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Ochratoxin A Induces Apoptosis in LLC-PK1 Cells via JNK and p38 MAPK Activation*

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Ochratoxin A (OTA) is a potential inducer of a tubular-interstitial nephropathy in humans and animals. In our study we addressed the question of involvement of apoptosis in the development of OTA-provoked nephrotoxicity. LLC-PK1 kidney cells were treated with nanomolar and micromolar concentrations of OTA for different lengths of time. The apoptotic process was estimated by morphological (haematoxylin/eosin staining, fluorescent staining of DNA free ends – TUNEL assay) and biochemical (MAPKs and Hsps) changes of cells. Forty-eight hours of treatment with 5×10^{-6} M OTA significantly decreased cell viability and induced apoptosis in 30.7 % of cells. In addition, a transient activation of ERK was observed as well as a strong and prolonged activation of stress kinases, JNK and p38 MAPK, after 12 and 48 hours of treatment. Expression of Hsp72 and Hsp27 was not affected by OTA. The results suggest that apoptosis mediated by activation of JNK and p38 MAPK might play an important role in OTA-induced nephrotoxicity in LLC-PK1 cells.

Keywords
OTA
LLC-PK1 cells
apoptosis
MAPKs
Hsps

INTRODUCTION

Ochratoxin A (OTA) is produced as a secondary fungal metabolite of some *Aspergillus* and *Penicillium* strains and is a frequent contaminant of feed and foodstuffs.[1,2] OTA is hazardous to humans and animals because of its nephrotoxic, hepatotoxic, immunosuppressive, teratogenic, and carcinogenic effects.[3–8] Kidneys are the main target of OTA.[9] OTA is known to affect multiple sites of the nephron: acute exposure mainly affects the proximal parts, while chronic exposure leads predominantly to damage of the proximal tubule.[2] Epidemiological studies implicate OTA with endemic nephropathy (EN), a progressive renal fibrosis, and tumours of the urinary tract.[10,11] It has been shown in animals that OTA induces a tubular-interstitial nephropathy similar to EN as well as renal adenoma and carcinoma.[12,13]

Some aspects of OTA toxicity have been attributed to the phenylalanine moiety responsible for the inhibition of protein synthesis by competitive inhibition of phenylalanil-tRNA synthase. The open lactone moiety of OTA molecule is structurally analogous to active sites of some mitochondrial enzymes, thus OTA may interfere with oxidative processes such as reactive oxygen species (ROS) formation and lipid peroxidation (LPO).[14–16] OTA toxicity has been also related to the impairment of intracel-

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lular calcium handling, competitive inhibition of transport carrier proteins located in the inner mitochondrial membrane (ATPase, succinate dehydrogenase, and cytochrome c oxidase), inhibition of incorporation of orotic acid into the RNA, and interference with signal transduction in some cell types.[10,16,17]

Therefore, exposure to OTA can cause cell deregulation and death: either through overproduction of ROS and LPO, and disruption of the Ca^{2+} transport, or through inhibition of protein synthesis and inhibition of phenylalanine metabolism leading to the reduction of phosphoenolpyruvate carboxykinase and gluconeogenesis, ultimately inducing severe cell disturbances.[18–21]

Cells typically die either by apoptosis or necrosis.[22] Although the two types of cell death can be differentiated biochemically and morphologically, these processes are more similar than previously thought.[23] In necrosis the cell membrane loses its integrity and the cell content is released causing an inflammatory response, while in apoptosis the cell content remains »well-packed« in the apoptotic bodies and no inflammation occurs. However, both types of cell death can be induced by the same pathophysiological signals, can be prevented by anti-apoptotic mechanisms, and once initiated can switch to the other type of cell death.[23]

OTA has been reported to activate apoptotic responses in several cell types, including Hak, HeLa cells, MDCK-C7, MDCK-C11, OK, and NRK-49F fibroblasts.[24–26] Apoptosis of liver and kidney cells has been observed after OTA administration to mice and rats *in vivo*.[27,28] Induction of apoptosis via the JNK pathway has been suggested for some OTA-induced changes in the renal function as well as for part of its teratogenic action.[9,29] The carcinogenic potential of OTA has been also correlated with its capacity to induce apoptosis and affect cell signalling in gap junctional communication-proficient rat liver and human kidney epithelial cells grown *in vitro*.[10] However, the exact molecular mechanisms involved in the induction of apoptosis by OTA have not yet been fully clarified.

