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Combinatory effects of citrinin and ochratoxin A in immortalized human proximal tubule cells

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Abstract

Ochratoxin A (OTA) and citrinin (CIT) are two mycotoxins often occurring together in grains and cereals. Although both are nephrotoxic and can induce apoptosis, combination effects have not been examined up to now. Therefore, the aim of this study was to take a close look at the interactions of citrinin and OTA in cultured human proximal tubule-derived cells (IHKE cells). The cytotoxicity of both mycotoxins was studied, measuring the metabolic activity and the cell number. Furthermore, caspase 3-activation as a marker for apoptosis was examined for both mycotoxins alone and in combination. The results show that citrinin had an antagonistic effect on ochratoxin A induced caspase 3-activation in concentrations of 2.5 and 5 µmol/l. Higher concentrations (7.5 and 15 µmol/l) lead to additive effects, lower citrinin concentrations (0.25 and 1 µmol/l) did not show any effect at all. The observed decrease in caspase 3-activity was specific for the combination with OTA, since the combination of citrinin with cisplatin did not show any effect. Citrinin did not influence the OTA-induced apoptosis when added two hours after applying ochratoxin A. Also, the combination of both toxins decreased the uptake of OTA into the cells which might be an explanation for the antagonistic effect of citrinin in certain concentrations. However, the transport into cells can not be the only explanation, so further examinations are necessary.

Keywords: citrinin, ochratoxin A, apoptosis, cytotoxicity, cell culture

Introduction

Contamination of food and feed with mycotoxins is an enormous problem for human and animal health. The various mycotoxin-producing molds can form a great variety of toxins such as ochratoxin, citrinin, aflatoxin, patulin, trichothecenes and fumonisins. However, toxicological data are mostly available for single mycotoxins only. In spite of the fact that contaminated materials often contain two or more mycotoxins, little is known about their interaction. For example, citrinin has been found together with ochratoxin A in feeds (1). Also, Vrabcheva *et al.* (2) reported findings of contaminated grains containing ochratoxin A as well as citrinin in villages with an increased incidence of Balkan Endemic Nephropathy (BEN). Both mycotoxins are most likely involved in BEN, an illness in whose etiology apoptosis has been suggested to take part (3).

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Apoptosis, also known as programmed cell death, was shown to be induced in various cells by many different substances. In contrast to necrotic cell death, apoptosis is characterized by caspase-activation, DNA ladder formation and cytosolic as well as nuclear shrinkage. Caspases are proteolytic enzymes which play a major role in apoptosis. Caspase 3-activity can be measured specifically with the substrate Ac-DEVD-AFC, which is cleaved into the fluorescing AFC (7-Amino-4-trifluoromethylcoumarin) and the tetrapeptide Ac-DEVD. The classical biochemical marker of apoptosis is the presence of oligonucleosome-sized fragments of DNA, generated by caspase 3-dependent endonucleolytic activity, leading to the typical DNA ladder when analyzed by agarose gel electrophoresis.

Ochratoxin A (OTA), a nephrotoxin produced by several *Penicillium* and *Aspergillus* species, is found mainly in cereals and cereal products, but also in coffee, beer, peanuts and various other food. Among others, it has carcinogenic, teratogenic and embryotoxic properties (4). Schwerdt *et al.* showed that OTA activates caspase 3 in IHKE cells at concentrations as

low as 10 nmol/l; DNA ladder is formed at concentrations of 30 nmol/l (5). OTA also activates caspase 3 in MDCK and OK cells, but at higher concentrations (5, 6). In general, ochratoxin A is more cytotoxic than citrinin (7-10), with EC_{50}-values as low as 7.9 µmol/l in HL-60 cells (7) and 1.2 µM in porcine urinary bladder epithelial cells (PUBECs) (10).

Citrinin, another nephrotoxin, is produced by *Penicillium*, *Aspergillus* and *Monascus* spp. and found in grains mainly. It is frequently formed in combination with OTA. Citrinin has antibiotic and bacteriostatic properties. Its cytotoxicity is well examined, with EC_{50}-values ranging from 5 µmol/l to more than 1 mmol/l, depending on cell lines, incubation time and endpoint (7-11). Citrinin has also been found to induce apoptosis in HL-60 cells with a minimal effective dose of 40 µmol/l (7).

