Ochratoxin A Induces JNK Activation and Apoptosis in MDCK-C7 Cells at Nanomolar Concentrations

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Accepted for publication March 1, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

Ochratoxin A (OTA) is a ubiquitous fungal metabolite with nephritogenic, carcinogenic, and teratogenic action. Epidemiological studies indicate that OTA may be involved in the pathogenesis of different forms of human nephropathies. Previously we have shown that OTA activates extracellular signal-regulated kinases 1 and 2, members of the mitogen-activated protein kinases (MAPK) family, in the C7-clone but not in the C11-clone of renal epithelial Madin-Darby canine kidney (MDCK) cells. Here we show that nanomolar concentrations of OTA lead to activation of a second member of the MAPK family, namely, c-jun amino-terminal-kinase (JNK) in MDCK-C7 cells but virtually not in MDCK-C11 cells, as determined by kinase assay and Western blot. Furthermore, OTA potentiated the effect of tumor necrosis factor-α on JNK activation. In parallel to its effects on JNK, nanomolar OTA induced apoptosis in MDCK-C7 cells but not in MDCK-C11 cells, as determined by DNA fragmentation, DNA ladder formation, and caspase activation. In addition, OTA potentiated the proapoptotic action of tumor necrosis factor-α. Our data provide additional evidence that OTA interacts in a cell type-specific way with distinct members of the MAPK family at concentrations where no acute toxic effect can be observed. 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.

Ochratoxin A (OTA) is a secondary fungal metabolite that has been detected in a variety of animal chow and human food (Kuiper-Goodman and Scott, 1989; Gekle and Silbernagl, 1996). Kidneys represent the main target of OTA. Epidemiological studies provided evidence for a correlation between high OTA levels in food and blood samples, respectively, and the incidence of human nephropathies and renal tumors (Simon, 1996). Furthermore, OTA seems to be involved in the pathogenesis of Balkan endemic nephropathy, chronic interstitial nephritis, and karyomegalic interstitial nephritis (Kuiper-Goodman and Scott, 1989; Simon, 1996) and exerts teratogenic effects (Kuiper-Goodman and Scott, 1989). In addition, it has been reported that OTA may induce apoptosis (Seegers et al., 1994). However, the concentrations used in this study were in the high micromolar range. Due to its ubiquitous occurrence, the complete avoidance of OTA exposure is impossible (Kuiper-Goodman and Scott, 1989; Simon, 1996). Thus, studies on the toxicodynamics of OTA are highly relevant for both animal and human health.

We have recently demonstrated that long-term incubation of principal cell-like Madin-Darby canine kidney (MDCK)-C7 cells, which represent one of two MDCK cell clones recently isolated in our laboratory (Gekle et al., 1994), with OTA leads to the activation of two mitogen-activated protein kinases (MAPKs), namely, of extracellular signal-regulated kinase (ERK)1 and ERK2, associated with an epithelial dedifferentiation of these cells (Schrammek et al., 1997c). This was not the case in MDCK-C11 cells. The phenotypic alterations resemble those recently described in alkali-dedifferentiated MDCK-C7F cells (Wünsch et al., 1995; Schrammek et al., 1997b) and in MDCK-C7 cells stably expressing a constitutively active mutant of MEK1, the activator of ERK1 and ERK2 (Schrammek et al., 1997a).

MAPKs are important intracellular signaling pathways that transduce signals from the plasma membrane into the nucleus and consist of a serial sequence of protein kinases that phosphorylate and activate the respective downstream kinase, leading to the activation of the respective MAPK (Blumer and Johnson, 1994). Different MAPK isoforms phosphorylate specific substrates either in the cytosol (e.g., cyto-
solic phospholipase A$_2$, at the plasma membrane (e.g., EGF-receptor), or in the nucleus (e.g., the transcription factor Elk1). In addition, several members of the MAPK family, such as c-Jun N-terminal kinases (JNK) and different isoforms of p38 MAPK appear to be involved in the transduction of stress signals (Seger and Krebs, 1995).

