The Nephrotoxin Ochratoxin A Induces Key Parameters of Chronic Interstitial Nephropathy in Renal Proximal Tubular Cells

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Key Words

Ochratoxin A • ERK1/2 • Renal disease • Interstitial nephropathy • Apoptosis • Necrosis • Epithelial tightness • Inflammation • Fibrosis • Epithelial-to-mesenchymal transition

Abstract

Ochratoxin A (OTA) is a nephrotoxic and cancerogenic mycotoxin. There is epidemiological evidence that OTA exposition leads to cortical interstitial nephropathies in humans. However, virtually no data are available investigating the effect of OTA on renal cortical cells with respect to induction of nephropathy. Thus, we investigated whether OTA is able to induce changes of cellular properties potentially leading to interstitial nephropathy, using proximal tubular cell lines (OK, NRK-52E). OTA decreased cell number and cell protein time and dose dependently. Accordingly we investigated the effect of 100 nM or 1000 nM OTA. The decline of cell number after OTA exposure is due to necrosis and apoptosis, as measured by LDH release or DNA ladder formation and caspase-3 activation, respectively. OTA incubation of proximal tubular cells also resulted in a loss of epithelial

tightness as determined by diffusion of FITC labeled inulin. Inflammation, fibrosis and epithelial-to-mesenchymal transition are described in chronic interstitial renal disease. Therefore, we also investigated the effect of OTA on NF κ B activity, collagen secretion and generation of α smooth muscle actin. OTA alone was sufficient to induce the latter parameters in proximal tubular cells. Finally, OTA is a nephrotoxic substance and elevated activity of mitogen activated protein kinases (MAPK) is described in nephropathies. As we investigated the effect of OTA on activity of ERK, JNK and p38 by ELISA, we found that OTA activates the MAPK measured dose dependently. In summary, OTA induced phenomena typical for chronic interstitial nephropathy, like loss of cells and epithelial tightness, necrosis and apoptosis as well as markers of inflammation, fibrosis and epithelial-to-mesenchymal transition in proximal tubular cells. Thus, we could show for the first time that OTA is able to induce key parameters of nephropathy in proximal tubular cells in culture. Moreover OTA interacts with MAPK and thus may exert its specific toxic actions.

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Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite that is found in a variety of animal chow and human food [1, 2]. OTA enters the food chain by cereals and its products, coffee, beer, wine, poultry and pork. Due to its extreme stability and its almost ubiquitous incidence as food contaminant, complete avoidance of dietary intake of OTA is impossible [3].

OTA was shown to be a nephrotoxic substance since substantial time [4] with an elimination half-life of around 840 h in humans [5]. Numerous studies showed the occurrence in human blood serum and human kidney. 50 - 100 % of the human samples were positive for OTA (reviewed in [6]). There is evidence that OTA is involved in the pathogenesis of Balkan endemic nephropathy [7], chronic interstitial nephritis in northern Africa and karyomegalic interstitial nephritis [8]. The toxicological profile of OTA includes teratogenesis, nephrotoxicity and immunotoxicity [6]. Moreover, OTA was classified as being carcinogenic in animals [9] and as a possible carcinogen in humans [10].

Virtually all progressive renal diseases are characterized by cell death, inflammation, fibrosis [11-13] and epithelial-to-mesenchymal transition [14]. Balkan endemic nephropathy, which is qualified by the WHO as "...progressive and very gradually developing renal failure with insidious onset... The last stage shows marked fibrosis ..." is characterized by tubular degeneration, interstitial fibrosis and impaired renal function [7]. This is in line with numerous animal studies, showing development of renal disease accompanied by proximal tubular atrophy and cortical interstitial fibrosis and inflammation after exposition to OTA [15, 16].

