DOI 10.1002/mnfr.200400062 Mol. Nutr. Food Res. 2005, *49*, 118 – 130

Review

Ochratoxin A at nanomolar concentrations: A signal modulator in renal cells

Michael Gekle, Christoph Sauvant and Gerald Schwerdt

Physiologisches Institut der Universität Würzburg, Würzburg, Germany

Ochratoxin A (OTA) is a ubiquitous fungal metabolite with nephrotoxic, carcinogenic, and apoptotic potential. Toxicokinetics make the kidney the primary target organ for OTA. Due to its widespread occurrence in improperly stored foodstuff the complete and safe avoidance of OTA for humans is impossible. There are several reports showing a significant correlation between OTA exposure and certain forms of nephropathies. At nanomolar concentrations OTA leads to specific changes of function and phenotype in renal cells. The toxin interacts with certain cellular "key-molecules" (*e.g.*, mitogen-activated protein (MAP) kinases, Ca^{2+}), thereby disturbing cellular signalling and regulation events as well as mitochondrial function. Moreover, OTA has the ability to modulate physiological signals (*e.g.*, angiotensin II or TNFα) and thereby influences cell function and cell growth and may even stable re-program the cells (*e.g.*, altered distribution of chromosomes). This review concentrates on the effects of OTA in the nanomolar range and its interactions with cellular signalling networks in different renal cells proposing OTA to act as a signal modulator.

Keywords: Cellular signalling networks / Ochratoxin A / Renal cells / Review

Received: August 31, 2004; revised: November 16, 2004; accepted: November 18, 2004

Contents

Correspondence: Prof. Dr. med. Michael Gekle, Physiologisches Institut, Universität Würzburg, Röntgenring 9, D-97070 Würzburg
E-mail: michael.gekle@mail.uni-wuerzburg.de
Fax: +49-931-312741

Abbreviations: BEN, Balkan endemic nephropathy; **ERK**, extracellular signal-regulated kinase; **IHKE cells**, immortalized human kidney epithelial cells; **JNK**, c-jun amino terminal kinase; **MAPK**, mitogen-activated protein kinase; **MDCK cells**, Madin-Darby canine kidney cells; **MEK**, MAP/ERK kinase; **NTP**, National Toxicology Program; **OTA**, ochratoxin A

1 Introduction

During the late 90s of the last century European public got acquainted with a food contaminant, so far only known to specialists, by several publications in newspapers and magazines: the mycotoxin ochratoxin A. Initiated by statements of national food safety authorities, like the German Bundesinstitut für Risikobewertung (www.bgvv.de), which started to recommend maximum contamination levels for food, public interest in the toxicology of OTA as well as the concern regarding food safety increased. In order to provide satisfactory answers to consumer questions information regarding food contamination but also regarding the mechanism of action of any toxin is necessary. Several studies have been performed using OTA concentrations in the micro- or even higher molar range showing that OTA can disturb various cellular processes as *e.g.*, macromolecule

synthesis, lead to oxidative stress or altered protein expression pattern [1 – 3]. However, these high concentrations are to be expected only after artificially induced laboratory conditions and do not represent naturally occurring conditions. Therefore, in this review we want to focus on the mechanisms of action of OTA in the nanomolar or low micromolar concentration range. This concentration range refers to total OTA (= protein-bound + free). In most cell culture studies serum-free conditions were used and therefore the fraction of protein-bound OTA is neglectable under these conditions.

2 What is OTA?

In 1965, van der Merwe *et al.* [4] described the formation of a toxic metabolite by fungi of the species *Aspergillus ochraceus Wilh*. and called it ochratoxin A (OTA). In the same year they described the physicochemical properties of OTA as well as of its analogues ochratoxin B and C [5]. OTA consists of a dihydroisocoumarin moiety linked to one molecule L-phenylalanine by a peptide bond. Hydrolysis of this bond yields ochratoxin d and L-phenylalanine. OTA is a weak organic acid with a pK_a value of ~7.1 [18]. Thus, in the nondissociated form OTA is lipophilic and probably well membrane-permeable, whereas the dissociated form (2/3 at pH 7.4) is hydrophilic. The potential significance of a metabolite produced by *Aspergillus ochraceus* results already from the widespread natural occurrence of this fungus. *Aspergillus ochraceus* can be found, *e.g.*, in soil, mould, and improperly stored grain [4]. Another possible way of exposure was its use as flavor enhancer for coffee or fish [4]. Later on it was shown that *Aspergillus ochraceus* is not the only source of OTA, but that other species, like, *e.g.*, *Penicillium viridicatum*, also produce OTA [6].

Soon after the first description of OTA several groups started to investigate its possible toxic actions in different animal models [7 – 9]. The purpose of those studies was to determine the main target of the toxin within the organism. Several studies focused on the liver, being the main metabolic organ [10 – 12]. Although all of those studies showed that OTA might affect certain organs no specific profile of toxicity could be established. Generalized affection of different parts of the organism due to unspecific cell damage seemed to prevail. Yet, studies investigating the function of different organ systems in detail after exposure to naturally occurring doses were still missing. Only in 1974, Krogh *et al.* [13] succeeded to show that administration of OTA at naturally occurring doses leads to selective damage of the kidney and its function. Together with others (*e.g.*, [14 – 16]) this study unmasked OTA as a nephrotoxin. Yet, explanations for its organ specificity and mechanism of action were still missing. Furthermore, Krogh *et al.* could show that OTA contamination was responsible for the long known Danish porcine nephropathy that has caused great losses to Scandinavian farmers.

