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Exposure to nephrotoxic Ochratoxin A enhances collagen secretion in human renal proximal tubular cells

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Ochratoxin A (OTA) is a nephrotoxic mycotoxin. There is evidence that OTA leads to cortical interstitial nephropathies in humans, associated with fibrosis. No data are available on the effect of OTA-induced collagen secretion from renal cortical cells. As kidney cortex mainly consists of proximal tubules, we investigated the effect of OTA on particular collagens (I, III, IV) in a well-established proximal tubular cell line (opossum kidney (OK) cells) and in primary cultured human renal proximal tubular epithelial cells (RPTECs). In fibroblasts, OTA neither exerted toxic effects nor induced collagen secretion, most probably due to the absence of suitable uptake mechanisms. OTA exerted time- and dose-dependent toxicity in both OK cells and human RPTECs. Moreover, OTA induced collagen secretion in a time- and dose-dependent manner in both cell types. In opposite to transforming growth factor β1 (TGF-β1), OTA incubation induced increased apical secretion of the basement membrane collagen IV. This might be evidence for a loss of cellular polarity after OTA incubation. We conclude that in proximal tubular cells, OTA is able to induce extracellular matrix deposition. As collagen secretion was also inducible in primary cultured human RPTECs, we hypothesize that OTA-induced extracellular matrix deposition by proximal tubular cells may be of importance in generation of renal diseases in humans which are under suspicion of being induced by OTA.

Keywords: Collagen secretion / Fibrosis / Ochratoxin A / Renal disease / Renal proximal tubule

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1 Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite that is found in a variety of animal feed and human food [1, 2]. OTA enters the food chain by cereals and its products, coffee, beer, wine, poultry, and pork. Due to its stability and its almost ubiquitous occurrence as food contaminant, complete avoidance of dietary intake of OTA is impossible [3, 4]. Ever since the first studies, OTA was shown to be a nephrotoxic substance [5] with an elimination half-life of around 840 h in humans [6]. Numerous studies showed the occurrence in human blood serum and human kidney. 50–100% of the human samples were positive for OTA (reviewed in [7–9]). There is evidence that OTA is involved in the pathogenesis of Balkan endemic nephropathy where contamination with high amounts of OTA is described [9, 10]. The toxicological profile of OTA includes teratogenesis, nephrotoxicity and immunotoxicity [7]. Moreover, OTA was classified as being carcinogenic in animals [11–13] and as a possible carcinogen in humans [1, 14].

Virtually all progressive renal diseases are characterized by fibrosis [15–17] and so is 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 ...". Balkan endemic nephropathy is characterized by tubular degeneration, interstitial fibrosis and impaired renal function [18, 19]. This is in line with numerous animal studies, showing development of renal disease accompanied by proximal tubular atrophy and cortical interstitial fibrosis after exposition to OTA [8, 18, 20]. Fibrosis is excess production of extracellular matrix. Sustained excess extracellular matrix production is building up additional resistance hindering transport processes across epithelial barriers. Exchange of solutes and water between tubular lumen and renal interstitium is the major process in urine formation. Thus, sustained excess extracellular matrix formation will impair renal function. Collagens are the major constituents of extracellular matrix [21].

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Abbreviations: OK cells, opossum kidney cells; **OTA**, ochratoxin A; **RPTEC**, renal proximal tubular epithelial cell

Most surprisingly, there are no experimental data available on the effect of OTA on renal cortical cells (proximal tubular cells) with respect to collagen secretion. Therefore, we investigated the effect of OTA on secretion of selected collagens from a well-established proximal tubular cell line (OK cells). As there is also evidence for fibrotic effects of OTA in humans, we also measured OTA-induced collagen secretion in primary cultured human renal proximal tubular epithelial cells (RPTECs). In the following study, we could show for the first time that OTA induced increased secretion of collagens I, III, and IV in proximal tubular cells from animal and humans. This indicates that OTA exposure of proximal tubular cells alone is able to induce extracellular matrix deposition and thus supporting renal cortical fibrosis.