Damage of proximal tubular cells is thought to be a motor driving tubulointerstitial disease [17]. Therefore, it is most surprising, that almost no experimental data are available on the effect of OTA on renal cortical cells (proximal tubular cells) with respect to the mentioned key parameters of chronic interstitial nephritis. As we could show previously that OTA induces apoptosis in proximal tubular cells (IHKE cells), we hypothesized that maybe other typical parameters of chronic renal disease will be affected by OTA exposure, too. Therefore we investigated the effect of OTA on selected parameters of chronic renal cortical disease in proximal tubular cell lines, i.e.: cell number, necrosis and apoptosis, fibrosis, inflammation and epithelial-to-mesenchymal transition. Moreover, as there is evidence that OTA exposition leads to activation of mitogen activated protein kinases

(MAPK) in renal cells [18-20] we also investigated the effect of OTA on activation of the antagonistic MAPK ERK1/2 on the one hand and p38 and JNK on the other hand. In the following study, we present in vitro data showing for the first time that exposure of proximal tubular cells to OTA alone is able to, all at once, induce a bundle of effects typical for chronic interstitial cortical renal disease in animals and humans.

Materials and Methods

Cell culture

OK cells were obtained from Dr. Biber, Dept. of Physiology, University of Zürich. Cells were maintained in culture at 37°C in a humidified 5% CO₂, 95% air atmosphere. The growth medium was minimal essential medium (MEM), pH 7.4, supplemented with Earl's salts, non-essential amino acids, 10 % (v/v) fetal calf serum (Biochrom KG, Berlin, Germany) and 26 mmol/l NaHCO₃. NRK-52E cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. NRK-52E cells were cultured in DMEM medium, enriched with 26 mmol/l NaHCO₃ and 5 % (v/v) fetal calf serum. NRK-52E cells were cultured on petri dishes or permeable supports (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). The cells were maintained at pH 7.4 and 37°C, and gassed with 95% O₂ / 5% CO₂. All studies were performed between passage 60 and 100. The seeding density was 0.4·10⁶ cm⁻². The medium was changed every third day and the cells were used for experiments at day 10 after seeding. All experiments were performed with cells that were serum starved for 24 hours before the experiments. Cells were seeded on 6well or 24well plates, petri dishes or permeable supports as appropriate.

pMAPK-ELISA

Quantification of MAPK-phosphorylation was done by ELISA according to Versteeg et al [21] with minor modifications described previously [22]. Cells were seeded in 96-well plates (Maxisorp, Nunc) and serum-starved for 24 h prior to the experiment. After stimulation as indicated in the text, the cells were fixed with 4% formaldehyde in PBS and washed three times with PBS containing 0.1 % Triton X-100. Cells were blocked with 10% fetal calf serum in PBS/Triton for 1 h and incubated overnight with the primary antibody (1:1000). After three washes cells were incubated with the secondary antibody (peroxidase-conjugated mouse anti-rabbit antibody, dilution 1:10000) in PBS/Triton with 5% BSA for 1 h at room temperature and washed three times with PBS/Triton for 5 min and twice with PBS. Subsequently, the cells were incubated with 50 µl of a solution containing 0.4 mg/ml o-phenylenediamine, 11.8 mg/ml Na₂HPO₄, 7.3 mg/ml citric acid and 0.015% H₂O₂ 15 min at room temperature in the dark. The resulting signal was detected at 490 nm with a multiwell multilabel counter (Victor², Wallac, Turku, Finland). Finally, protein content in the wells was determined with Trypan Blue solution. Primary antibody against p-ERK1/2, p-p38 and p-JNK were from cell signaling technology®, Beverly, USA.

Caspase-3 activity assay

Caspase-3 activity was measured according to the manufacturer's instructions (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications [23]: cells were washed once with PBS buffer (4°C) and incubated with 150 µl cell lysis buffer for 10 min on ice, harvested, and centrifuged at 16000g for 10 min at 4°C. 60 µl of the supernatant was incubated with 50 µmol/l DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; end-concentration) for 60 min at 37°C, and fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Victor², Wallac, Turku, Finland). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. As control, cell extracts were incubated as described above but in the presence of caspase-3 inhibitor zDEVD-CHO. No activity could be found under these conditions. Protein content was determined with bicinchoninic acid assay (Pierce) using bovine serum albumin as standard.