Once ingested OTA is rapidly reabsorbed in the jejunum, reaching a bioavailability of 97% [17]. Half life of OTA in serum ranges from 120 h in rats to 840 h in monkeys [18]. Responsible for this long half life and the retention in serum is the strong binding to serum proteins, mainly to albumin (association constant = 7×10^4 M^{-1} – 3×10^6 M^{-1}) [18 – 20]. Due to this protein binding less than 0.2% of OTA are free and thus able to leave blood vessels or undergo glomerular filtration. In the gastrointestinal tract OTA is hydrolyzed to ochratoxin α to some extent depending on the physiological germ flora. In ruminants 80% are hydrolyzed whereas in rats less than 50% undergo hydrolysis [17, 21]. Toxicity of OTα is very low as compared to OTA. To a small extent (1–4%) OTA is hydroxylated and conjugated in liver cells but the importance of these processes needs still further clarification [22, 23]. OTA and OTα are excreted with feces and urine, the latter being quantitatively more important [17, 18].

3 Is OTA relevant for human health?

OTA itself has been detected in a variety of animal chow and human food, like for example bread, cereals, red wine, coffee, and beer. Because it is a very stable molecule OTA remains in foodstuff even during certain refinement processes like brewing. The incidence of OTA in human food ranges >80% of the samples examined in countries like USA, Canada, UK, Germany, Poland, and others [17, 18]. The degree of contamination ranged from 0.4 μg/kg to 69 mg/kg of sample (for a detailed review on the occurrence of OTA see [18]). Furthermore, OTA has been detected in slaughtered animals. 76% of serum samples of slaughtered pigs in Canada contained OTA in concentrations up to 573 nmol/L [18]. The high degree of food contamination already points to a high risk of exposure for humans. According to a European survey the mean plasma concentration of OTA in adults is ~0.875 nmol/L ranging from 0.53 nmol/L to 2.75 nmol/L [24]. In quantitative terms the most important source of OTA are cereals contributing to ~50% of our daily intake, followed by wine, coffee, spices, and beer. The average daily intake of an adult European is 1.044 ng per kg body weight (1.71 – 0.168 ng/kg bw). The intake can sum up to 3.55 ng /kg bw due to different food profiles [24]).

The importance of OTA for human health arises from its already mentioned widespread occurrence and the resulting high risk of exposure. In view of the nephrotoxic action of OTA in different animal models, a potential impairment of human renal function has to be considered. On the other hand OTA gained importance for human health due to the suspicion that it is involved in the pathogenesis of Balkan

Table 1. OTA concentrations in human blood serum of patients suffering different renal diseases.

	Mean (nmol/L)	SEM	*n*	$p < 0.05$ *versus* control	Fold increase over control
Control	0.69	0.12	10		1
Morbus Wegener	1.23	0.15	25		1.78
Diabetic nephropathy	1.20	0.27	8		1.74
Interstitial nephropathy	1.38	0.21	9	Yes	2.00
Suspect of BEN	3.67	2.00	3	Yes	5.32
Chronic glomerulonephritis	1.61	0.34	12	Yes	2.33
Other glomerulonephritidis	0.93	0.09	60		1.35
Other renal diseases[a)]	0.87	0.14	22		1.26

a) Pyelonephritis, analgesic-induced nephropathy, chronic renal insufficiency, cystic kidney disease
Blood samples were collected in the Departments of Nephrology, Universities of Würzburg and Erlangen (Prof. Dr. C. Wanner and Prof. Dr. R. Schmieder). OTA was extracted and concentration determined as described in [36, 37].

endemic nephropathy (BEN) [18, 25] and of certain other forms of interstitial nephritis [26–30]. Recently, increased concentrations of OTA in blood and urine samples of patients suffering form glomerulonephritis or nephrotic syndrome have been reported [31]. Furthermore, the incidence of BEN, that is associated with an increased risk of urogenital adenoma and carcinoma [32, 33], is high in areas with high OTA contamination of human food [25]. Of course, the final prove has still to be provided that OTA causes these diseases. It is noteworthy that 50% of human end-stage renal diseases are of unknown origin and current consensus suggests that chemicals, including mycotoxins, such as OTA, probably play a significant role in those cases where no etiology is documented [34]. Thus, studies on the toxicokinetics and toxodynamics of OTA are relevant for human health, because the complete avoidance of OTA-exposure is impossible [18, 30, 35].

Recently, we performed a small study in order to gain initial information whether enhanced blood OTA concentrations can be detected in patients with renal diseases in Germany (Table 1). The preliminary data show that in some, but not all, groups of renal diseases OTA levels are increased. Thus, renal disease *per se* does not lead to elevated OTA levels. These data support the hypothesis that OTA may contribute to the development to certain diseases. Of course the sample number is small and therefore the data have to be interpreted with caution. In summary, OTA is a nephrotoxic substance with probable relevance for human health.

4 Why the kidneys?

Although it has been shown that possible targets of OTA are the liver, the immune system, and brain cells [38–40], the kidney is the main target of OTA. This high susceptibility of the kidney is, at least in part, the result of OTA-toxicokinetics. Renal blood flow per tissue weight is extremely high, resulting in the delivery of relative large amounts of OTA as compared to other organs. Furthermore, free OTA is secreted in the proximal tubule (Fig. 1) and subsequently reabsorbed, mainly in the proximal straight tubule, the thick ascending limb of the loop of Henle and the collecting duct [36, 41–46]. Mechanisms involved in reabsorption are, *e.g.*, H^+-dipeptide-cotransporter(s) and nonionic diffusion [42, 45, 46]. These toxicokinetic features result in an accumulation of OTA in renal tissue, where the highest concentrations have been detected in the papilla and the inner medulla [47].