In this study, the molecular mechanism of OTA toxicity was investigated in the LLC-PK1 epithelial kidney cells. The ability of different OTA concentrations to induce apoptotic signals was estimated by biochemical and morphological changes in LLC-PK1 cells.

EXPERIMENTAL

Cell Culture

A pig kidney cell line corresponding to the proximal epithelial cells, LLC-PK1, was cultured at 37 °C in a 5 % CO_2 atmosphere in MEM culture medium supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 100 IU/ml (≈ 60 μg/ml) penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 290 μg/ml L-glutamine and 2.5 mg/ml sodium hydrogen carbonate. Cells were plated in 6 well plates, usually at a plating density of 1–3 x 10^6 cells/ml.

Cells were harvested by removing the medium, washing in phosphate-buffered saline (PBS; 20 mmol dm^{-3} phosphate buffer, 150 mmol dm^{-3} NaCl, pH = 7.2), treating with trypsin (0.25 % solution in PBS) for 5 min, and centrifuging at 600 *g* at 4 °C for 5 min.

Treatment

LLC-PK1 cells were treated with 0.10, 0.25, 0.50, 1.00, and 5.00 x 10^{-6} M OTA for 2, 4, 6, 12, 24 or 48 hours. OTA was dissolved in ethanol at a concentration of 5 x 10^{-6} M, and the final concentration was obtained by appropriate dilution in the culture medium. The final ethanol concentration in the medium was 0.025 %. Ethanol was added to the control cells in a final concentration of 0.025 %.

Trypan Blue Exclusion Assay

To assess cell viability, 0.5 ml of 0.4 % Trypan blue solution was added to the test tube. 0.3 ml PBS was added to 0.2 ml cell suspension, the suspension was mixed and then incubated for 10 min. Non-viable cells (dye-stained) as well as viable cells (unstained) were counted under light microscopy.

Whole Cell Lysis

Treated and untreated LLC-PK1 cells were washed twice with cold PBS and lysed with 100 μL of ice-cold whole-cell lysis buffer (50 mmol dm^{-3} Tris-HCl pH = 8.0, 137 mmol dm^{-3} NaCl, 1 % Nonident P-40, 10 % glycerol, and a »Complete protease inhibitor« cocktail tablet). After 20 minutes, cell lysates were centrifuged at 4 °C at 14000 rpm. Samples were denatured by boiling for 3 minutes with 6 × Laemmli sample buffer (0.375 M Tris-HCl pH = 6.8, 12 g/100 ml SDS, 3 % glycerol, 0.2 g/100 ml bromophenol blue, 12 % β-mercaptoethanol in distilled water).

Haematoxylin/Eosin Staining of Cells

In order to visualize and estimate apoptotic cells, LLC-PK1 cells were stained with haematoxylin/eosin following OTA treatment. For this purpose, LLC-PK1 cells were cultured on coverslips in small Petri dishes. Confluent cells were treated with OTA as described in *Treatment*. After removal of culture medium, cells were stained with haematoxylin and eosin using Kiernan's method.[30]

Fluorescent Staining of DNA Fragments (TUNEL Assay)

Terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling of DNA (TUNEL assay) was used for visualizing and estimating apoptotic cells. The assay was carried out using dUTP-fluorescein isothiocyanate (FITC) according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Boehringer Mannheim, Germany).

Western Blotting

Western blot analysis was performed in order to assess ERK1/2, JNK 1/2, p38, Hsp27 and Hsp72 protein expression as well as ERK1/2, JNK 1/2 and p38 activation (phosphorylation).