JNK, also known as stress-activated protein kinases, represents a group of enzymes activated by exposure of cells to cytokines and environmental stress (Whitmarsh and Davis, 1996). 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 (Gupta et al., 1996). JNK activation is mediated by dual phosphorylation on Thr and Tyr residues by the MAPK kinases MKK4 and MKK7 (Ip and Davis, 1998). The JNK signaling pathway causes activation of the transcription factor AP-1, a process that has been previously implicated in oncogenic transformation (Whitmarsh and Davis, 1998). The JNK signaling pathway causes activation of the transcription factor AP-1, a process that has been previously implicated in oncogenic transformation (Whitmarsh and Davis, 1998).

In summary, OTA: 1) is a known stressor of renal cells, 2) has been suggested to induce apoptosis, and 3) has been shown to activate MAPK. Based on these facts, it was the aim of this study to test the hypothesis that nanomolar concentrations of OTA are able to simultaneously activate JNK and induce apoptosis. We used the two well established renal epithelial cell lines MDCK-C7 and MDCK-C11, which have been shown to be valuable models, to investigate differential effects on MAPK activation (Schramek et al., 1997c). Here we report that nanomolar concentrations of OTA lead to the activation of JNK in MDCK-C7 cells but not in MDCK-C11 cells. In parallel to its effects on JNK, nanomolar OTA induced apoptosis in MDCK-C7 cells but not in MDCK-C11 cells. Furthermore, OTA potentiated the proapoptotic effect of TNF-α.

Materials and Methods

Cell Culture. Cells were seeded in plastic culture dishes (growth area = 75 cm$^2$; Nunc, Wiesbaden, Germany) in 10 ml of minimum essential medium (MEM) with Earle’s salts, nonessential amino acids, and l-glutamine (Biochrom KG, Berlin, Germany), and cultured under standard cell culture conditions (37°C, 5% CO$_2$). The MEM was supplemented with 10% fetal calf serum (Biochrom KG) and 24 mmol/l NaHCO$_3$. Media were changed three times a week and the cells were subcultivated once a week. In this study, we used two subtypes of MDCK cells that were cloned in our laboratory recently (Gekle et al., 1994). These two cell types give us the opportunity to study separately two homogenous cell populations derived from a single parent cell line. During the exposure to OTA, only one of the two cell types (MDCK-C7 or MDCK-C11) was present in the Petri dish. MDCK-C7 cells consist of only one cell type and resemble the principal cells of the collecting duct. MDCK-C11 cells resemble the intercalated cells of the collecting duct.

Western Blot Analysis. MDCK-C7 and MDCK-C11 cells were washed three times with ice-cold PBS and lysed in ice-cold Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 1% Triton X-100) for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. The protein content was determined using a microbicinchoninic acid assay (Pierce, Rockford, IL) with BSA as standard. Cell lysates were matched for protein and precleared with 2% BSA as the standard. Cell lysates were matched for protein and precleared with 2 μl of preimmune serum preadsorbed to 50 μl of protein A-Sepharose-coated beads for 1 h at 4°C. The precleared supernatants were incubated overnight with 20 μl of a JNK1-specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), preadsorbed to protein A-Sepharose. Immunocomplexes were then used to measure JNK1 activity.

JNK1 Activity Assay. For measurement of JNK1 activity, the respective immunocomplexes were collected by centrifugation, washed four times with a washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EGTA, 0.5% Triton X-100) and once with kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl$_2$, 1 mM dithiothreitol, 10 mM p-nitrophenylphosphate), and were resuspended in a final volume of 40 μl of kinase buffer containing 5 μg of glutathione-S-transferase (GST)-c-Jun (Santa Cruz Biotechnology), 50 μM ATP, and 10 μCi [γ$^32$P]ATP. The reaction was initiated by incubation at 30°C and continued for 10 min. Thereafter, 40 μl of 2× Laemmli sample buffer was added to terminate the reaction. Samples were then boiled for 3 min and subjected to SDS-12% polyacrylamide gel electrophoresis. The gels were stained in Coomassie brilliant blue, dried, and exposed for 1 to 2 h to Amershams Hyperfilm MP at −70°C with intensifying screens. In addition, kinase activity was determined by cutting the GST-c-Jun bands and measuring the radioactivity in a liquid scintillation counter.