DNA ladder formation assay

DNA ladders were measured as described in [23]. Cells in culture medium were collected by brief centrifugation. Seeded cells on the Petri dish were harvested in cell lysis buffer (5 mmol/l Tris, 20 mmol/l EDTA pH 8.0, 0.5% Triton X-100), briefly incubated on ice and together with the previous cell pellet centrifuged (20 min, 16000xg, 4°C). 50 µg/ml proteinase K and 40 µg/ml RNase A were added to the supernatant and incubated for 60 min at 37°C. DNA was extracted by adding the same volume phenol/chloroform/isoamylalcohol [25:24:1 in TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0)]. After shaking and centrifugation (30 min, 3420 x g) the upper phase was collected. One tenth volume 3 mol/l sodium acetate pH 5.2 and two volumes ice cold (-20°C) ethanol were added and samples were left overnight at -20°C. After centrifugation (30 min, 16000xg, 4°C), pellet was washed with 70% ice cold ethanol and dried. DNA was solved in water. DNA concentration was measured at 260 nm in a photometer. DNA ladder was visualized in 1.5% agarose gel.

Determination of collagen secretion

In order to determine whether OTA induces collagen I, III or IV secretion in our cells we performed enzyme-linked immunosorbent assay (ELISA) as described in [24]. Media and collagen standards (Sigma, Deisenhofen, Germany) were incubated for 24 h in 96-well Nunc-Immuno Maxisorb plates (Nalge Nunc International, Naperville, IL) followed by washing and blocking with 2% bovine serum albumin in phosphate buffered saline. Subsequently the wells were incubated with rabbit antibody against collagen I, III or collagen IV (1:1000; Biotrend, Köln, Germany) for 1h at room temperature. After three washes with 0.05% Tween in phosphate buffered saline, HRP-conjugated secondary antibody (1:5000; Biotrend, Köln, Germany) was applied for 1 h at room temperature. After three washes with 0.05% Tween in phosphate buffered saline the wells were incubated with o-phenylenediamine (Sigma, Deisenhofen, Germany) and the reaction was stopped after 15

min with 1 N H₂SO₄. The absorbance at 490 nm was determined using a multiwell-multilabel reader.

RT-PCR

RNA from cells was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). In brief, RT-PCR was performed according to Superscript One-Step RT-PCR system protocol (Invitrogen, Carlsbad, USA). cDNA was generated at 55°C for 15 min and then the samples were denatured at 94°C for 2 min. PCR amplification was performed in 35 cycles of 94°C for 15 s, then 55°C for 30 s and 72°C for 30 s. For α-SMA, the primers were 5' - gat cac cat cgg gaa tga acg - 3' (sense) and 5' - ctt aga agc att tgc ggt gga - 3' (antisense), resulting in an 389 bp RT - PCR - product, according to [25].

Determination of epithelial tightness

For measurement of epithelial tightness, cells confluent grown on filter membranes (6well, Falcon, USA) were incubated with 100mg/l FITC marked inulin at 4°C for up to 55 min at the basolateral side. At 5, 25, 55 min 200 µl of the apical buffer (PBS) were analyzed for FITC fluorescence using a multiwell and multilabel counter (Victor², Wallac, Turku, Finland). The expired volume was immediately replaced by fresh buffer in order to avoid hydrostatic effects.