35 μg of total proteins was loaded for each sample onto a 12 % polyacrylamide gel usually run at 100 V. Transfer onto nitrocellulose membrane was conducted at 250 mA for 90 minutes. Membranes were blocked for one hour with blocking buffer containing 4 g BSA in 100 ml of TBS+T (25 mmol dm^{-3} Tris pH = 7.6, 150 mmol dm^{-3} NaCl, 0.05 % Tween 20). Membranes were probed overnight at room temperature with either anti-JNK-P antibody (Promega) diluted 1:5000 in blocking buffer, anti-ERK-P antibody (Promega) diluted 1:5000 in blocking buffer, anti-p38-P antibody (Promega) diluted 1:2000 in blocking buffer, anti-JNK_1(FL) antibody (Santa Cruz Biotechnology) diluted 1:800 in blocking buffer, anti-ERK_1(C-16) antibody (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer, anti-p38(C-20) antibody (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer, anti-Hsp27 antibody (Stressgene) diluted 1:5000 in blocking buffer, anti-Hsp72 antibody (Stressgene) diluted 1:1000 in blocking buffer or anti-actin antibody (Sigma) diluted 1:1000 in blocking buffer. A horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1:4000 in the solution of 4 g BSA in 100 ml of TBS+T was used to allow detection of the appropriate bands using an enhanced chemiluminescence reagent and film. All experiments were repeated at least three times and representative blots were analyzed using the ScionImage software for Windows (Scion Corporation).

Statistical Analysis

Data from this study were analyzed by a one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Dunnett's test). The level of $P<0.05$ was considered statistically significant for all the tests performed. We also performed Levene's test and established homogeneity of variance among all the groups of data assessed. The SPSS computer program for MS Windows, release 6.0, was used for statistical analysis.

RESULTS AND DISCUSSION

Time- and Dose-dependent Viability of LLC-PK1 Cells Treated with OTA

The fungal metabolite OTA has been shown to exert chronic damaging effects in mammals at low concentrations.[2,31] Chronic exposure to OTA leads to impairment of renal function and morphology as well as to increased incidence of renal adenoma and carcinoma. The mechanism of action of low OTA concentrations (<1 μmol dm^{-3} for most cultured kidney epithelial cell lines) has not yet been unveiled satisfactorily. It seems that the disruption of cell viability by necrosis is associated with impaired protein synthesis and/or ROS generation caused by higher OTA concentrations (>1 μmol dm^{-3} for most cultured kidney epithelial cell lines).[29,31,32] It has been reported that OTA affects multiple sites of the nephron; acute exposure mainly affects the proximal parts, while chronic exposure affects predominantly proximal tubules. LLC-PK1 cells are therefore widely used as the *in vitro* model of cultured tubular epithelial cells, since they retain several characteristics of the proximal tubule cells.[2,33]

To investigate whether OTA impairs cell viability, we incubated LLC-PK1 cells with different OTA concentrations for 12, 24, and 48 hours. As shown in Figure 1, 5 μmol dm^{-3} OTA significantly decreased viability of LLC-PK1 cells after 12 (by 50 %), 24 (by 76 %), and 48 (by 84 %) hours of treatment, while 1 μmol dm^{-3} OTA significantly decreased cell viability only after 24 (by 45 %) and 48 (by 67 %) hours of treatment. Lower OTA concentrations (100–500 nmol dm^{-3}) affected significantly the viability of LLC-PK1 cells only if the cells were treated with OTA for 48 hours.

We addressed the question whether the observed decrease in viability of LLC-PK1 cells reflects necrotic or apoptotic cell death. We therefore estimated the morphology of adherent LLC-PK1 cells exposed to the same OTA treatment by haematoxylin/eosin staining and TUNEL assay. Our results demonstrate that both low (250–500 nmol dm^{-3}) and high (1–5 μmol dm^{-3}) OTA concentrations significantly increased the number of LLC-PK1 cells undergoing apoptosis after 24 and 48 hours of treatment, as summarized in Figure 2.