2 Materials and methods

2.1 Cell culture

OK cells were obtained from Dr. Biber, Dept. of Physiology, University of Zürich. 3T3 mouse fibroblasts were obtained from American Type Culture Collection (ATCC). Cells were maintained in culture at 37°C in a humidified 5% CO_2, 95% air atmosphere. The growth medium was minimal essential medium (MEM), pH 7.4, supplemented with Earl's salts, nonessential amino acids, 10% v/v fetal calf serum (Biochrom, Berlin, Germany), and 26 mmol/L $NaHCO_3$. All studies were performed between passage 60 and 100. The seeding density was 0.4×10^6 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 deprived for 24 h before the experiments. Cells were seeded on Petri dishes or 24-well plates. RPTECs were obtained from Cell Systems Biotechnologie Vertrieb (St. Katharinen, Germany). RPTECs were cultured in REBM™ medium, enriched with REGM™ BulletKit® 10 µg/mL human EGF (0.5 mL/500 mL), 5.0 mg/mL insulin (0.5 mL/500 mL), 0.5 mg/mL hydrocortisone (0.5 mL/500 mL), 50 mg/mL gentamicin (0.5 mL/500 mL), 0.5 mg/mL epinephrine (0.5 mL/500 mL), 6.5 µg/mL triiodothyronine (0.5 mL/500 mL), 10 mg/mL transferrin (0.5 mL/500 mL), and 5% v/v fetal calf serum. REBM™ medium and REGM™ BulletKit® was obtained from Cell Systems Biotechnologie Vertrieb. The cells were maintained at pH 7.4 and 37°C, and gassed with 95% O_2/5% CO_2. All studies were performed between passage 2 and 10. The seeding density was 2.5×10^3 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 deprived for 4 h before the experiments. Cells were seeded on Petri dishes or 24-well plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA).

2.2 Determination of collagen secretion, cell number, and protein content

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 [22, 23]. In brief, media and collagen standards (Sigma, Deisenhofen, Germany) were incubated for 24 h in 96-well Nunc-Immuno-Maxisorb plates (Nalge Nunc International, Naperville, IL, USA) 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 1 h at room temperature. After three washes with 0.05% Tween in phosphate-buffered saline, horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 5000; Biotrend) 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) and the reaction was stopped after 15 min with 1 N H_2SO_4. The absorbance at 490 nm was determined using a multiwell-multilabel reader (Victor^{2} ™ Wallac, Turku, Finland). Cell number was determined using a Coulter Counter™ Z2 particle counter and size analyzer (Coulter Electronics, Luton, Beds., England). Protein content was measured by BC Assay™ protein assay (Pierce, Rockford, JL, USA). If not stated otherwise, all other chemicals were from Sigma.

2.3 Data analysis

Data are presented as means ± SEM, respectively. The *n*-value is given in the text or in the figures. 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 analysis of variance (ANOVA) as appropriate. Differences were considered statistically significant when $P < 0.05$.

3 Results

As already mentioned, OTA is thought to be associated with the generation of renal cortical fibrosis [8]. Thus, we investigated the effect of OTA on the secretion of collagen I, III, and IV by use of a specific ELISA technique (see Section 2). First, we investigated the effect of OTA on OK proximal tubular cells. OTA led to a time- and dose-dependent decrease of OK cell number, as indicated in Fig. 1. OTA exerted marked effects on cell survival after 24 h. After 48 h incubation with OTA cell number was decreased to 70% or 30% of control for 100 nM or 1000 nM OTA, respectively. The effect of 10 nM OTA on the cell number did not differ from 100 nM OTA over time. For both 100 nM and

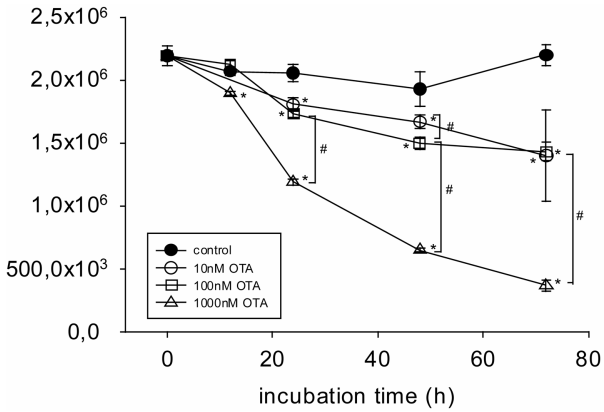

Figure 1. Effect of OTA incubation on OK cell number. OK cells grown on Petri dishes were incubated with different concentrations of OTA (10 nM, 100 nM and 1000 nM) for up to 72 h. Cell number was measured using a coulter counter, as described in Section 2. *n* = 6 for every other data point, respectively. *, Statistical difference from control; #, statistical difference to a different concentration.