SEAP-Assay detection of NFκB

Cell signaling was assessed by the Mercury™ Pathway Profiling reporter gene assay system from Clontech Inc. using secretory alkaline phosphatase (SEAP) as reporter and pEGFP-C1 (Clontech Inc, San Francisco, CA) as transfection efficiency control. SEAP-activity in the media was determined with the AttoPhos® System from Promega (Mannheim, Germany) and the cellular EGFP content was determined using a microplate spectrofluorometer (Victor², Wallac, Turku, Finland). SEAP activity is always expressed as activity corrected for transfection efficiency, as determined by EGFP, and normalized to cell protein.

Other methods

Cell number and cell volume was determined using a Coulter Counter. Protein content as measured by BCA protein assay (Pierce, Rockford, USA) (see the results section or the figure legends for additional details on experimental protocols). LDH release was measured according to [26] at room temperature in a photometer.

Data analysis

Data are presented as means ± SEM respectively. The *n* value is given in the text or in the figures and stands for the number of supports used for the respective experiments (wells, filters, petri dishes). For all experiments, *n* equals the number of culture plates or filters used to perform the measurements. Statistical significance was determined by unpaired Student's *t*-test or ANOVA as appropriate. Differences were considered statistically significant when *P* < 0.05.

Materials

If not stated otherwise, chemicals were from Sigma.

Fig. 1. Effect of OTA incubation on OK cell number. Effect of incubation with OTA (10 nM, 100 nM and 1000 nM) for up to 72 h on total OK cell number. $n = 3 - 6$ for every data point. Statistical significance is indicated by * or # respectively.

Results

We investigated the effect of OTA on cell number in two distinct cell lines of proximal tubular origin: OK cells (opossum) and later on NRK-52E cells (rat). OTA led to a time- and dose-dependent decrease of OK cell number, as indicated in figure 1. After 48 h incubation with OTA cell number was decreased to 30 % or 70 % of untreated controls for 100 nM or 1000 nM OTA, respectively. The effect of 10 nM OTA on cell number did not differ from 100nM OTA over time. For both 100 nM and 1000 nM OTA, the half maximal effect occurred after 24 h incubation. Therefore, all subsequent experiments using OK cells were performed using a 24 h incubation period.

Substantial loss of epithelial cells should lead to leakage of epithelia. Therefore, we measured diffusion of FITC labeled inulin through OK cell epithelia incubated with 100 nM or 1000 nM OTA. As presented in figure 2A, incubation with 100 nM or 1000 nM OTA for 24 h increased FITC inulin diffusion through OK cell epithelia to a similar extent. Thus, in the concentrations tested OTA induced detectable leakage in proximal tubular epithelium.

To determine whether the decrease in cell number described above is due to necrosis, we measured the effect of OTA on LDH release (Fig. 2B). Cytosolic LDH is released into the medium if integrity of the cell membrane is deteriorated in cells suffering from necrotic cell death. In OK cells 1000 nM OTA led an increase of

Fig. 2. Effect of OTA on epithelial tightness and necrosis after 24h. Epithelial tightness was determined by diffusion of fluorescence labeled inulin for up to 60 min through incubated OK cell epithelia. All data were normalized to % of control at $t = 5$ min. Increase of fluorescence in the apical compartment is given as increase in % of control at $t = 5$ per minute. Diffusion is given as pmol fluorescein per minute and per filter. $n = 19 - 22$ for every bar. * indicates significant difference from control. Necrosis was determined by release of cytosolic lactate dehydrogenase (LDH) into cell culture medium. LDH release is given as % LDH activity in the supernatant in relation to total LDH activity of supernatant and cell lysat set as 100 %. Control values given in units enzymatic activity are $9,89 \cdot 10^{-5} \pm 2,17 \cdot 10^{-5}$ for supernatant and $261,9 \cdot 10^{-5} \pm 40,5 \cdot 10^{-5}$ for lysat. $n = 5$ for all bars. * indicates significant difference from control.

LDH release after 24 h, whereas 100 nM OTA did not increase LDH release. Thus, necrotic cell death in OK cells after 24 h is induced by OTA in the micromolar concentration range, whereas nanomolar concentrations exerted no effect.