OTA is bound to plasma proteins, namely albumin, for more than 99% [20]. Therefore, glomerular filtration contributes only to a minor extent to the load of free OTA in the tubular lumen. As discussed later in detail, OTA is a substrate for the proximal tubular organic anion transport system and thus actively secreted into the proximal tubular lumen. In contrast, mass reabsorptive transport of water takes place in the proximal tubule, leading to the reabsorption of around 70% of filtered water. In summary, these mechanisms potentially increase the concentration of free OTA in the proximal tubular lumen more than 10-fold as compared to total OTA in serum [41], before it is partially reabsorbed in post-proximal parts of the nephron, especially the thick ascending limb of the loop of Henle and the collecting duct [42]. As discussed later on, nephrotoxic changes induced by OTA are found frequently in the cortical parts of the kidney, which consist predominantly of proximal tubules. Moreover, human nephrotoxicities suspiciously induced by OTA are of the cortical interstitial type and thus also located in the part of the kidney that consists predominantly of proximal tubules. Taken together, OTA is concentrated in proximal tubular cells mainly due to secretory transport and in collecting duct cells due to reabsorptive transport, making these two sites major targets for OTA toxicity. For yet unknown reasons morphological changes are detected mostly in and close to proximal tubules. Thus, toxicokinetics contributes, at least in part, to the explanation of kidney specificity of OTA toxicity.

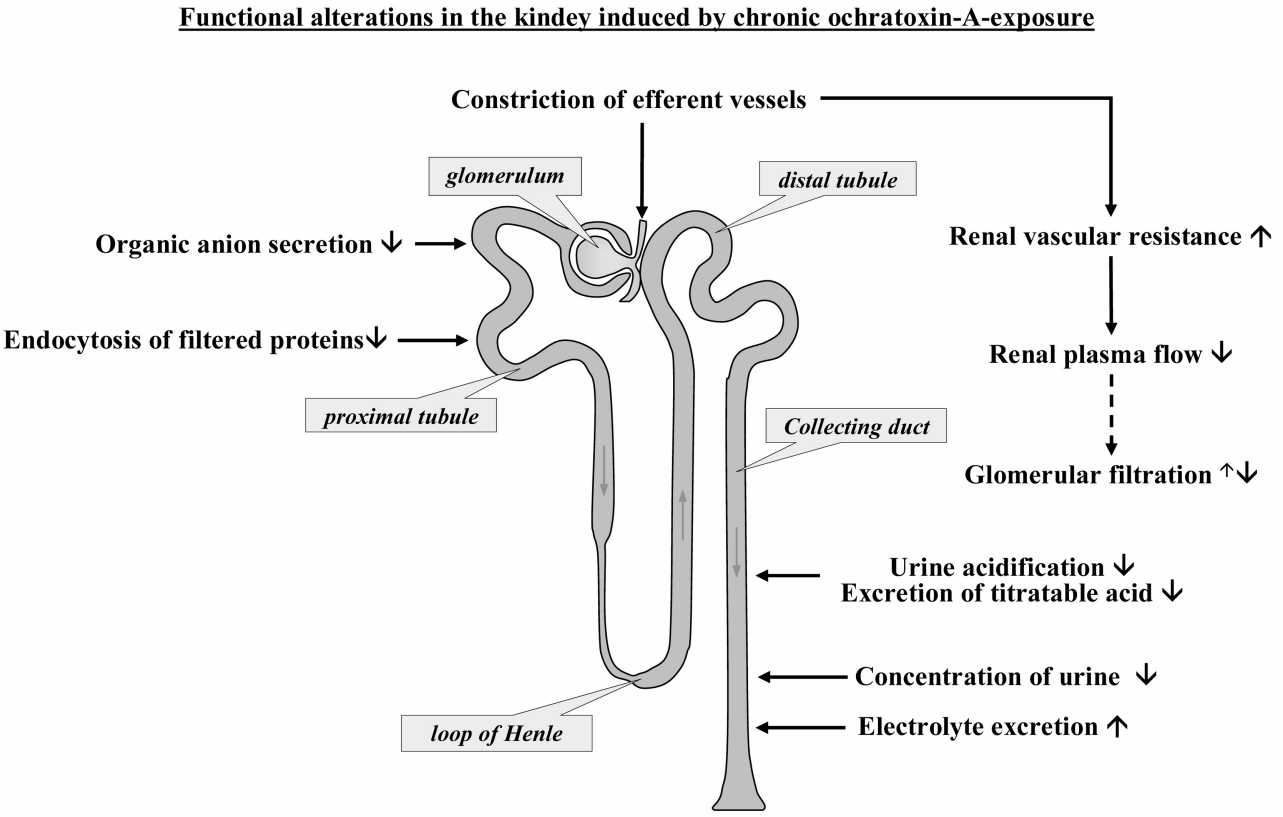

Figure 1. Scheme of a nephron showing the different segments and the respective effects of low-dose chronic OTA exposure. In the glomerulum OTA leads to a preferential constriction of the efferent vessel, thereby increasing at least transiently the hydrostatic pressure in glomerular capillaries, potentially leading to hyperfiltration. Finally, glomerular filtration drops because glomerular capillaries are damaged by the hypertension and because renal blood flow is reduced to an extent that limits filtration despite enhanced hydrostatic pressure. In the proximal tubule organic anion secretion and reabsorptive endocytosis of filtered proteins is reduced. In the thick ascending limb of the loop of Henle OTA may impair electrolyte transport and therefore the establishment of a proper osmotic gradient required for urine concentration. In the collecting duct OTA exposure results in reduced excretion of titratable acid, impaired transepithelial electrolyte transport and concentrating ability.