Mixtures of both mycotoxins were also examined regarding the cytotoxicity with trypan blue exclusion only. The results show that the combinations are more toxic than each one of the single compounds (12-14). In PUBECs, no additive or synergistic effect was observed (10).

However, nothing is known about combinatory effects when looking at the caspase 3-activation. Since the mode of action concerning the induction of apoptosis of these mycotoxins is still not very clear, examinations of the combination effects caused by citrinin and OTA might be helpful.

Materials and Methods

Chemicals

DMEM/Ham F-12 media and fetal calf serum were obtained from Biochrom KG (Berlin, Germany). Ochratoxin A and Citrinin were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany), mouse epidermal growth factor from BD Biosciences (Heidelberg, Germany). All other chemicals were obtained from VWR International GmbH (Darmstadt, Germany) and Sigma-Aldrich.

Cell culture

Immortalized human proximal tubule cells (IHKE cells, passage 150-175) were cultured as described by Tveito (15) in DMEM/Ham's-F12 medium (100 µl/cm^2) enriched with 13 mmol/l $NaHCO_3$, 15 mmol/l HEPES, 36 µg/l hydrocortisone, 5 mg/l human apotransferrin, 5 mg/l bovine insulin, 10 µg/l mouse epidermal growth factor, 5 µg/l Na-selenit, 10% fetal calf serum and in addition 1% penicillin/streptomycin under standard cell culture conditions (37 °C, 5% CO_2).

Cytotoxicity assays

Cytotoxicity was measured using the CASY cell-counting System Model TT (Schärfe System GmbH, Reutlingen, Germany) and the CCK8-assay (by Dojindo Laboratories, Japan; obtained from probior GmbH, München, Germany) according to the manufacturer's instructions. For details of the CCK-8 assay see also (16). Before incubation with citrinin and OTA, cells were incubated in serum-free medium for 24 h in Petri dishes (CASY) or 96-well plates (CCK8-assay). For the assay, cells were incubated with different concentrations of citrinin and OTA for 24 h.

Caspase 3 assay

Before incubation with the substances, approximately 5*10^6 cells were incubated in serum-free medium for 24 h in Petri dishes. The chemicals were incubated for 24 h. Caspase 3-activity was measured according to the manufacturer's instruction (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications. In brief, cells were incubated with 100 µl cell lysis buffer for 30 min on ice, harvested and centrifuged at 13000 g for 10 min at 4 °C. Supernatant was incubated with the caspase 3 substrate Ac-DEVD-AFC for 60 min at 37 °C and fluorescence of the cleaved product was measured at 405 nm excitation and 520 nm emission wavelength in an microtiter-plate-reader (FLUOstar Optima, BMG LABTECH GmbH, Offenbach, Germany). Cleaved AFC was quantified by a calibration curve using known AFC-concentrations. Protein content was determined with bicinchonic acid assay from Sigma.

DNA ladder formation assay

Culture medium was collected and cells were separated by centrifugation. Seeded cells on Petri dishes were harvested in cells lysis buffer (10 mM TRIS-HCl, 150 mM NaCl, 2 mM EDTA, 1% SDS, adjusted to pH 8.0 + 0.5 mM guanidin hydrochloride), incubated on ice together with the previously obtained cell pellet and centrifuged. RNase (30 µg/ml) and Proteinase K (60 µg/ml) were added to the supernatant successively and incubated for

30 minutes each. DNA was extracted by using DNA-Wizard Clean-up-Kit (Promega GmbH, Mannheim, Germany) according to the manufacturer's instruction. DNA ladder formation was analyzed in 1.5% agarose gel.

Uptake of ^{3}H-OTA and determination of intracellular radioactivity

The uptake experiments and determination of intracellular radioactivity were performed according to (17).