Activation of MAPKs by OTA

Schramek *et al.* have shown that OTA has the ability to activate members of the mitogen-activated protein kinase (MAPK) family.[29] MAPKs are important participants

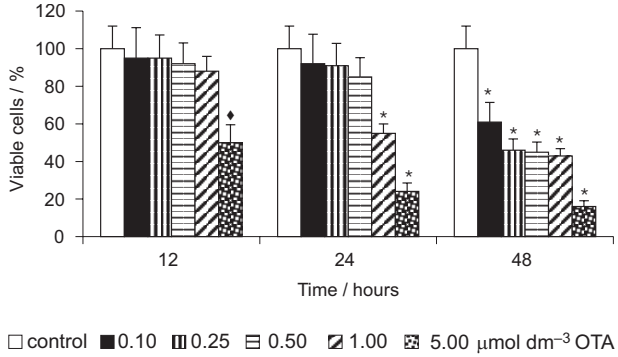

Figure 1. Effects of OTA on LLC-PK1 cell viability. Cells were treated with OTA in a concentration range from 100 nmol dm^{-3} to 5 μmol dm^{-3}. Cell viability was examined after 12, 24 and 48 hours by the Trypan blue exclusion assay. Values for untreated control cells were taken as 100 % and values for OTA-treated cells were taken as the decrease of the number of viable (unstained) cells expressed in percents. Data represent the mean + SD of three replicates for each experimental condition. Significantly different from control values, ◆$P<0.05$; significantly different from control and itself, *$P<0.05$.

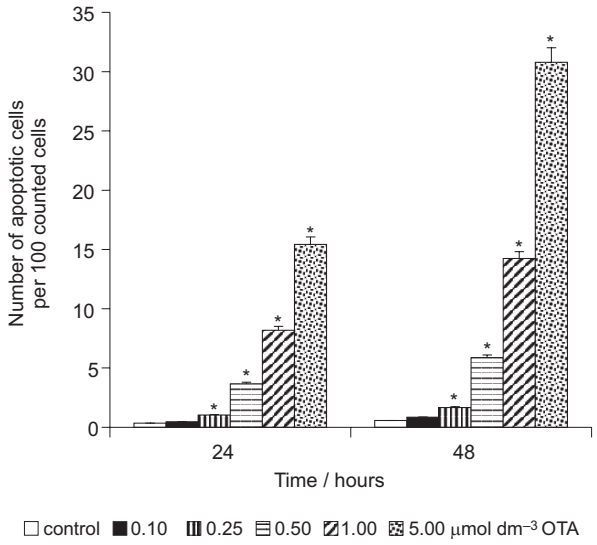

Figure 2. Apoptosis induction by OTA. LLC-PK1 cells were treated with OTA in a concentration range between 100 nmol dm^{-3} and 5 μmol dm^{-3} for 24 and 48 hours. Cells were stained with haematoxylin/eosin and TUNEL assay, and quantification of apoptotic cells was performed. Data represent the mean + SD of six replicates for each experimental condition. Significantly different from control values, $^*P<0.05$.

in the intracellular signalling pathways that transduce signals from the plasma membrane into the nucleus, thereby influencing various cellular responses such as cell growth and transformation, as well as differentiation and invasion.[34–36] There are four major classes of MAPKs, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 MAPK, and big MAPK (BMK)/ERK5. MAPKs are activated by phosphorylation of both threonine and the neighbouring tyrosine regulatory site by a specific upstream protein kinase.[37]

Upon activation, MAPKs phosphorylate other cytoplasmatic proteins and translocate from the cytoplasm to the nucleus to regulate the activity of transcription factors, thus modulating the expression of different genes.

It is known that many mitogens and growth factors activate ERK1/2 pathways to evoke cell survival and proliferation.[38,39] However, stress kinases, JNK and p38 MAPK, are involved in the transduction of pathological signals and respond to proinflammatory and stressful physical or chemical stimuli.[40,41] JNK activation has been shown to be associated with apoptosis in some cell types by induction of AP-1-dependent gene expression.[42–44] On the other hand, p38 MAPK is necessary for stress-induced expression of proinflammatory cytokines and growth factors.[45,46] The role of p38 MAPK in terms of cell death was mostly considered to be sensitizing to or enhancing apoptosis.[47] It has been reported that OTA induces JNK activation and apoptosis in MDCK-C7 cells, another well established renal epithelial cell line.[9] To investigate whether OTA affects MAPKs activation in LLC-PK1 cells, we monitored the expression and activation of ERK1/2, JNK1/2 and p38 MAPK after 2, 4, 6, 12 and 24 hours of treatment. Summarized results of these experiments are shown in Figure 3. Two hours of OTA treatment caused activation of ERK1/2 in a dose-dependent manner (Figure 3a). This activation was transient; 4- and 6-hour OTA treatments did not activate ERK1/2 (data not shown). However, we observed strong activation of JNK and p38 upon 6 (Figure 3b; we got the same expression pattern after 6 and 12 hours of treatment), 12 (Figure 3b) and 24 hours (Figure 3c) of OTA treatment. It is known that a dynamic balance between the anti-apoptotic ERK pathway and the pro-apoptotic JNK/p38 MAPK pathways is important for determining whether a cell survives or undergoes apoptosis.[48] Our results suggest that this balance was disrupted. Pro-apoptotic JNK/p38 MAPK signals directed cells towards programmed cell death while a transient activation of ERK1/2 pathway was not strong enough to stimulate cell survival.