5 Renal sites of action

The functional unit of the kidney, the nephron, can be subdivided into various segments (Fig. 1). Roughly, the nephron "starts" with a glomerulum where the formation of primary urine takes place. It is also the site of renin formation and therefore crucially involved in blood pressure regulation. Next, there is the proximal tubule, which reabsorbs the major part of electrolytes and valuable organic molecules, like glucose, amino acids, and proteins. Furthermore, the proximal tubule secrets xenobiotics (including OTA) and metabolites, thereby rendering the kidney the most important excretory organ together with the liver. The loop of Henle is the motor of urine concentration, since here NaCl but not H_2O is reabsorbed, generating a hypertonic interstitial space. The final adjustment of urinary electrolytes, H^+ and H_2O takes place in the collecting duct. This process is highly regulated by hormones, like aldosterone or vasopressin. OTA acts mainly on three sites along the nephron (Fig. 1). It impairs renal blood flow and glomerular function [48]. Furthermore, OTA-exposure alters tubular function [49]. With respect to this action on tubular epithelia the two most affected segments of the nephron are the proximal tubule and the collecting duct. Possibly, the thick ascending limb of the loop of Henle is also affected, explaining the urine-concentrating deficiency. Furthermore, OTA increases the incidence of renal adenoma and carcinoma [18]. Thus, OTA impairs the formation of primary urine, secretion of waste, and the fine adjustment of water and electrolyte homeostasis.

6 Structural alterations induced by OTA

Nephrotoxic effects after naturally exposure have been described mainly in pigs and poultry, whereas the effects of experimental exposure have been studied predominantly in

rats and mice [17, 18]. Histopathological degenerative, atrophic, and hypertrophic alteration of proximal tubular epithelium and glomeruli, as well as interstitial fibrosis were described. Furthermore, cellular condensation and basal membrane-thickening were observed. Biochemical studies (reviewed in [17]) on the cellular level showed the interaction of OTA with mitochondrial respiration and transport [50], synthesis of macromolecules [1], and glycogen metabolism [10, 51].

Recent data [52] indicate that collagen secretion is markedly increased by OTA in cultivated proximal tubular cells. Elevated secretion levels were found for collagen types typically located to the interstitial or to the basal membrane, which suits well with the fibrotic changes described *in vivo* (see above). This is evidence that tubulo-interstitial fibrotic alterations in the kidney induced by OTA are, at least in part, due to the interaction of OTA with proximal tubular cells.

7 Functional alterations induced by OTA

7.1 Glomeruli

Acute application of OTA had no measurable effect on hemodynamics [36, 53]. Yet, after chronic application the situation is different with a dramatic decrease of renal blood flow (RBF) and an increase of total renal vascular resistance. Thus, the observed reduction in glomerular filtration rate (GFR) is, at least in part, the result of altered hemodynamics (Fig. 1). Furthermore, chronic application of OTA led to a predominant increase of the resistance in the efferent arterioles (R_{eff}) [48], thereby increasing at least transiently the hydrostatic pressure in glomerular capillaries, potentially leading to hyperfiltration. Finally, glomerular filtration drops because glomerular capillaries are damaged by the hypertension and because RBF is reduced to an extent that limits filtration despite enhanced hydrostatic pressure. It was shown that the effect of OTA on renal hemodynamics is mediated by angiotensin II. Most probably, OTA increases angiotensin II levels with subsequent functional and structural changes of renal hemodynamics [54]. In conclusion, chronic OTA exposure leads to reduction of RBF and GFR whereas acute exposure does not affect renal hemodynamics. The underlying mechanism is an increase of efferent arteriolar resistance, probably mediated by angiotensin II. Consequently, glomerular capillary pressure rises possibly leading to structural damage of the glomeruli.

7.2 Proximal tubule

Acute exposure to OTA has no measurable effect on proximal tubular function [36, 53], similar to renal hemodynamics. Investigation of net-secretion of *para*-aminohippuric acid (PAH), the classical substrate for the organic anion transport system, *in vivo* revealed a decrease of the transport maximum for PAH by 84% after 6 days [36]. Thus, the transport system for organic anions represents a target for chronic but not for acute OTA exposure (Fig. 1). The exact mechanism leading to the impairment of organic anion secretion remains until now unresolved.

The impairment of secretion of organic anions by OTA seems to be to some extent specific, because other transport processes in the proximal tubule, like amino acid or glucose reabsorption, undergo no or only minor changes under the same experimental conditions [36, 48]. This conclusion is supported by a study showing downregulation of three proteins involved in organic anion secretion (bile salt export pump, organic anion transporter K1, and renal organic anion transporter 1 [2]) after OTA exposure. Basolateral uptake of organic anions from blood into proximal tubular cells is generally thought to be the rate limiting step of organic anion secretion [55]. This step is mediated by two tertiary active transport proteins, namely OAT1 [56] and OAT3 [57, 58]. Recent data from rabbit proximal tubule indicate that OAT1 and OAT3 both contribute to basolateral uptake of OTA into proximal tubules by around 50% [59]. No quantitative data are available on the respective effect of chronic OTA exposure on OAT1 or OAT3 or others. Nevertheless, the above-mentioned data clearly indicate downregulation of proximal tubular organic anion transport proteins by chronic OTA exposure. Inhibition of the organic anion carrier by OTA has in addition severe implications for toxicokinetics [36]. Because renal elimination of OTA depends crucially on the secretion *via* the organic anion carrier, prolonged exposure of OTA hinders its own renal excretion.

Some studies reported that OTA exposure might increase urinary protein excretion [13, 14]. In liver cells it has been observed that OTA may impair the uptake of albumin into phagolysosomes [60]. Since proximal tubular protein endocytosis prevents the excretion of filtered proteins, interaction of OTA with this process could lead to tubular proteinuria. This hypothesis was confirmed in cultured proximal tubular cells [61]. Very recent data also showed that exposure of proximal tubular OK cells to OTA induces characteristic cellular effects also described for chronic renal disease, which are accompanied by proteinuria. These effects include induction of inflammatory signalling pathways, enhanced collagen secretion and epithelial to mesenchymal transition [62].