Data analysis

All data are given as mean values ± SEM. The significance of the differences was determined using the unpaired Student's t-test. To compare the measured values with the expected, the latter were calculated by the addition of the mean values obtained for the single incubation:

$$\text{Expectation value } [\%] = \text{mean}_{\text{substance 1}}\ [\%] + \text{mean}_{\text{substance 2}}\ [\%] - 100\%$$

The SEM was calculated as follows:

$$\text{SEM}_{\text{expected}} = \sqrt{(\text{SEM}_{\text{substance 1}})^2 + (\text{SEM}_{\text{substance 2}})^2}$$

The determination of the significance was calculated using suitable tables and the following equation:

$$t\text{-test value} = \frac{|\text{mean}_{\text{measured}} - \text{expectation value}|}{\sqrt{(\text{SEM}_{\text{measured}})^2 + (SEM_{\text{expected}})^2}}$$

Results and Discussion

Combination of Citrinin and OTA

Since citrinin has not been tested in IHKE cells yet, cytotoxicity was examined using the CCK8-assay, which measures the metabolic activity, and the CASY cell counter. The median effective concentration (EC_{50}) values after 24 h incubation time were determined to be 56.3 ± 1.6 µmol/l for the CCK8-assay and 27.7 ± 0.8 µmol/l when using the cell counter. EC_{50}-values for OTA were 69.1 ± 7.3 nmol/l with the CCK8-assay and 450.5 ± 6.6 nmol/l with the CASY system. These results are in accordance with other experiments in which OTA was more cytotoxic compared to citrinin (7-10).

Since there are no data for citrinin concerning the induction of apoptosis in IHKE cells, the caspase 3-activation caused by citrinin was measured. When IHKE cells were incubated for 24 h with 0.25-15 µmol/l citrinin, a significant increase in the caspase 3-activity was observed at concentrations over 5 µmol/l (Figure 1, grey bars). The citrinin-induced apoptotic effect, measured as caspase 3-activation, was confirmed by DNA ladder formation using OTA as positive control (data not shown). In order to study combination effects of both toxins, citrinin concentrations ranging from 0.25 µmol/l up to 15 µmol/l were combined with 25 nmol/l and 50 nmol/l ochratoxin A, respectively (Figure 1, white bars). As the results in Figure 1 show, 2.5 and 5 µmol/l citrinin had an antagonistic effect on the caspase 3-activation induced by 25 and 50 nmol/l OTA. The caspase 3-activity for these combinations is significantly lower as the calculated expectation values (Figure 1 A and B, black bars). At higher concentrations (7.5 and 15 µmol/l) the effect was additive, lower concentrations (0.25 and 1 µmol/l) had no influence on the OTA-induced caspase 3-activation.

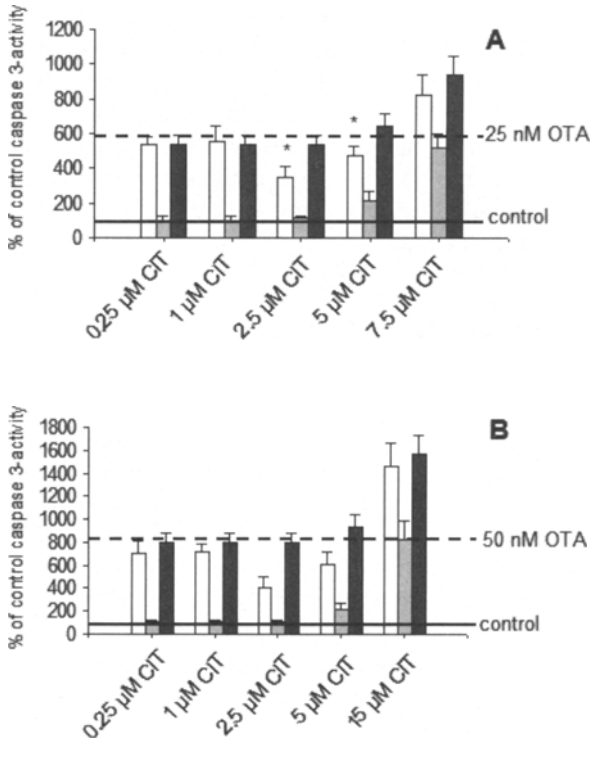

Figure 1. Caspase 3-activity in human proximal tubule-derived cells (IHKE cells) after 24 h exposure to the indicated substances and concentrations. White bars: combination of 25 nmol/l OTA (Fig. 1 A) or 50 nmol/l OTA (Figure 1 B) with the indicated citrinin (CIT) concentrations. Grey bars: caspase 3-activity induced by citrinin alone. Black bars: calculated expectation values (for the calculation of the expectation values see materials and methods). Experiments were performed as triplicates with three passages at least (N >9). * p <0.01 compared to calculated expectation values

