells functionally express this transporter [20]. Furthermore, the basal GSH levels in the two cell types were substantially different. While NAC treatment resulted in a 10-fold increase in the GSH levels of PT cells, it did not increase the levels in LLC-PK_1 cells. The high basal GSH levels in LLC-PK_1 cells most likely prevent a further increase in response to NAC exposure by feedback inhibition of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis (for a review, see Ref. [38]).

In addition to the anti-oxidative effect of NAC and GSH, the protective effect of NAC may be explained by the nucleophilicity of GSH. Covalent binding of OTA to GSH could inactivate the mycotoxin and promote its excretion

from the tubular cell. Our results, therefore, point toward a central role for GSH in the protection against OTA-induced cellular toxicity.

We are the first to show that OTA induces the formation of 8-oxoguanine in vitro, providing unequivocal evidence of oxidative DNA damage. Oxidative base modifications may result in mutations, whereas oxidation of deoxyribose moieties may induce base release or DNA strand breaks (Ref. [24] and references therein). Singlet oxygen selectively reacts with guanine and predominantly forms 8-oxoguanine, which is also one of the products formed after attack of DNA by the hydroxyl radical [24,27].

In contrast, Gautier et al. [39] found no evidence for 8-oxoguanine formation in rats treated with OTA (up to 2 mg/kg body wt). The latter authors ascribe the discrepancy between the generation of ROS in vitro and in vivo and the resultant oxidative damage to the protective activity of GSH and TOCO. Furthermore, those authors suggest that the maximal plasma concentrations (15 μM) were below the minimal OTA concentration (30 μM) required to induce LPO in vitro and were consequently too low to induce 8-oxoguanine formation in the rats.

Formation of 8-oxoguanine adducts occurred in a time-dependent manner, which correlates well with the OTA-induced production of ROS in these cells. Using the same method as described in this study, Morin et al. [30,31] showed the formation of 8-oxoguanine in cell cultures treated with pro-oxidants. The OTA concentrations elevating the ROS levels and inducing the formation of 8-oxoguanine were of the same order of magnitude. Recently, Griffith [38] showed that OTA in the presence of Fe(III)-porphyrin facilitates single-strand cleavage of supercoiled plasmid DNA through the production of ROS (i.e. hydroxyl radical $HO^{\cdot}$). The author proposes that OTA is oxidized to a (hydro-) quinone. Quinones are known to redox cycle, thus creating free radicals that bind covalently to a variety of cellular macromolecules [40]. The formation of the (hydro-) quinone of OTA implicates the reported involvement of oxidative metabolism in the genotoxicity of OTA [41] and could help to explain the previously described CYP450-dependent mutagenicity of OTA and its metabolites [42]. Redox cycling causes the oxidation of reducing equivalents in the cell, including GSH, and may thus contribute to the observed depletion of GSH in the present study. Moreover, Gautier et al. [43] have shown that most, if not all, of the DNA adducts attributed to OTA and its metabolites do not contain an OTA-moiety, further implicating oxidative stress as important determinant in the OTA-induced DNA damage. OTA and its metabolites have also been observed to induce the formation of DNA adducts in rats and mice [34,44–46]. Interestingly, DNA adduct formation was reduced by pre-treatment of mice with vitamins A, C and E, indicating the involvement of oxidative stress in OTA-mediated DNA damage [46].

The results of the present study show that OTA induced a comparable response in the two PT cell models, the primary PT- and the continuous LLC-PK_1 cells. In addition, the effects of TOCO and NAC on the OTA-mediated toxicity were similar and differed only quantitatively. Particularly, NAC showed a more potent protection in PT cells that can be explained by its more pronounced effect on the GSH levels in the primary cells. This suggests that the primary PT cells are slightly less sensitive to the actions of OTA, as judged by the lower maximal ROS response, the higher IC_{50} and the more potent NAC protection in the primary PT cells.

In conclusion, cultured renal tubular cells respond to OTA exposure by elevation of ROS levels, depletion of GSH levels and an increase in oxidative DNA damage, which are indicators of oxidative stress. Taking our results obtained with TOCO and NAC into account, it may be hypothesized that the OTA-induced oxidative stress contributes to both the DNA damage and cytotoxicity. The effects of TOCO indicate that OTA-induced LPO does not contribute profoundly to the observed loss of cell viability. The strong protective effect of GSH indicates that oxidative-stress-related protein damage is a major determinant in OTA-mediated toxicity. To which extent the ROS formation contributes to other toxic effects, in particular by disrupting protein synthesis, affecting intracellular calcium handling, or inducing mitochondrial dysfunctioning etc., requires further exploration. Furthermore, OTA-related ROS formation may induce epigenetic effects.

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