7.3 Postproximal segments

Certain functional alterations point to an involvement of postproximal parts of the nephron (Fig. 1), *e.g.*, increased

Table 2. Described effects of OTA on proximal tubular and collecting duct cells in the low dose ($\leq 10^{-6}$ mol/L) and the high dose ($>10^{-6}$ mol/L) range.

	Proximal tubule	**Collecting duct**
Low dose	Noncompetitive inhibition of the organic anion transport system [36, 44] Inhibition of the organic cation transport system [44] Cell proliferation and hypertrophy [43, 67] Impaired protein reabsorption [13, 61] Apoptosis [68, 69] Mitochondrial hyperpolarization, "stimulation" [70] MAPK activation and apoptosis [62]	Blockade of plasma membrane anion channels and Cl^-/HCO_3^--exchange [53, 71, 72] Alterations of cellular pH- and Cl^--homeostasis and of transepithelial H^+- and electrolyte transport [53, 65, 66, 71, 73] Activation of extracellular-signal-regulated-kinases (ERK 1/2) [73] Cell dedifferentiation [71, 73] JNK activation and apoptosis [68, 74] Mitochondrial hyperpolarization, "stimulation" [75]
High dose	Decreased cell viability [43, 44, 67] Leaky plasma membrane [44, 67] Impaired DNA synthesis [44, 67] Impaired protein synthesis [44, 61] Reduced cell growth [44, 67] Cell detachment and general decrease of transport activity [44, 61, 67, 75] Necrotic cell death [38, 39] Mitochondrial inhibition [76]	Decreased cell viability [53, 73] Leaky plasma membrane [73] Impaired DNA synthesis [1, 73] Impaired protein synthesis [1] Reduced cell growth [73] Cell detachment and general decrease of transport activity [53, 73, 75]

ERK, extracellular signal-regulated kinase; JNK, c-jun amino terminal kinase; MAPK, mitogen-activated protein kinase

urine flow rate, reduced osmolality, and altered electrolyte excretion [13, 14, 63]. Of special interest is a national toxicology program (NTP)-study where rats were fed µg/kg body weight doses of OTA over a period of 2 years [56]. Besides an increased incidence in renal adenoma and carcinoma, the functional changes observed included urine hypoosmolality, emphasizing the involvement of postproximal parts of the nephron. In contrast to renal hemodynamics and the proximal tubule, acute effects on electrolyte and acid excretion attributable to postproximal parts of the nephron have been detected [53]. OTA induced an increase of NaCl excretion and a decrease of K^+ excretion. The pattern of the OTA-induced alterations clearly points to the distal tubule or collecting duct as site of action. The behavior of urine pH and titratable acid points also to the collecting duct as site of action since the final adjustment of pH occurs there [64 – 66]. One underlying mechanism is most probably interference with channels involved in electrolyte transport. Investigation in collecting-duct-derived Madin-Darby canine kidney (MDCK) cells revealed that OTA acts as a blocker of plasma membrane Cl^- conductance [53]. The concentration required for half-maximum inhibition was 30 nmol/L and well within the concentration range found during natural exposure [18]. Chronic application of OTA led to a different pattern. Absolute excretion of Na^+ was in tendency but not statistically different from control. Fractional excretion of Na^+ and K^+ was significantly increased. A significant increase in absolute excretion was most probably masked by the parallel reduction of GFR. In contrast to the cations, absolute and fractional excretion of Cl^- was still significantly elevated [13, 53]. Another important malfunction after chronic OTA exposure was the reduced concentrating capability of the kidney, leading to polyuria [13, 14, 18]. OTA seems to impair regulating mechanisms for urine concentration in the thick ascending limb of the loop of Henle, reducing maximum achievable osmolality [53]. Consequently, chronic OTA exposure would lead to (i) increased water excretion under physiological conditions and to (ii) reduced excretion during high water intake.

8 Which mechanisms mediate OTA-toxicity?

The majority of earlier studies used concentrations which are not observed during naturally occurring exposure (high-dose range, Table 2). The mechanisms observed in these studies – *e.g.*, direct and unspecific inhibition of macromolecule synthesis [1], DNA adduct formation [77], lipid peroxidation [78], oxidative damage [3], uncoupling of mitochondria [76] – occur only at very high (experimental) levels of exposure and thus represent a so-called "primary nonspecific" action. These high doses certainly can lead to general and unspecific cell perturbations, cell transformation, and subsequent tumor formation. However, these high concentrations do not occur by every-day exposure and therefore these high concentration-mediated actions have only minor toxicological relevance. Relevant, *i.e.*, nanomolar, concentrations do not exert a "classical" toxic action,

like for example leakage of cytosolic proteins (indicating unspecific cell perturbation and necrotic cell death) or inhibition of mitochondrial function [70, 79]. Thus, in this concentration range, OTA seems not to be a classical toxin.

9 The role of cell signalling

As listed in Table 2, cells react in a very specific manner to OTA in the low-dose range. Changes in cell function or phenotype ("specific action") can be observed without alterations of cell viability or integrity. Of course, this does not exclude the possibility that the "specific" action of OTA (as, *e.g.*, disturbance of pH-homeostasis) leads to a subsequent reduction of cell viability ("secondary nonspecific" action). Our current working hypothesis for the "specific" actions of OTA in the low-dose range is the following: OTA interacts with certain cellular key targets (*e.g.*, enzymes regulating second messengers) and thereby leads to the disturbance of cellular signalling and regulatory events (*e.g.*, carrier inhibition [44, 71], MAPK activation [73]). Those changes in cellular signalling and/or regulation result in specific changes of cell function and/or phenotype, leading to changes in renal function and finally to the possible disturbance of the whole-organism homeostasis. The determination of the key targets can improve our understanding of mycotoxicosis substantially. In the following we will discuss the role of signalling in more detail. Of course there are many signalling pathways in every cell and the OTA-induced disturbances of the signalling network are so far only partially understood.