1000 nM OTA, the half-maximal effect occurred after 24 h incubation, whilst after 48 h 80% of the maximal effect occurred for the respective concentration. As loss of cell number correlated with loss of protein content (data not shown), we used cell protein determination as equivalent in the following experiments.

As the half-maximal effect on cell number occurred after ~24 h, we decided to investigate collagen secretion of OK cells after incubation with up to 1000 nM OTA for 24 h. As indicated in Fig. 2A, 100 nM OTA did not induce collagen secretion after 24 h in OK cells, whereas 1000 nM OTA induced significant secretion of collagens I, III, and IV, which was approximately twofold. In order to investigate whether in OK cells OTA induction of collagen secretion is also time-dependent, we investigated the collagen secretion in OK cells after 48 h OTA incubation. As shown in Fig. 2B, OTA incubation of OK cells for 48 h started to induce significant secretion of collagens I, III, and IV at 60 nM. Moreover, collagen secretion increased dose-dependently up to 1000 nM, reaching approximately 7-fold stimulation. Thus, in OK cells OTA induced collagen secretion in a time- and dose-dependent manner. This means that secretion of extracellular matrix in OK cells, as reflected by the measured collagens, is induced by OTA incubation.

Fibroblasts are also present in the kidney cortex and are thought to participate in excess production of extracellular matrix in renal disease [24, 25]. Therefore, we wanted to investigate whether exposure of isolated fibroblasts (3T3 mice fibroblasts) to OTA is sufficient to induce collagen secretion. As shown in Fig. 3, incubation of 3T3 fibroblasts to up to 1000 nM OTA neither reduced cell protein (3A) nor increased secretion of either collagen (3B). Thus, OTA did

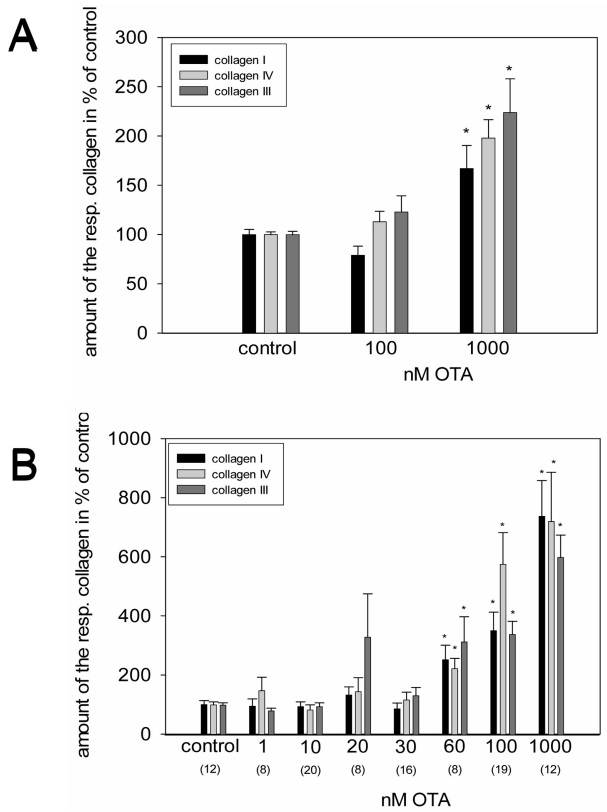

Figure 2. Collagen-ELISA detecting the amount of secreted collagens I, III, IV by OK cells after incubation with OTA. (A) Collagen synthesis in OK cells after treatment for 24 h with increasing amount of OTA. Control secretion of collagens after 24 h: collagen I, 1226 ± 41 ng/mg; collagen IV, 41 ± 8 ng/mg; collagen III, 66 ± 7 ng/mg. *n* = 13 for control bars and *n* = 9 for all other bars. Significance is indicated by asterisks. (B) Collagen synthesis in OK cells after treatment for 48 h with increasing amount of OTA. Control secretion of collagens after 48 h: collagen I, 4243 ± 2095 ng/mg; collagen IV, 144 ± 21 ng/mg; collagen III, 141 ± 18 ng/mg. *n* is given in brackets below the concentration values. *n* is equal for every experimental group. Significance is indicated by asterisks.

not induce secretion of collagens I (exemplary for interstitial collagen type, as is also collagen III) or IV (exemplary for basement collagen type) in the 3T3 fibroblast cell line.