Expression of Hsp72 and Hsp27 Is not Affected by OTA

Highly conserved heat shock proteins (Hsps) accumulate in cells responding to heat and to a variety of other stressful stimuli. Hsps, which function mainly as molecular chaperones, allow the cells to adapt to changes in their environment and to survive under lethal conditions. There is interplay between the molecules taking part in cell stress and cell death responses, and Hsps appear to function at key regulatory points in the control of apoptotic process. Hsp72 (inducible member of the Hsp70 family) and Hsp27 (member of the small Hsps family) are known as regulators of the intrinsic pathway of apoptosis operating at several control points, including inhibition of apoptosis inducing factor (AIF), inhibition of apoptosome formation and thereby prevention of caspase-9 maturation, and inhibition of caspase-3.[49–53] In addition, Hsp70 and Hsp27 modulate the extrinsic pathway of apoptosis by inhibiting the Daxx pathway and JNK activation.[54,55] We have previously reported that the expression of Hsp70 family members, Hsp72 and Hsc73, in LLC-PK1 and MDCK cells as well as in rat kidney tissue is not affected by OTA treatment.[56] In this study, we monitored the expression of Hsp72 and Hsp27 in LLC-PK1 cells exposed to high and low OTA concentrations for different periods of time. As shown in Figure 4, OTA did not affect the expression either of Hsp72 or Hsp27. Hsp genes are regulated at the transcriptional level by the heat shock factor (HSF), which binds to the heat shock promoter element (HSE).[57,58] Under stressful conditions, HSF1 is hyperphosphorylated on multiple serine residues in a ras-dependent manner by the MAPK family members and the resulting phosphorylated trimers have a capacity to bind DNA and translocate from the cytoplasm to the nucleus.[59] Under normal growth conditions, HSF1 activity is repressed and exists in an inert monomeric state