As 100 nM OTA induced cell loss after 24 h without inducing necrosis, we wanted to know what the mechanism of this particular cell loss could be. As OTA

Fig. 3. Effect of 24 h OTA incubation on apoptosis in OK cells. DNA ladder formation in OK cells after 24 h exposure to OTA. Each lane was loaded with 40 μ g DNA. n is 3 for every bar. * indicates significant difference from control and # indicates significant difference from the lower OTA concentration used. Insert showing typical gel from DNA ladder formation. Gels were scanned and the area under curve (AUC) was determined for every experimental condition and normalized to controls (control set as 1). Control AUC was 4033 $\pm$ 1198 in density units under curve. Caspase-3 activity in OK cells after 24 h exposure to OTA. n is 9 - 12 for every bar. Control value is 11219 $\pm$ 7035 in AFC counts per mg cell protein. * indicates significant difference from control and # indicates significant difference from the lower OTA concentration used.

is known to induce apoptosis in renal cells [20], we investigated the effect of OTA incubation on apoptosis. Apoptosis was determined using two independent assays. First, we measured the formation of DNA ladders in OK cells undergoing the already mentioned incubation scheme. As indicated in figure 3A, incubation with OTA for 24 hours induced DNA ladder formation dose dependently

Fig. 4. Effect of OTA on parameters of fibrosis and inflammation after 24h. (A) Collagen-ELISA detecting the amount of secreted collagens I, III, IV by OK cells after incubation with OTA. Collagen synthesis after 24 h OTA is shown. Collagen secretion was determined as described in the methods section and normalized to the respective control values (set as 100 %). Control values are as follows: collagen I: 1226 $\pm$ 41, collagen IV: 41 $\pm$ 8, collagen III: 66 $\pm$ 7; all given in ng of the respective collagen per mg cell protein. n is 9 - 13 for every bar. Significant difference from controls is indicated by asterisks. (B) Effect of 24 h OTA incubation on NFkB activity in OK cells. NFkB activity was detected by SEAP assay as described in the methods section. Control value is 4,27 $\pm$ 0,27 in SEAP counts per μ g cell protein. n = 5 for all bars. * indicates significant difference from control and # indicates significant difference to the lower OTA concentration used.

in OK cells. Second, we assayed apoptosis measuring activation of the apoptotic enzyme caspase-3 (Fig. 3B). Similar to what was measured for DNA ladder formation, OTA dose dependently increased caspase-3 activity in OK cells after incubation for 24 h. In summary, apoptotic cell death in proximal tubular OK cells is induced dose dependently by OTA. Moreover, apoptosis already occurs

Fig. 5. Effect of OTA incubation on NRK-52E cell number and epithelial - mesenchymal - transition in NRK-52E cells. Effect of incubation with OTA (100 nM, 1000 nM and 10000 nM) for up to 96 h on total cell number of NRK-52E cells. $n = 3 - 6$ for every data point. Effect of OTA incubation on generation of α SMA in NRK-52E cells. α SMA - mRNA was detected by multiplex RT - PCR using GAPDH as internal control. RT - PCR

products were separated in agarose gels and were analyzed densitometrically. Control values (in AUC density counts) are 491 ± 166 for α SMA and 4983 ± 539 for GAPDH. The control ratio was set as 100 %. The graph represents data from 5 different sets of experiments, RNA extraction and subsequent RT - PCR (as indicated in the bars). * indicates significant difference from control.

after OTA incubation in the nanomolar concentration range explaining cell loss without necrosis.

OTA is thought to be associated with generation of renal cortical fibrosis [27]. Thus, we investigated the effect of OTA on the secretion of collagen I, III and IV from OK cells after 24 h by specific ELISA technique (Fig. 4A). 100 nM OTA did not increase the secretion of any collagen tested. 1000 nM OTA stimulated secretion of collagen I, III and IV. Thus, secretion of extracellular matrix in OK cells is increased after OTA exposure.