The OK cell line is an immortalized cell line from the opossum proximal tubule. It is a well-established model system for proximal tubular cells, which is described in numerous publications [26, 27]. Nevertheless, it is not of human origin. Moreover, immortalized cell lines often show altered properties as compared to native cells. Therefore, we decided to use human RPTECs in primary culture to gain information more likely transferable to the human system. First, we incubated RPTECs with increasing concentrations of OTA for up to 72 h. As indicated in Fig. 4, OTA decreased the total protein content of RPTECs in a time-

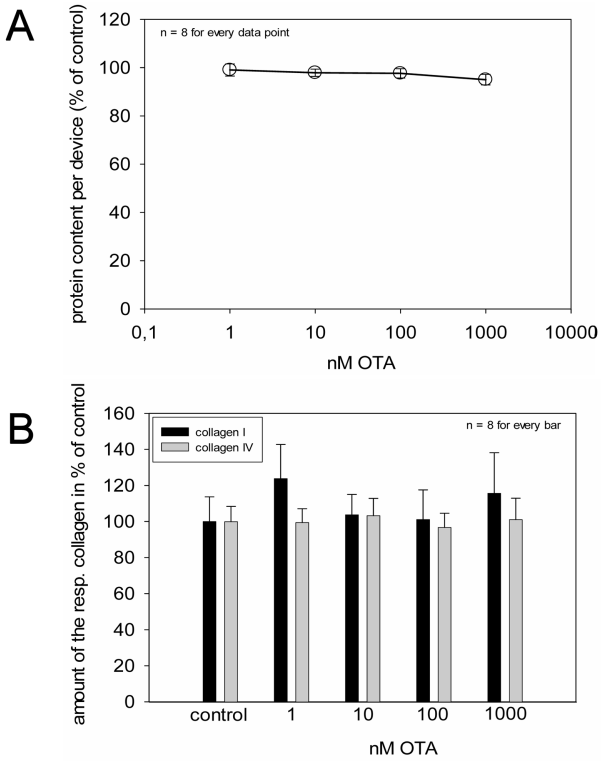

Figure 3. Effect of OTA on collagen secretion from 3T3 fibroblasts. (A) Protein content per well (24-well plates) of 3T3 fibroblasts after 48 h incubation with increasing concentrations of OTA (up to 1000 nM). (B) Collagen synthesis (I, IV) of 3T3 fibroblasts after 48 h incubation with increasing concentrations of OTA (up to 1000 nM). Control secretion of collagens after 48 h: collagen I, 9654 ± 2122 ng/mg; collagen IV, 471 ± 66 ng/mg. *n* is given in the respective figures. Significance is indicated by asterisks.

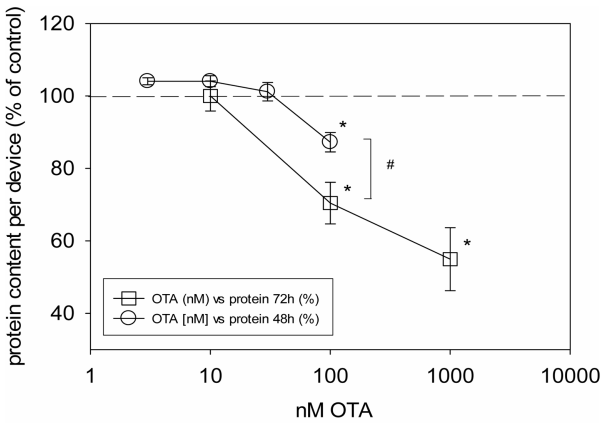

Figure 4. Effect of OTA on RPTEC protein content. RPTECs were incubated with increasing concentrations (up to 1000 nM) of OTA after 48 h or 72 h. *n* = 3 for every 48 h data point and *n* = 4 for every 72 h data point. Significant difference from control is indicated by asterisks, whereas significance between data points is indicated by #.