4,6-Diamidino-2-phenylindole (DAPI) Staining. To visualize chromatin condensation and fragmentation in whole cells, we used the fluorescent dye DAPI. In brief, cells were seeded on glass cover slips and incubated with the respective media, as indicated in the text. Subsequently, the cells were washed with PBS (10 min), fixed with ice-cold methanol/acetic acid (3:1), and washed again with PBS. Then, the cells were incubated for 15 min in DAPI solution (5 mg/l DAPI, 0.1% Triton X-100, 2 mmol/l MgCl$_2$, 100 mmol/l NaCl, 10 mmol/l PIPES, pH 6.8) and washed again. DAPI fluorescence was visualized using an inverted microscope (Axiovert 100; Zeiss, Göttingen, Germany) at an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

Determination of DNA-Ladder Formation. Cells were lysed (0.5 ml of 5 mmol/l Tris, 20 mmol/l EDTA, pH 8, 0.5% Triton X-100) and centrifuged for 20 min at 13,000 rpm (4°C). The supernatant was removed and incubated with protease K and RNase H for 60 min at 37°C. Subsequently, DNA was extracted using phenol/chloroform/isoamyl alcohol (centrifugation for 30 min at 6000 rpm). DNA was precipitated overnight with 0.1 volume of 3 mol/l sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at −20°C. After a 30-min centrifugation at 13,000 rpm, the pellet was washed with 0.1 ml of 70% ethanol.
ethanol and dried. After resuspension of the pellet, the DNA concentration was determined and equal amounts were loaded onto a 1.4% agarose gel. The bands were visualized using ethidium bromide.

**Quantification of DNA Fragmentation.** DNA fragmentation was quantified with the diphenylamine method (Sandau et al., 1997). Cells were lysed as described above and centrifuged for 30 min at 14,000g to separate intact chromatin and DNA fragments. DNA was precipitated with 500 μl of 10% trichloroacetic acid overnight at 4°C. The pellet was incubated at 100°C for 15 min in 350 μl of 5% trichloroacetic acid and centrifuged for 5 min at 4000g thereafter. Subsequently, 350 μl of diphenylamine reagent (1.5% diphenylamine + 0.01% paradehyde in acetic acid + 15 ml/H₂SO₄) were added to the supernatant and incubated for 12 to 24 h at 30°C in the dark. O.D.₅₅₀ of the supernatants were determined, and the percentage of fragmented DNA was calculated.

**Determination of Caspase Activity.** Caspase-3 activity was determined with the Caspase-3 Activity Assay from Boehringer Mannheim GmbH (Mannheim, Germany) according to the manufacturer’s instructions. Activity was determined as the cleavage of the fluorescent substrate 7-amino-4-trifluoromethyl-coumarin (picomoles per hour per milligram of cell protein).

**Other Measurements.** Cell number was determined by a Coulter-counter (Coulter Electronics, Krefeld, Germany). Lactate dehydrogenase (LDH) release and protein were determined as described previously (Schwerdt et al., 1997).

**Chemicals.** If not stated otherwise, all substances were of the highest purity available and were purchased from Sigma (Munich, Germany).

**Statistics.** The data are presented as mean values ± S.E. Significance of difference was tested by t test or by ANOVA in combination with the Scheffe F test for multiple comparison of means, as appropriate. Differences were considered significant if P < .05. n represents the number of Petri dishes tested. Cells from at least two different passages were used for all experiments.