Inflammation is thought to be a hallmark in development of chronic renal disease and additionally is strongly associated with renal fibrosis. As fibrosis was induced by OTA, it therefore seemed reasonable to investigate whether in OK cells inflammation is also promoted by OTA. NF κ B is an important central inducer of inflammation [28]. Therefore, we investigated the effect of OTA on NF κ B activity using a specific SEAP assay. As indicated in figure 4B, OTA dose dependently led to an increase in NF κ B-activity in OK cells after 24 h. Thus, OTA alone is able to induce the inflammatory cascade in proximal tubular cells.

Transformation of tubular epithelial cells into a myofibroblast like phenotype is described to occur in renal disease [29], which leads to loss of epithelial polarity.

Together with cellular transformation, expression of α -smooth muscle actin (α SMA) takes place which is not present in intact renal tubular epithelial cells [30]. The rat is a well defined organism with respect to DNA-sequence, whereas almost no sequence data is available on OK cells (opossum). As RT-PCR using OK cell RNA was not successful, we decided to perform RT-PCR experiments using RNA from NRK-52E (renal cortical epithelial cells from rat) cells. Therefore, we first investigated the effect of OTA on NRK-52E cell survival. In comparison to OK cells, OTA showed decelerated onset of action on cell number in NRK-52E cells and less sensitivity to OTA (Fig. 5A). Nevertheless, after 48 h incubation a significant difference in cell loss induced by 100 nM or 1000 nM OTA occurred, which is similar to what was shown in OK cells after 24 h. As half maximal effect of OTA takes place after 48 h, this time point was chosen for incubation of NRK-52E cells with OTA. Figure 5B shows that exposition of NRK-52E cells to 100 nM OTA did not increase the amount of α SMA RNA present, whereas incubation with 1000 nM OTA did. Thus, OTA induced epithelial-to-mesenchymal transition in renal proximal tubular NRK-52E cells.

OTA is classified as nephrotoxic substance and it is known that OTA induces activation of ERK1/2 and JNK

in a renal cell line derived from the collecting duct (MDCK) [19, 20]. As there is evidence that ERK1/2 activity is elevated in interstitial nephropathy (see discussion) we wanted to know whether the mitogen activated protein kinases (MAPK) ERK1/2 and its counterparts p38 and JNK are activated by OTA in a proximal tubular derived cell line (OK cells). OTA, given for 24 h, activated ERK1/2, JNK and p38 in a dose dependent manner (Fig. 6). As MAPK are known to regulate cellular fate, we speculate that their differential activation may be involved in OTA toxicity.

Discussion

We present experiments testing whether the well known nephrotoxin OTA is able to induce key parameters associated with chronic interstitial renal disease, as there are: necrosis and programmed cell death (apoptosis), inflammation, fibrosis and epithelial-to-mesenchymal transition.

Most of the experiments were performed using OK cells, which are an accepted and well established model system representing proximal tubular cells. Hence, these cells suffer from being offspring of opossum kidney, a species which is not well described with respect to its genomic sequence. Thus, we decided to perform the RT-PCR experiments (detection of α SMA RNA) using the rat proximal tubular cell line NRK-52E. OTA incubation of both cell lines exerted a well comparable effect on cell number, with only the onset of action being delayed in NRK-52E cells as compared to OK cells. Moreover, the effect of 100 nM and 1000 nM on NRK-52E cell number after 48 h showed comparable pattern as presented for OK cells after 24 h. This is evidence that the observed phenomena are not due to cell culture artifacts and moreover shows that two proximal tubular cell lines from different species behave similarly to the experimental maneuvers performed herein.