10 Ca^{2+}-signalling and modulation of physiological signals

Cytosolic Ca^{2+} is a modulator of numerous cellular events, like metabolism, transport, and gene expression. The influence of OTA on cellular Ca^{2+} homeostasis has been investigated most thoroughly in immortalized human proximal tubular cells [79]. Within seconds OTA evokes reversible and concentration-dependent cytosolic Ca^{2+} oscillations with a threshold concentration of $\leq$0.1 nmol/L. These oscillations are abolished by removal of extracellular Ca^{2+}, by Ca^{2+}-channel blockade and by inhibition of phospholipase C. Furthermore, OTA stimulates the endoplasmic Ca^{2+}-ATPase activity and increases the filling state of Ca^{2+}-stores. Exposure to OTA also increases the cellular cAMP content in a dose-dependent manner. In addition, OTA-induced changes of cytosolic Ca^{2+} are reduced significantly by protein kinase A inhibition. Finally, OTA potentiates the effects of angiotensin II and epidermal growth factor (EGF) on cytosolic Ca^{2+} homeostasis. In addition, the toxin potentiates EGF- and angiotensin-II-induced cell proliferation in a Ca^{2+}-dependent manner. A decrease in cell viability can be observed only after 24 h exposure with threshold concentrations >10 nmol/L. This reduction of cell viability is independent of Ca^{2+}. These data show that OTA impairs cellular Ca^{2+}- and cAMP-homeostasis already at low nanomolar concentrations, resulting in dose-dependent cytosolic Ca^{2+} oscillations. Furthermore, OTA interferes with hormonal Ca^{2+} signalling, thereby leading to altered cell proliferation.

11 pH- and energy-homeostasis

The effect of nanomolar concentrations of OTA on cellular pH- and energy-homeostasis and the possible involvement of mitochondria was investigated in immortalized human kidney epithelial cells [70]. Within seconds OTA evokes a decrease of pH_c with a threshold concentration of 0.1 nmol/L, followed by sustained alkalinization. When Ca^{2+}-entry across the plasma membrane is prevented, virtually no OTA-induced pH-changes can be observed. Na^+/H^+-exchange (NHE) or the vacuolar-type H^+-ATPase are not involved in OTA-induced acidification. On the contrary, OTA stimulates NHE in a Ca^{2+}-dependent manner. Furthermore, OTA exposure does not increase lactic acid production, indicating that anaerobic glycolysis is not enhanced. Inhibition of complex I, III, and IV of the mitochondrial electron transport chain, with rotenone, antimycin A, and CN^- prevents OTA-induced acidification almost completely, indicating that activation of mitochondria provoked OTA-induced cytosolic acidification. Moreover, OTA induces a hyperpolarization of the mitochondrial membrane potential in a Ca^{2+}-dependent manner [79]. Finally, OTA exposure results in a mitochondria-dependent increase of cellular ATP content. Therefore, OTA can activate mitochondria and NHE *via* its interference with cellular Ca^{2+}-homeostasis.

Mitochondria are "stimulated" by nanomolar OTA concentrations and therefore the toxin decreases the rate of anaerobic glycolysis. In contrast, inhibition of mitochondria forces the cell to increase the glycolysis rate in order to maintain the cellular ATP level as long as possible. Interestingly, enhanced anaerobic glycolysis due to inhibition of mitochondria reduces the apoptosis-inducing effect of OTA in immortalized human kidney epithelial (IHKE) cells [80]. Therefore, nonrespiring mitochondria can protect certain cell types from apoptotic cell death. The increased use of glycolysis leads to increased formation of lactic acid which acidifies the cytosol and finally the extracellular surrounding of the cell. Extracellular acidification either provoked or artificially induced protects IHKE cells from apoptosis by an as yet not understood mechanism. Taken together, OTA-induced stimulation of mitochondria reduces lactic

acid formation and thereby acidification which ultimately can favor apoptotic cell death leading to atrophy. On the other hand, if cells suffering from OTA-induced genomic alterations enhance anaerobic glycolysis (which is a feature of many tumor cells), this would prevent apoptotic death of these transformed cells and may support tumor formation.

12 Mitogen-activated protein kinases

12.1 Extracellular signal-regulated kinases, ERK1/2

MAPKs are serine/threonine kinases activated by dual phosphorylation on both a tyrosine and a threonine residue [81 – 83]. These enzymes have emerged as components of one of the most important membrane-to-nucleus signalling pathways in eukaryotes. A MAPK cascade consists of a module of at least three kinases: a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a dual-specificity MAPK kinase (MAPKK), which in turn phosphorylates and activates a MAPK. The classical and best-studied MAPK cascade consists of Raf kinase (as a MAPKKK), MAP/ERK kinase (MEK1) or MEK2 (as MAPKK) and ERK1 or ERK2 (the respective MAPK). The Raf/MEK/ERK signalling cascade is activated in response to a variety of mitogenic stimuli operating through different mechanisms, *e.g.*, receptor tyrosine kinases, certain G protein-coupled receptors, or cytokine receptors. Recently, several additional MAPK cascades have been characterized. One of them consists of JNK (or SAPK1 for stress-activated protein kinase 1). Unlike the classical MAPK pathway, the JNK module is only modestly activated by growth factors and phorbol esters but is instead strongly activated by cellular stresses, including heat shock, UV irradiation, protein synthesis inhibitors, and inflammatory cytokines. This is also the case for another MAPK called p38 (or SAPK2 for stress activated protein kinase 2) [84].