**Results**

**Nanomolar OTA Concentrations Activate JNK1 in MDCK-C7 Cells but Not in MDCK-C11 Cells after Long-Term Incubation.** To investigate whether OTA is able to induce JNK1 activity in MDCK-C7 cells and/or MDCK-C11 cells, which resemble principal cells and intercalated cells, respectively, we immunoprecipitated JNK1 and measured its enzymatic activity in a kinase assay by liquid scintillation counting using GST-c-Jun as a substrate. After 24 h of serum deprivation, basal JNK1 activity was 2.5 ± 0.3-fold higher in intercalated cell-like MDCK-C11 cells when compared with principal cell-like MDCK-C7 cells (n = 5; the mean counts for C7 and C11 were 6641 and 16946 dpm, respectively). Incubation in the presence of OTA for 8 h led to a clear concentration-dependent stimulation of JNK1 in MDCK-C7 cells but not in MDCK-C11 cells, whereas incubation with 100 nmol/l of the protein synthesis inhibitor anisomycin for 20 min markedly activated JNK1 in both cell clones (Fig. 1). The anisomycin-stimulated JNK1 activity was 26.5-fold in MDCK-C7 cells and 10.3-fold in MDCK-C11 cells (Fig. 1). The concentration-dependent OTA-induced JNK1 activation in MDCK-C7 cells after 8 h was 1.3-, 2.3-, and 13.4-fold at concentrations of 10 nM, 100 nM, and 1 μM, respectively (Fig. 1). In MDCK-C11 cells, quantification of the OTA-induced JNK1 activity by liquid scintillation counting revealed either no increase of JNK1 activity (at 10 nmol/l OTA) or only a 1.3- and 2.2-fold stimulation at 100 nM and 1 μM OTA, respectively (Fig. 1). Thus OTA, when applied for 8 h at nanomolar concentrations, shows a capacity for JNK1 stimulation that is selective for MDCK-C7 cells over MDCK-C11 cells.

**Short-Term Effects of TNF-α on JNK1 Activity in MDCK-C7 versus MDCK-C11 Cells.** To investigate whether the selective effect of OTA on JNK1 activity in MDCK-C7 cells is indeed specific for this mycotoxin, we investigated the effects of TNF-α, a well known potent activator of the JNK signaling pathway in several cell types. After short-term incubation (20 min) of both MDCK cell clones in the presence of 50 μg/l TNF-α, and in contrast to OTA, we obtained a comparable JNK1 activation in both MDCK-C7 and MDCK-C11 cells. Stimulation of the cells with TNF-α for 20 min revealed a 5.38 ± 1.01- and 4.82 ± 0.89-fold increase in JNK1 activity in MDCK-C7 and MDCK-C11 cells, respectively (n = 5). As depicted for MDCK-C7 cells in Fig. 2, this TNF-α-induced JNK1 activation was both time- and concentration-dependent. Stimulation of MDCK-C7 cells by 50 μg/l TNF-α led to a 2.2-, 3.6-, and 4.5-fold increase in JNK1 activity after 5, 10, and 20 min, respectively (Fig. 2). After 40 min of incubation in the presence of 50 μg/l TNF-α, JNK1 activity again decreased (2.3-fold increase when compared with unstimulated MDCK-C7 cells). In addition, TNF-α-induced JNK1 activity after 20 min of stimulation was highest (7.4-fold) with a concentration of 100 μg/l TNF-α (Fig. 2). Altogether TNF-α has the ability to activate JNK1 after short-term incubation in both MDCK-C7 and MDCK-C11 cells. This is in contrast to OTA, which does not stimulate

![Fig. 1. Basal and OTA-induced JNK1 activity in MDCK-C7 versus MDCK-C11 cells.](image1)

![Fig. 2. TNF-α-induced JNK1 activity in MDCK-C7 and MDCK-C11 cells.](image2)
JNK phosphorylation in either MDCK-C7 or MDCK-C11 cells after 20-min incubation (Fig. 3A).