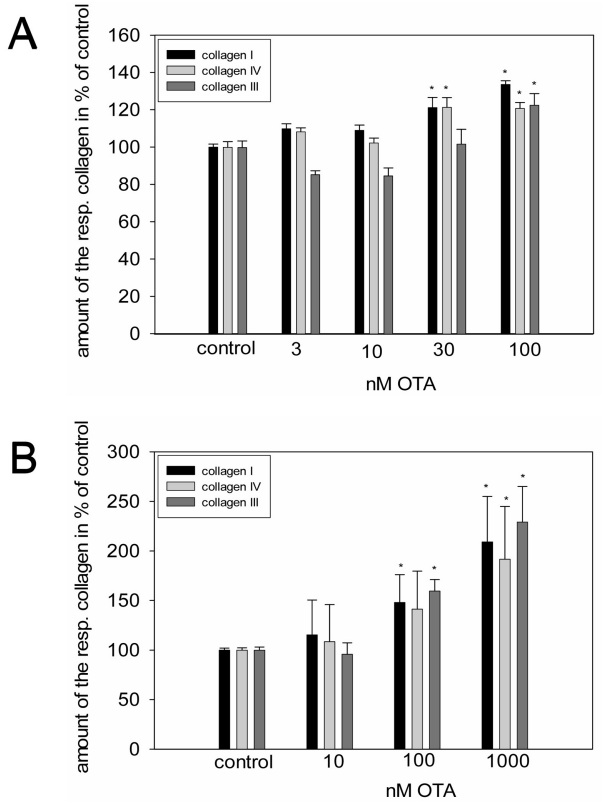

Figure 5. Effect of OTA incubation on collagen I, III, IV synthesis in RPTECs. (A) Collagen synthesis in RPTECs after 48 h incubation with OTA. Control secretion of collagens after 48 h: collagen I, 9809 ± 160 ng/mg; collagen IV, 474 ± 15 ng/mg; collagen III, 534 ± 19 ng/mg. *n* = 3 for 100 nM bars and *n* = 4 for all other bars. (B) Collagen synthesis in RPTECs after 72 h incubation with OTA. Control secretion of collagens after 72 h: collagen I, 89681 ± 2044 ng/mg; collagen IV, 2299 ± 76 ng/mg; collagen III, 10621 ± 386 ng/mg. *n* = 6 for every bar. Significant difference from control is indicated by asterisks.

and dose-dependent manner, indicating general toxicity also to proximal tubular cells from humans.

Forty-eight hours incubation with OTA led to a dose-dependent increase of collagen secretion in RPTECs, 100 nM leading to an increase to 130% for collagen I and to 120% for collagens III and IV (Fig. 5A). As stimulation of collagen secretion was less as compared to OK cells, we decided to extend the incubation period to 72 h. Incubation of RPTECs with OTA for 72 h led to a dose-dependent increase of secretion for collagen I, III, or IV. As indicated in Fig. 5B, 100 nM OTA increased collagen secretion ~1.5-fold, whereas 1000 nM OTA doubled secretion of collagens I, III, and IV in RPTECs. Thus, OTA induced collagen secretion in human RPTECs. Therefore, secretion of extracellular matrix in human RPTECs is induced by OTA incubation, too.

4 Discussion

This study shows for the first time that in proximal tubular cells OTA in the high nanomolar concentration range is able to induce extracellular matrix deposition which is typical for the chronic renal diseases induced by OTA. The fact that OK cell number decreases time- and dose-dependently after incubation with OTA, reflects the well-known fact that OTA is a nephrotoxic substance [7, 28, 29]. The same was found for the human proximal tubular RPTECs, which supports the idea that OTA is also nephrotoxic in humans [7, 8, 14]. By contrast, incubation of fibroblasts with OTA did not lead to reduction in cell protein even at concentrations and exposure times that exerted a toxic effect in proximal tubular cells (as indicated by loss of cell number respectively cell protein). Even after 72 h incubation, no effect of OTA on fibroblast cell number was detected even at 1000 nM (data not shown). As mentioned in Section 3, onset of collagen secretion is associated with decline in cell number in OK cells or in RPTECs. Therefore, substantial induction of collagen secretion seems highly unlikely in fibroblasts even after 72 h incubation with OTA. OTA is known to be transported in substantial amounts by organic anion transport proteins present in proximal tubular cells [30] and H^+-dipeptide-cotransporter [31] which are not present in fibroblasts. Moreover, there is evidence that uptake into the cells is a prerequisite for OTA toxicity [32] and thus OTA does not act *via* interaction with structures at the outer cellular surface. Thus, the lack of OTA toxicity to fibroblasts in our setup indicates that less OTA enters the cells due to the absence of suitable transporters. This is reflected by *in vitro* studies, showing a more than 12-fold accumulation of OTA in renal tissue and in cultured renal epithelial cells [33], indicating that accumulation of OTA in renal tissue is the reason for its nephrotoxicity.