The fact that both OK and NRK-52E proximal tubular cell number decreases after incubation with OTA, reflects the well known fact that OTA is a nephrotoxic substance [6]. 100 nM OTA alone are sufficient to decrease proximal tubular epithelial tightness so that more inulin (5000 Da) passes the OK cell epithelium paracellularly as compared to untreated controls. 100 nM OTA alone only moderately reduced cell number, but induced epithelial leakage to the same extent as 1000 nM OTA did. Thus, the mechanism involved must be, at least in part, independent from the decline in cell number. This is in agreement with data

Fig. 6. ELISA detecting the relative amount of activated (phosphorylated) MAPK per cell protein after incubation with OTA for 24 h. Data is given in % of control with control values set as 100 %. (A) Relative amount of phosphorylated ERK1/2 (p-ERK) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is $0,53 \pm 0,10$. (B) Relative amount of phosphorylated JNK (p-JNK) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is $0,25 \pm 0,03$. (C) Relative amount of phosphorylated p38 (p-p38) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is $0,23 \pm 0,05$. n is given in the respective bar. * indicates significant difference from control and # indicates significant difference to the lower OTA concentration used.

from [18] showing impaired cell-cell contact after non cytotoxic doses of OTA. Additionally, recent data indicate a role of ERK1/2 on renal paracellular permeability [31], which is in agreement with the described activation of ERK1/2 by OTA. Increase in paracellular permeability does not differ between 100 nM and 1000 nM OTA, although 1000 nM OTA decreased OK cell number far more than 100 nM OTA. Coulter counter measurements indicate increased cell volume in the resting cells after 1000 nM OTA (data not shown). The hypertrophy could be due to cell spreading in response to cell loss in order to maintain tightness of the epithelial barrier. This would represent a suitable mechanism to even out a loss in cell number.

Loss of cell number induced by a toxic stimulus can be due to necrosis or apoptosis. As both necrosis [32] and apoptosis [18, 20, 33] is described to be induced by OTA in renal epithelial cells, we investigated the effect of OTA on both parameters by measurement of LDH release (necrosis) or DNA ladder formation and caspase-3 activity (apoptosis). OTA induced necrosis at 1000 nM and had no effect at 100 nM. Apoptosis is induced by 100 nM OTA and 1000 nM OTA in either assay. Thus, the observed decrease in cell number is due to apoptosis and necrosis, whereas necrosis takes place only at higher concentrations. Therefore, cell loss induced by 100 nM OTA seems to be exclusively due to apoptosis.

OTA is supposed to be involved in the development of renal disease of the chronic interstitial type in endemic areas, namely Balkan endemic nephropathy [27]. In general, chronic renal interstitial disease is characterized by inflammation, fibrosis and epithelial-to-mesenchymal transition [12, 13, 29]. Therefore, we investigated the effect of OTA on the mentioned parameters. OTA clearly induced the pro-inflammatory mediator NF κ B (at 100 nM and 1000 nM). Thus, OTA induces NF κ B in proximal tubular cells, thereby most probably leading to inflammation. NF κ B is also known as inductor of renal fibrosis [34]. In this respect, it is not surprising that OTA also enhances secretion of collagenes (I, III, IV) in OK cells.

According to our data, constant exposition to OTA leads to proximal tubular cells exerting elevated ERK1/2 activity levels. Proximal tubular damage drives chronic interstitial nephropathy [17]. There is experimental evidence that ERK1/2 activation takes place in proximal tubules in interstitial renal disease [35, 36]. As already mentioned, OTA induces nephropathies of the interstitial type. Thus ERK activation might serve as cellular switch

which is responsible for induction of key parameters of cortical nephropathy as shown above.