**Long-Term Effects of OTA and TNF-α on p46 and p55 JNK Phosphorylation in MDCK-C7 Cells when Compared with MDCK-C11 Cells.** To investigate the effects of both OTA and TNF-α on the JNK signaling pathway during long-term incubation experiments, we performed Western blot studies using an antibody that recognizes only the phosphorylated forms of p46 and p55 JNK (Fig. 3). After 24 h of serum deprivation, MDCK-C7 cells showed a higher basal phosphorylation of p55 JNK2 (see Control lanes in Fig. 3A, top), whereas in MDCK-C11 cells a higher basal phosphorylation state of p46 JNK1 was obtained consistently (see Control lanes in Fig. 3A, bottom). In MDCK-C7 cells, 1 μM OTA led to a strong, time-dependent increase in both p46 and p55 JNK phosphorylation, which started after 3 h and was highest after 24 h (Fig. 3A, top). In contrast to OTA, 50 μg/l TNF-α led to an increased JNK phosphorylation of both isoforms as early as 20 min after incubation (Fig. 3A, top). Furthermore, the TNF-α-induced JNK phosphorylation in MDCK-C7 cells showed the tendency toward a biphasic effect with decreases in JNK phosphorylation after 1 and 24 h of incubation (Fig. 3A, top). In MDCK-C11 cells, 1 μM OTA hardly increased p46 or p55 JNK phosphorylation. Increases in OTA-induced JNK phosphorylation were only detected after 6 and 12 h, which decreased again after 24 h (Fig. 3A, bottom). In the presence of 50 μg/l TNF-α, however, JNK phosphorylation in MDCK-C11 cells was highest after 20 min of stimulation, which started to decrease after 1 h and was decreased toward basal levels after 6 h of incubation (Fig. 3A, bottom). Thereafter, at 12 and 24 h, again a slight increase in p46 JNK1 phosphorylation was detected when compared
with unstimulated controls (Fig. 3A, bottom). Furthermore, the effect of TNF-α on phosphorylation of both p46 JNK and p55 JNK after 12 h in MDCK-C7 cells was potentiated in the presence of OTA (Fig. 3B). This effect was also observed in MDCK-C11 cells, although it seemed to be less pronounced (Fig. 3B).

OTA Induces Apoptosis at Nanomolar Concentrations. It has been reported that JNK is activated by many proapoptotic stimuli. Thus, we investigated whether nanomolar OTA concentrations can induce apoptosis in MDCK-C7 cells. Figure 4, A and B, shows the staining pattern of MDCK-C7 nuclei under control conditions and after 24-h incubation with 100 nmol/l OTA. Exposure to OTA led to the appearance of a heterogeneous staining pattern as compared with control. The small bright spots indicate chromatin condensation or fragmentation, a typical feature of apoptosis. As shown in Fig. 4, C and D, no such changes could be observed in MDCK-C11 cells, indicating that OTA did not induce DNA fragmentation in MDCK-C11 cells. To visualize DNA fragmentation directly, the formation of a DNA ladder in an agarose gel, a typical feature of apoptosis (Quarrie et al., 1995; Imura et al., 1997), was determined. Figure 5 shows that 24-h exposure to 100 nmol/l OTA induced DNA ladder formation in MDCK-C7 cells but not in MDCK-C11 cells. Thus, our data show that the cell type, which shows more JNK activation during OTA exposure, also responds with apoptosis to OTA exposure.

Figure 6A shows the dose-response curve for OTA-induced DNA-fragmentation in MDCK-C7 and MDCK-C11 cells. DNA fragmentation behaves similarly to JNK activation (Fig. 1) because MDCK-C7 cells are more sensitive to OTA-induced DNA fragmentation than MDCK-C11 cells. Figure 6B shows that DNA fragmentation increases with time, at least over a period of 72 h. Thus, not only the concentration of OTA is of importance but also the time of exposure. To confirm the induction of apoptosis by another, independent method, we determined the activation of caspase-3, which represents an early hallmark during apoptosis (Kroemer et al., 1995; Patel et al., 1996; Enari et al., 1998). As shown in Fig. 7, OTA exposure led to an activation of caspase activity. Again, MDCK-C7 cells reacted much more sensitively than MDCK-C11 cells. Thus the data on caspase activation confirm our hypothesis that OTA-induced JNK activation leads to apoptosis.