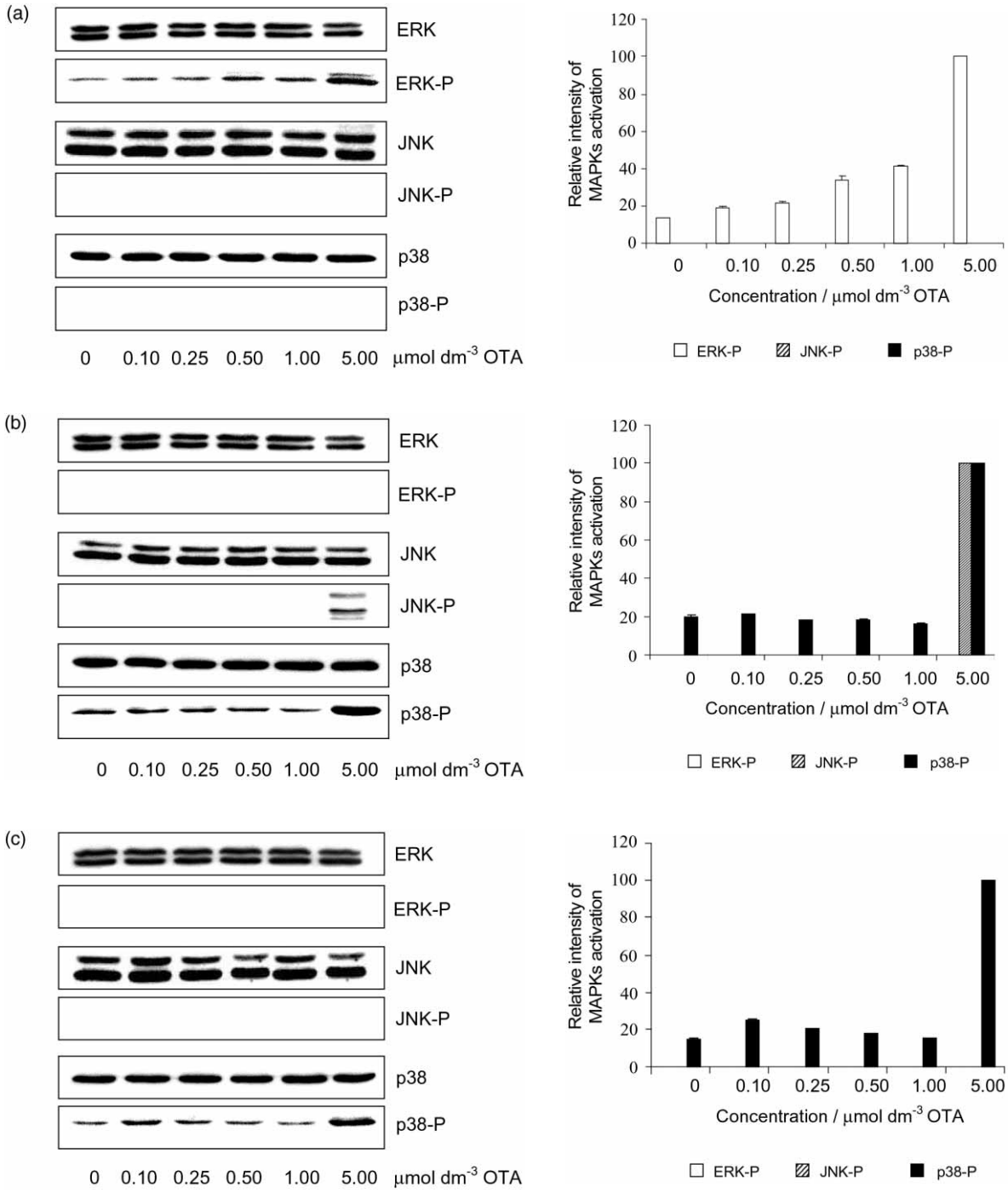

Figure 3. OTA provokes activation of MAPKs. LLC-PK1 cells were exposed to OTA in a concentration range from 100 nmol dm^{-3} to 5 μmol dm^{-3} for 2 (a), 6 and 12 (b), and 24 (c) hours. Western blot analysis was performed as described in Experimental. Representative blots for expression and activation of ERK, JNK and p38 MAPK are shown. Data represents the mean + SD of relative intensity of MAPKs activation.

characterized by constitutive phosphorylation on critical serine residues located in the regulatory domain.[60] JNK has been reported to be the MAPK kinase in mammals that binds HSF-1 in its conserved domain and phosphorylates HSF-1 within its regulatory domain. Phosphorylation of HSF-1 by JNK leads to suppression of its transcriptional activity by rapidly clearing HSF1 from sites of transcription.[61,62]

We suggest that OTA-induced JNK activation prevented *hsp* genes overexpression in LLC-PK1 cells by a