In MDCK-C7 cells, but not in MDCK-C11 cells, OTA leads to a time- and concentration-dependent increase in ERK1/2 phosphorylation [73]. OTA-induced ERK1/2 phosphorylation in MDCK-C7 cells occurs at concentrations of 500 nmol/L, starts after 2 h, and is maximal after 8 h. Furthermore, after 8 h incubation, 500 nmol/L and 1 μmol/L OTA significantly increase ERK1/2 activity in MDCK-C7 but not in MDCK-C11 cells. This OTA-stimulated ERK1/2 phosphorylation and ERK1/2 activation in MDCK-C7 cells is partially inhibited by the synthetic mitogen-activated protein kinase kinase (MKK or MEK) inhibitor PD098059. Transepithelial resistance and lactate dehydrogenase release remain unaltered after incubation in the presence of 1 μM OTA for 8 h or of 100 nM OTA for 24 h, so it is unlikely that the effects on ERK1/2 are due to secondary toxic effects of the mycotoxin. Interestingly, OTA-induced long-term activation of ERK1/2 in MDCK-C7 cells is associated with epithelial dedifferentiation and transformation [85]. In contrast, MDCK-C11 cells, which do not show significant increases in ERK1/2 phosphorylation and ERK1/2 activity in response to OTA, retain their epithelial phenotype under identical experimental conditions. Taken together, the data indicate that OTA-induced ERK1/2-activation can result in cell dedifferentiation or even transformation. Thus, OTA-induced activation of ERK1/2 could be an important intracellular signalling pathway that mediates some of the mycotoxin's effects on renal epithelia. This assumption is supported by recent data showing dose-dependent activation of ERK1/2 by OTA (concentrations = 1μM for 24 h) in proximal tubular cells (OK cells) [62].

12.2 c-jun amino terminal kinase, JNK1/2

JNK, also known as stress-activated protein kinases, represents a group of enzymes that are activated by exposure of cells to cytokines and environmental stress [86, 87]. Transcripts derived from the *jnk* genes are alternatively spliced to create several JNK1, JNK2, and JNK3 isoforms, which are expressed as 46 kDa (JNK1) and 55 kDa (JNK2, JNK3) protein kinases. The JNK signalling pathway causes activation of the transcription factor AP-1, a process that has previously been implicated in oncogenic transformation. Furthermore, it has been reported that JNK might play a role in both, tumor growth and tumor suppression [88]. The fact that many pro-apoptotic stimuli (*e.g.*, UV radiation or TNF-α treatment) activate JNK provides evidence for a role for the JNK signalling pathway in apoptosis. Furthermore, it is possible that JNK may provide a protective signal, as suggested by studies in fibroblasts and thymocytes [89]. Nanomolar concentrations of OTA lead to activation of JNK in MDCK-C7 cells but virtually not in MDCK-C11 cells, as determined by kinase assay and Western blot [74]. Furthermore, OTA potentiats the effect of tumor necrosis factor α (TNFα) on JNK-activation. In proximal tubular OK cells, nanomolar concentrations of OTA were also shown to activate JNK after 24 h exposure. This was also the case for another stress activated kinase called p38, which exerts actions similar to JNK [62]. Thus, OTA interacts in a cell type-specific way with distinct members of the MAPK family at concentrations where no acute toxic effects can be observed.

12.3 ERK-JNK-(p38)-crosstalk

In general, ERK1/2 is supposed to act promitotic, whereas JNK and p38 are kinases supporting apoptosis, fibrosis, and inflammation [84, 90]. As already mentioned, OTA induces stimulation of ERK1/2 and JNK in renal cells derived from collecting duct. OTA (concentrations ≤ 1 μM for 24 h) also

activates ERK1/2, JNK, and p38 simultaneously in proximal tubular OK cells [62]. Thus, in principle OTA is able to induce MAPK activities promoting opposing effects in renal cells. There is evidence that it is the balance between ERK1/2 on the one hand and JNK (and therefore most likely p38, too) on the other hand that determines cellular fate. This was also shown for renal proximal tubular cells [91]. Therefore, an increase of apoptosis, inflammation, and collagenosis in renal cells could be the result of a shift of kinase balance away from ERK1/2 on to JNK and p38.

Moreover, the simultaneous activation of ERK1/2 and JNK gives rise to an attractive hypothesis on the increased incidence of renal tumors and urinary tract tumors associated with OTA-induced nephropathies in animals and BEN in humans [92]. Constant OTA-exposure of the kidney may result in an increased fraction of cells with elevated ERK1/2 activity due to clonal selection. ERK1/2 activation was shown to be increased in a number of tumors from various organs and was also shown to be activated in around 50% of all renal neoplasias [93]. In opposite, activation of JNK was shown to induce apoptosis in human cancer cells [94]. Thus, the tumor-inducing capacity of OTA can depend on the balance of ERK1/2 activation on the one hand and on the activation of JNK on the other hand. If cells are exposed to OTA, any stimulus shifting balance of MAPKs on to ERK1/2 as compared to JNK (and p38) will inhibit apoptosis and thus potentially induce tumor growth.