However in general, ERK1/2 is supposed to act pro-mitotic, anti-fibrotic and anti-inflammatory, whereas JNK and p38 are kinases supporting apoptosis, fibrosis and inflammation [37, 38]. OTA was shown to induce stimulation of extra cellular regulated kinases 1 and 2 (ERK1/2) in the human kidney epithelial cell line SB3 [18] and in the collecting duct derived cell line MDCK-C7 [19]. In the same cell line OTA also induced activation of c-jun amino terminal kinase (JNK) [20]. We could show that this is also the case in proximal tubular OK cells and that p38 is also activated by OTA exposure. Thus, in principle OTA is able to induce MAPK supporting apoptosis, fibrosis and inflammation, too. Furthermore, there is evidence that it is the balance between ERK1/2 on the one hand and JNK and p38 on the other hand that determines cellular fate, which was also shown for renal proximal tubular cells [39]. Therefore, increased apoptosis, collagen secretion and NF κ B activation in OK cells could be due to activation of pro-fibrotic JNK or p38, shifting balance away from ERK1/2 influence on to influence of JNK or p38. Future studies will have to investigate this phenomenon in more detail.

Epithelial-to-mesenchymal transition leads to generation of myofibroblasts out of renal epithelial cells. Myofibroblast activation is thought to be a key event in progression of chronic renal disease [29]. α SMA RNA was induced in proximal tubular cells after exposure to OTA. From our data we propose, that proximal tubular epithelial-to-mesenchymal transition can be induced by OTA. Only recently, evidence was obtained that epithelial-to-mesenchymal transition is induced by ERK activation in a model for diabetic nephropathy using NRK-52E cells [40]. As OTA also activated ERK1/2, we hypothesize that OTA may exert epithelial-to-mesenchymal transition by activation of ERK1/2.

The resulting myofibroblasts are cells probably lacking polarity. OTA induced collagen secretion was increased to a similar extent for collagen I, III or IV in OK cells. As collagen I and III are located in the interstitial space and collagen IV is located in the basement membrane, OTA seems to induce collagenes independently of their extra cellular target site, which is in opposite to what was shown for the prototypical pro fibrotic cytokine TGF- β 1 [24]. Thus, if the basement membrane collagen IV is elevated in the apical compartment, cells seem to have somehow lost correct polarity which may be explained by epithelial-to-mesenchymal transition.

In summary, we could show for the first time that OTA alone is able to induce cell loss, fibrosis, inflammation and epithelial transition in proximal tubular cells all at once. We show that, in vitro, OTA changes several renal cell functions as described to take place in Balkan endemic nephropathy, and endemic forms of chronic interstitial nephritis and karyomegalic interstitial nephritis, which additionally supports epidemiological data indicating that these diseases are induced by OTA exposition. As OTA is found in plasma and urine of almost any individual [6], we hypothesize that this may also be of importance in humans.

Finally, our data give rise to an attractive hypothesis on the increased incidence of renal tumors and urinary tract tumors associated with Balkan endemic nephropathy [27]. According to our data, constant exposition of the kidney leads to proximal tubular cells exerting elevated ERK1/2 activity levels. ERK1/2 activation was shown to be increased in a number of tumors from various organs and also from kidney [41]. In opposite, activation of JNK and p38 was shown to induce apoptosis in human cancer cells [42]. Thus, tumor inducing capacity of OTA depends on the balance of ERK1/2 activation on the one hand and on the activation of JNK and p38 on the other hand. Thus, if cells are exposed to OTA, any stimulus shifting balance of MAPK on to p-ERK1/2 compared to p-JNK and p-p38 will inhibit apoptosis and thus potentially induce tumor

growth. Future studies will have to investigate this interesting phenomenon in more detail.

Abbreviations

EGF (epidermal growth factor); ERK1/2 (extracellular regulated kinase (isoforms) 1 and 2); JNK (c-jun asmino terminale kinase); MAPK (mitogen activated protein kinases); MEK (mitogen activated/extracellular-signal regulated kinase kinase); NRK-52E cells (normal rat kidney epithelial cells); OK cells (opossum kidney cells); OTA (ochratoxin A); PAH (para-amino hippurate); PKC (protein kinase c); P38 (extracellular regulated protein kinase 38).

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