Combination of citrinin and cisplatin

To examine if the antagonistic effect of citrinin (2.5 and 5 µmol/l) was specific for combinations with OTA, 2.5 and 5 µmol/l citrinin were combined with 20 µmol/l cisplatin. Cisplatin is a commonly used chemotherapeutic drug for treating solid tumors. It has been shown to induce apoptosis in kidney and proximal tubule cells (18, 19). Citrinin had an additive effect on cisplatin-induced caspase 3-activation (Figure 2), so this experiment demonstrated that the antagonistic effect was only specific for the combination of citrinin with ochratoxin A.

Uptake of OTA into IHKE cells in the presence of different citrinin concentrations

To analyze the cause of the antagonistic effect of 2.5 and 5 µmol/l citrinin, the uptake of tritium-labeled ochratoxin A (^{3}H-OTA) into IHKE cells was studied when applied alone and in combination with citrinin in concentrations ranging from 1 µmol/l to 15 µmol/l.

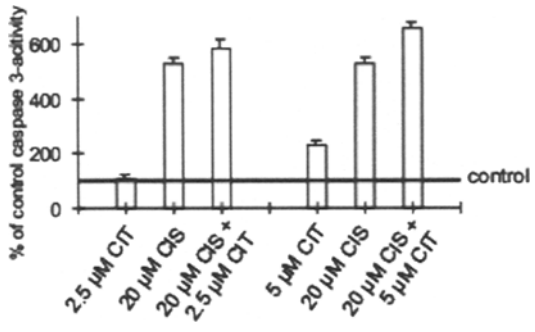

Figure 2. Caspase 3-activity in human proximal tubule-derived cells (IHKE cells) after 24 h exposure to the indicated substances and concentrations. cisplatin (CIS), citrinin (CIT). Experiments were performed as triplicates with three passages at least (N >9)

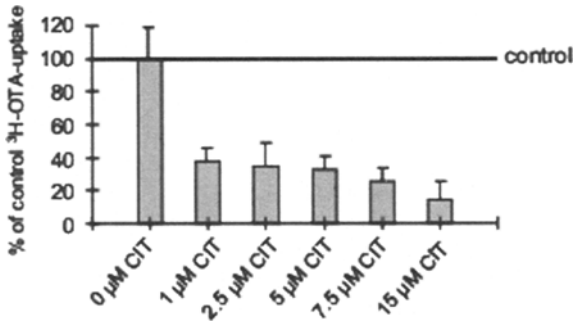

Figure 3. Uptake of tritium-labeled OTA (^{3}H-OTA) in human proximal tubule-derived cells (IHKE cells) applied alone and with the indicated citrinin concentrations. citrinin (CIT) (N=8)