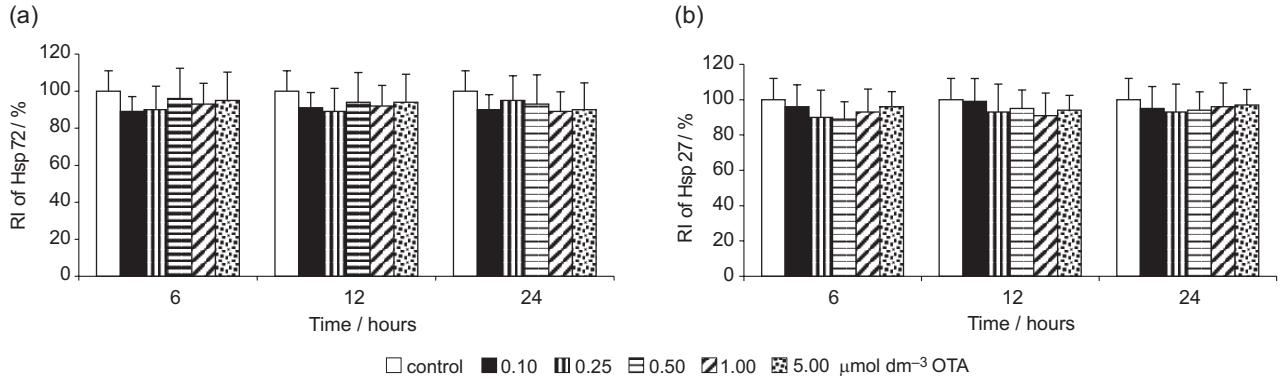

Figure 4. OTA does not affect expression of Hsp72 and Hsp27. LLC-PK1 cells were exposed to OTA in a concentration range from 100 nmol dm^{-3} to 5 μmol dm^{-3} for 6, 12, and 24 hours. Western blot analysis was performed as described under Experimental. Data represent the mean + SD of the relative intensity (RI) of Hsp72 (a) and Hsp27 (b) expression.

similar mechanism. Thus, Hsp72 and Hsp27 could not exert their protective function which, together with stress kinases activation, contributed to the induction of apoptosis in LLC-PK1 cells by OTA.

CONCLUSIONS

In this study, we have examined the effects of OTA on survival of LLC-PK1 cells, cultured kidney epithelial cells that have retained several characteristics of the proximal tubule cells.

The fungal metabolite OTA has been shown to exert chronic damaging effects in mammals at low concentrations. It seems that the disruption of cell viability by necrosis is associated with impaired protein synthesis and/or ROS generation caused by higher OTA concentrations (>1 μmol dm^{-3} for most cultured kidney epithelial cell lines). The mechanism of action of low OTA concentrations has not yet been unveiled satisfactorily. However, apoptosis has been suggested to be an important event in OTA-provoked cytotoxicity.

In our study, LLC-PK1 cells were treated with high and low OTA concentrations (from 100 nmol dm^{-3} to 5 μmol dm^{-3}) for different periods of time, and we found that the observed decrease in cell viability was associated with an increased number of apoptotic cells. Apoptosis, estimated by morphological and biochemical changes in LLC-PK1 cells, was mediated by strong and prolonged activation of JNK and p38 MAPK. In contrast, the activation of anti-apoptotic MAPK, ERK1/2, was transient. Moreover, the level of expression of Hsp72 and Hsp27, important inhibitors of the apoptotic process, was not affected by OTA. Therefore, we suggest that the signals transduced by those protective molecules were not strong enough to shift the anti-apoptotic/pro-apoptotic balance within LLC-PK1 cells towards cell survival.

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SAŽETAK

Okratoksin A izaziva apoptozu u LLC-PK1 stanicama aktivirajući JNK i p38 MAPK

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Okratoksin A (OTA) može izazvati tubularno-intersticijsku nefropatiju u ljudi i životinja. Cilj našeg istraživanja bio je ispitati ulogu apoptoze u nastanku nefrotoksičnosti koju uzrokuje OTA. Naš eksperimentalni model bile su LLC-PK1 bubrežne stanice izložene djelovanju OTA, od nanomolarnih do mikromolarnih koncentracija kroz različita vremenska razdoblja. Apoptoza je u stanicama utvrđena na temelju morfoloških (bojanje stanica hematoksilin/eozinom, fluorescentno bojanje slobodnih krajeva DNA tzv. TUNEL) i biokemijskih (MAP kinaze, Hsps) promjena. 48-satno izlaganje stanica 5 x 10^{-6} M OTA značajno je smanjilo staničnu vijabilnost i potaknulo apoptozu u 30,7 % stanica. Osim toga, utvrđena je kratkotrajna aktivacija ERK te snažna i dugotrajna aktivacija JNK i p38 MAPK nakon 6-, 12- i 48-satnoga tretiranja. OTA nije utjecao na razinu Hsp72 i Hsp27. Rezultati istraživanja ukazuju da je apoptoza posredovana aktivacijom JNK i p38 MAPK važan događaj u razvoju nefrotoksičnosti izazvanoj djelovanjem OTA na LLC-PK1 stanice.