Figure 8 compares the extent of OTA-induced DNA fragmentation with the extent of OTA-induced LDH release. LDH release represents a marker of plasma membrane integrity and can be used to estimate necrotic cell death. As shown in Fig. 8A, DNA fragmentation is affected to a much extent in MDCK-C7 cells compared to MDCK-C11 cells. This suggests that OTA induces apoptosis in MDCK-C7 cells, whereas MDCK-C11 cells remain viable despite OTA exposure.

**Fig. 4.** Exposure of MDCK-C7 cells to 100 nmol/l OTA for 24 h led to condensation of chromatin indicating apoptosis (A, control; B, OTA; bar, 10 μm). This was not the case in MDCK-C11 cells (C, control; D, OTA; bar, 10 μm). Representative images of three independent experiments.

**Fig. 5.** Exposure of MDCK-C7 cells to 100 nmol/l OTA for 24 h led to apoptosis-typical DNA fragmentation (DNA ladder formation). This was not the case for MDCK-C11 cells. Representative of three independent experiments. con, control.

**Fig. 6.** OTA induces a concentration-dependent increase in DNA fragmentation (A). MDCK-C7 cells responded ~5-fold more sensitively than MDCK-C11 cells. B, extent of DNA fragmentation increases with the time of exposure. *P < .05 versus control. Under control conditions, DNA fragmentation was 4.1 ± 0.5% in MDCK-C7 cells and 5.7 ± 0.5% in MDCK-C11 cells.
greater extent by OTA as compared with LDH release in MDCK-C7 cells. These data show that nanomolar OTA concentrations are able to induce apoptosis but not necrosis in MDCK-C7 cells. By contrast, in MDCK-C11 cells the changes in DNA fragmentation and LDH release were virtually proportional (Fig. 8B). Thus OTA seems to induce necrosis at higher concentrations in MDCK-C11 cells. The OTA-induced necrosis in MDCK-C11 cells was most probably accompanied by a slight increase in nonsystematic DNA fragmentation (i.e., no DNA ladder formation). The formation of reactive oxygen species seems not to be involved in OTA-induced apoptosis because neither 1 mmol/l N-acetyl cysteine nor 2000 U/ml catalase prevented OTA-induced caspase-3 activation or DNA fragmentation (data not shown).

Besides inducing apoptosis, OTA might also interfere with physiological proapoptotic stimuli such as TNF-α, which is known to induce apoptosis at least in part via the JNK pathway (Natoli et al., 1997). Thus we tested whether OTA modulates the proapoptotic action of TNF-α. As shown in Fig. 9, 24-h exposure to 50 μg/l TNF-α induced a slight but significant increase in DNA fragmentation in the absence of OTA in both cell lines. Caspase-3 activity was also increased slightly by TNF-α: 24-h exposure to TNF-α increased caspase-3 activity to 150 ± 9% of control in MDCK-C7 cells (P < .05 versus control, n = 6) and to 195 ± 16% of control in MDCK-C11 cells (P < .05 versus control, n = 6). In the presence of OTA, the effect of TNF-α on DNA fragmentation was significantly enhanced in both cell types (Fig. 9, A and B). As shown in Fig. 9C for MDCK-C7 cells, OTA potentiated the effect of TNF-α in a concentration-dependent manner. In addition, OTA potentiated the effect of TNF-α on caspase-3 activity. In MDCK-C7 cells, TNF-α (50 μg/l) increased caspase-3 activity by 4560 ± 430 pmol/h/mg (n = 6, P < .05) in the presence of 300 nmol/l OTA but by only 250 ± 50 pmol/h/mg (n = 6, P < .05) in the absence of OTA. In MDCK-C11 cells TNF-α (50 μg/l) increased caspase-3 activity by 2476 ± 326 pmol/h/mg (n = 6, P < .05) in the presence of 300 nmol/l OTA but by only 959 ± 106 pmol/h/mg (n = 6, P < .05) in the absence of OTA. These data show, furthermore, that the potentiating effect of OTA on TNF-α-induced caspase-3 activation was more pronounced in MDCK-C7 cells than with MDCK-C11 cells.