The first part of the experiments presented herein was performed using OK cells, which is an accepted and well-established model system representing proximal tubular cells. We could show previously that this is also true with respect to collagen secretion, as transforming growth factor β1 (TGF-β1) induced collagen secretion in OK cells after a 48 h incubation period [22]. In OK cells, exposure to OTA induced toxicity as assayed by the loss of cell number and led to secretion of collagens, which indicates the fibrotic potency of OTA. The same was observed for human RPTECs in principle: OTA exerted toxicity and induced secretion of collagens. In this respect it is notable, that in both OK cells and RPTECs, OTA induces marked collagen secretion in concentrations that exert only minor toxic effects (*e.g.*, 100 nM after 48 h in OK cells and after 72 h in RPTECs), thus reflecting the *in vivo* situation where renal fibrosis is a chronic event not or only minor associated with acute toxicity [15].

As shown in [22] the profibrotic cytokine TGF-β1 only slightly stimulated the apical secretion of the basement membrane collagen IV, whereas the interstitial collagen I is elevated to a greater extent. The same was found to be true in RPTECs where TGF-β1 (3 μg/L; 72 h) increased secretion of interstitial collagen I (191% ± 26%; mean ± SD) and collagen III (228% ± 37%; mean ± SD), whereas basement membrane collagen IV (167% ± 30%; mean ± SD) was not significantly increased (as compared to control). As TGF-β1 is a profibrotic cytokine, the preferential increase of collagen I and III (located in the interstitial space) as compared with collagen IV (located in the basement membrane) is not surprising [22]. Thus, the effect of the prototypical fibrotic cytokine TGF-ß1 on collagen secretion pattern is similar in proximal tubular OK cells and RPTECs, which indicates that both cell types are useful models for proximal tubular cells also with respect to collagen secretion studies.

However, OTA-induced collagen secretion was increased to the approximately same extent for collagen I, III, or IV in both, OK cells or RPTECs. As collagens I and III are located in the interstitial space and collagen IV is located in the basement membrane, OTA seems to induce collagens independently of their extracellular target site, which is in opposite to what was shown for TGF-β1. Thus, if the basement membrane collagen IV is elevated, cells seem to have somehow lost correct polarity. In general, chronic renal interstitial disease is not only characterized by fibrosis, but also by epithelial-to-mesenchymal transition leading to loss of epithelial polarity [16, 17, 34]. OTA seems to induce transformation of proximal tubular OK cells to myo-fibroblasts as indicated by expression of α-smooth muscle actin [35]. Moreover, OTA incubation of OK cells led to appearance of an apical organic anion transport activity normally strictly located to the basolateral membrane [36]. Both of the observations mentioned indicate a loss of normal cellular polarity due to OTA exposure. Polarity is a prerequisite for site-directed transport and is crucial for renal function [37]. Thus, the OTA-induced loss of cell polarity may be the reason for increased secretion of basement membrane collagen IV to the apical compartment to the same extent as interstitial collagens I and III.

Relative collagen secretion induced by the maximal amount of OTA used (1000 nM) is only 2- to 2.5-fold in RPTECs, whereas it is 6- to 7-fold in OK cells. This might simply reflect different sensitivities of OK cells and RPTECs to OTA. Another explanation might be that cell mass is much lower for RPTECs (for untreated controls ~70 μg/cm^2 as compared to 380 μg/cm^2 for OK cells). Collagen secretion in isolated proximal tubular cells is stimulated by autocrine action of TGF-β1 [38]. Thus, secreted TGF-ß1 levels in RPTEC media may be lower as compared to OK media, explaining the quantitative discrepancy in OTA-induced

collagen secretions. Whether these hypotheses hold true will have to be investigated in further studies.

OTA is supposed to be involved in the development of renal disease of the chronic interstitial type in endemic areas, namely Balkan endemic nephropathy [8]. In general, chronic renal interstitial disease is amongst others characterized by fibrosis and epithelial-to-mesenchymal transition [16, 17, 34]. However, up to now no *in vitro* data are available on OTA exposure of proximal tubular cells and collagen secretion. Thus, this paper represents the first report showing directly that OTA stimulates collagen secretion by RPTCs from animal and human origin. OTA is found in blood and urine of almost any individual (at least in Europe [39]), but the mean serum concentration is ~1 nM [40 – 42] which did not induce collagen secretion in our setup. Hence, in serum from individuals suffering from Balkan endemic nephropathy concentrations from 50 nM to 70 nM are described [43], which is well in the concentration range where OTA induces collagen secretion in human RPTECs. Therefore, we hypothesize that OTA-induced extracellular matrix deposition by proximal tubular cells may be of importance in humans, too. Moreover, we present evidence that OTA exposure may lead to a loss of polarity which is a sign of epithelial-to-mesenchymal transition.

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