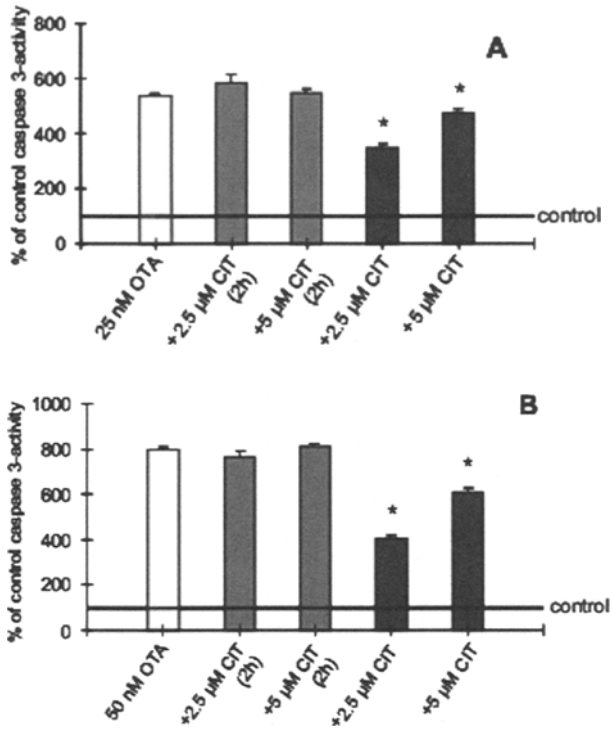

Figure 4. Caspase 3-activity in human proximal tubule-derived cells (IHKE cells) after exposure to Ochratoxin A (24 h) and citrinin (24 and 22 h respectively). ochratoxin A (OTA), citrinin (CIT). Grey bars (+2.5 µM CIT/+5 µM CIT): application of citrinin 2 hours after application of OTA. Black bars (+2.5 µM CIT/+5 µM CIT): simultaneous application of CIT and OTA. Experiments were performed as triplicates with three passages at least (N >9). *p <0.01 compared to calculated expectation values (for their calculation see materials and methods)

As shown in Figure 3, citrinin reduced the uptake of ochratoxin A by over 60% compared to the uptake of OTA without citrinin.

The citrinin concentration was of a minor importance only: 1 µmol/l citrinin lead to a reduced OTA-uptake of 37.1±8.7%; 2.5 and 5 µmol/l lead to a reduction to 35.0±13.9% and 32.8±8.3%, respectively. However, the antagonistic effect appeared with 2.5 and 5 µmol/l citrinin only, while 1 µmol/l did not affect the OTA-induced caspase 3-activation (see Figure 1). Since citrinin reduced the uptake of OTA in all tested concentrations (1-15 µmol/l), interferences with the uptake can not be the only explanation for the decreased caspase 3-activation with 2.5 and 5 µmol/l citrinin.

Effect of citrinin when applied with a two hour-delay

It is well known that OTA enters IHKE cells via the basolateral organic anion transport, the apical H^+-dipeptide-cotransport and non-ionic diffusion. The uptake can be divided into a

rapid phase during the first five minutes and a slow phase between 45 and 90 minutes (20). So it can be expected that the transport of OTA into the cells is completed after two hours. To examine if the decreased caspase 3-activity resulting from the combination of citrinin and OTA is due to the reduced uptake of OTA, citrinin was added to the cells two hours after the addition of OTA and the caspase 3-activation was measured. The antagonistic effect of 2.5 and 5 µmol/l citrinin did not appear when it was added two hours after OTA (Figure 4, grey bars). So it can be concluded that the disturbed accumulation of OTA in the cells is at least partly responsible for the antagonistic effect concerning the caspase 3-activation.

Conclusions

The results of this study clearly show the importance of studying combinatory effects of mycotoxins. In other studies analyzing cell viability with the trypan blue exclusion method, citrinin and ochratoxin A showed additive to synergistic effects. However, no variation of the ratio of OTA to citrinin was examined. The proportion of each toxin in the mixture was calculated according to their LC_{50} values (lethal cells dose of 50%), combining both compounds in the concentration of their individual LC_{50} values, resulting in ratios of 1:1 to 1:3 (µmol/l OTA : µmol/l CIT) (12, 14). The combination of ochratoxin A with certain concentrations of citrinin significantly lowered the caspase 3-activation and therefore the apoptotic cell death; so these citrinin concentrations (2.5 and 5 µmol/l) had a slightly protective effect. The concentration ratios used were oriented on ratios found in food (2). This study also points out the importance of concentrations and concentration ratios when examining combinatory effects. There may be a change in the mode of action of the toxicity depending on the ratio used in combinatory experiments. Further studies are necessary to explain the fact that 2.5 and 5 µmol/l citrinin had an antagonistic effect whereas 1 µmol/l did not affect OTA-induced caspase 3-activitation, although the uptake of OTA was already reduced at this concentration.

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