13 Cellular dedifferentiation

When it was shown that OTA activates ERK1 and ERK2 in the C7 clone but not in the C11 clone of renal epithelial MDCK cells, the question arose whether cellular dedifferentiation might result from this ERK activation [85]. OTA, at nanomolar concentrations leads to stable and irreversible phenotypical and genotypical alterations, resulting in sustained dedifferentiation of MDCK-C7 cells but not of MDCK-C11 cells. Dedifferentiated MDCK-C7 cells (OTA-C7 cells) display a distinct morphology from the parent cell line (spindle-shape, pleiomorphic, narrow intercellular spaces, increased cell size) and show a reduced proliferation rate and numerical chromosomal aberrations. Functionally, OTA-C7 cells are characterized by a dramatic reduction of transepithelial electrolyte transport and the complete loss of responsiveness to the mineralocorticoid hormone aldosterone. These data provide further evidence that OTA can lead to cell dedifferentiation and to transformation of cloned quiescent cells. The changes in phenotype due to this dedifferentiation could explain some of the OTA-induced changes in renal function, like altered renal electrolyte excretion. Only recently, OTA was shown to induce epithelial to mesenchymal transition in proximal tubular cells [62]. Epithelial to mesenchymal transition takes place in chronic renal disease and is associated with the expression of α-smooth muscle actin [62, 95], leading to generation of a fibroblast like cell type from former epithelial cells. The latter may explain the increase of collagen secretion during OTA exposure observed in proximal tubular cells [62], also from human origin [52].

14 Apoptosis

Cell perturbation caused by OTA can result in cell death. Principally, two possibilities of cell death exist, necrosis and programmed or regulated cell death (= apoptosis). Whilst necrotic cell death was long known, apoptosis came into the focus of interest only recently although described already in 1972 by Kerr *et al.* [96], an Australian pathologist. Increased apoptosis rates in the kidney may lead to polycystic kidney disease, glomerular sclerosis, or interstitial fibrosis [97 – 100]. The latter damages were found also in porcine nephropathy and BEN [97, 98, 101]. Therefore, the role of apoptosis in the etiology of BEN was suggested [102]. OTA in nanomolar concentrations leads to apoptosis in proximal tubule cells of human or marsupial origin (IHKE and OK cells, [62] and [69]) and in canine MDCK-C7 cells from the collecting duct [67]. Also in rat kidney apoptosis in the proximal tubule as well as in distal parts occurs after OTA administration together with oxidative damage [68]. Micronuclei formation, which indicates apoptotic cell death, occurs in human and rat kidney cells after OTA administration [103]. In IHKE and MDCK-C7 cells apoptosis induction is concentration-dependent with a maximum reached during exposure to 300 nmol/L OTA. In MDCK-C7 cells a sharp threshold exists because exposure to 100 nmol/L OTA leads to only minimal increase of caspase-3 activity whereas 300 nmol/L OTA leads to 14-fold increase of caspase-3 activity. This principle holds true also for the other cells but with different threshold concentrations. In addition, in IHKE cells a prolonged exposure (7 days) to only 5 nmol/L OTA leads to a significantly increased caspase-3 activity. This demonstrates that already low concentrations of OTA can lead to apoptosis when cells are exposed for prolonged time periods.

In parallel to its differential effects on JNK, nanomolar OTA induces apoptosis in MDCK-C7 cells but not in MDCK-C11-cells, as determined by DNA fragmentation, DNA ladder formation, and caspase-3 activation [74]. Thus, there exists a certain cell type specificity also with respect to OTA-induced apoptosis. In addition, OTA potentiates the proapoptotic action of TNFα and therefore interferes with endogenous signalling pathways. Induction of apoptosis *via* the JNK-pathway can explain some of the

OTA-induced changes in renal function as well as part of its teratogenic action.

Cytochrome *c* release from mitochondria was described frequently in the apoptotic process to precede the activation of caspase-3. The release of cytochrome *c* follows an imbalance of pro- and antiapoptotic proteins of the bax and bcl2-gene families [104, 105] in favor of apoptotic proteins which leads to the formation of the nuclear transition pore in the outer mitochondrial membrane [106]. Cytochrome *c* then binds to Apaf which together activate caspase-9 which in turn activates caspase-3 [107].

Although these are well-described phenomena, OTA exposure does not lead to cytochrome *c* release neither in IHKE nor in MDCK-C7 cells over an incubation period of 24 h, a time point at which caspase-3 activation as well as DNA ladder formation is clearly visible. This demonstrates that OTA does not lead to apoptosis using the cytochrome *c* and thus mitochondria-involving pathway. These data fit nicely to the mitochondria-stimulating action of OTA (see above). The exact mechanisms leading to increased apoptosis rates during OTA exposure remain still unknown.

15 Interaction with other nephrotoxins

The proapoptotic action of different substances in combination with OTA, including antibiotics, NO-donors, H_2O_2, $CdCl_2$, cisplatin, cyclosporine A, and different pH values, was tested by a rapid apoptosis screening assay [108]. With this test it was possible to demonstrate that the apoptotic potential of OTA can be influenced by other nephrotoxins or environmental factors. Depending on the combination used, additive and potentiating effects were detectable. For example, 1 nmol/L OTA antagonized the effect of 10 µmol/L cadmium on caspase-3 activity, whereas 100 nmol/L OTA potentiated it. In addition, the nature of interaction can be different from cell type to cell type. Notably, the effects of a combination of two different substances are not predictable because they can have opposite effects depending on their respective concentrations and the cell type used. Nevertheless, the nephrotoxic potential of OTA depends on the coexposure to other nephrotoxic agents, on their concentrations and on the cell line studied. Most probably, each scenario has to be evaluated separately.

16 Conclusions

At nanomolar concentrations OTA leads to specific changes of function and phenotype in renal cells but not to necrosis. The toxin interacts with certain cellular "key molecules" (*e. g.*, MAP kinases), thereby disturbing cellular signalling and regulation events as well as mitochondrial function. Moreover, OTA has the ability to modulate physiological signals (*e. g.*, angiotensin II or TNFα) and thereby influences cell function and cell growth and may even stably reprogram cells (*e. g.*, altered distribution of chromosomes). Thus, at toxicological relevant concentrations OTA may be regarded as a modulator of cellular signalling and not as a "classical" toxin.

Work performed in the authors' laboratory was supported by the Deutsche Forschungsgemeinschaft.

17 References

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