**Discussion**

The fungal metabolite OTA has been shown to exert chronic damaging effects in mammals at nanomolar concentrations (Kuiper-Goodman and Scott, 1989; Delacruz and Bach, 1990). Chronic OTA exposure leads to an impairment of renal function and morphology as well as to an increased incidence of renal adenoma and carcinoma. In addition, teratogenic effects have been described. The mechanism of action of nanomolar OTA concentrations has not yet been unveiled satisfactorily. Disruption of cell viability, in terms of necrosis, by impaired macromolecule synthesis or generation of reactive oxygen species seems to play a role only at micromolar or higher concentrations (Delacruz and Bach, 1990). Chronic OTA exposure leads to an impairment of renal function and morphology as well as to an increased incidence of renal adenoma and carcinoma. In addition, teratogenic effects have been described. The mechanism of action of nanomolar OTA concentrations has not yet been unveiled satisfactorily. Disruption of cell viability, in terms of necrosis, by impaired macromolecule synthesis or generation of reactive oxygen species seems to play a role only at micromolar or higher concentrations (Delacruz and Bach, 1990; Schramek et al., 1997c; Gekle et al., 1998).

Recently, we have shown that OTA has the ability to stimulate two members of the MAPK family, namely ERK1 and ERK2, in collecting duct-derived principle cell-like MDCK-C7 cells, but not in intercalated cell-like MDCK-C11 cells (Schramek et al., 1997c). MAPKs are important intracellular signaling pathways that transduce signals from the plasma membrane into the nucleus, thereby regulating various cellular functions such as cell growth and transformation (Lewis et al., 1998) as well as cell differentiation and

![Fig. 7. OTA exposure (24-h) leads to an increase of caspase-3 activation. MDCK-C7 responded much more sensitively than MDCK-C11 cells. n = 6 to 12 for each value plotted. *P < .05 versus control. Under control conditions, caspase-3 activity was 479 ± 130 pmol/h/mg in MDCK-C7 cells and 2915 ± 130 pmol/h/mg in MDCK-C11 cells.](image)

![Fig. 8. Comparison of DNA fragmentation with LDH release after 24-h exposure to 0, 100, 300, and 1000 nmol/l OTA. A, in MDCK-C7 cells, DNA fragmentation clearly precedes LDH release, supporting the hypothesis of apoptosis induction. B, in MDCK-C11 cells, DNA fragmentation and LDH release change almost proportionally, indicating that the majority of fragmentation results from necrosis. n = 6 to 12 each value plotted. The line without symbols represents an equal percentage of LDH release and DNA fragmentation. During necrosis, the experimental values should be close to this line.](image)
invasion (Schramek et al., 1997a; Lewis et al., 1998; Montessano et al., 1999). In addition, some MAPKs (JNK, p38 MAPKs) seem to be involved in the transduction of “pathophysiological signals” and respond to proinflammatory cytokines and stressful physical or chemical stimuli such as TNF-α, protein synthesis inhibitors, UV radiation, or hyperosmolality (Blumer and Johnson, 1994; Seger and Krebs, 1995).

One subfamily of MAPK, the c-Jun amino-terminal kinases (JNK), is activated by phosphorylation of Thr and Tyr at the -Thr-Pro-Tyr- phosphorylation motif via two MAP kinase kinases, MKK4 and MKK7 (Hibi et al., 1993; Derijard et al., 1994; Sanchez et al., 1994; Ip and Davis, 1998). JNK protein kinases phosphorylate the transcriptional activation domains of the transcription factors ATF2, ATF4, c-Jun, JunD, Elk-1, and Sap-1 (Gupta et al., 1996; Whitmarsh and Davis, 1996), leading, for example, to the formation of c-Jun/c-Fos heterodimers or c-Jun homodimers, which influence the transcriptional activity of a variety of genes (Davies, 1994). JNK is activated by many proapoptotic stimuli, for example, in response to UV radiation or to treatment of cells with TNF-α (Whitmarsh and Davis, 1996). Furthermore, JNK activation has been shown to be associated with apoptosis in some cell types (Xia et al., 1995; Butterfield et al., 1997). In the human myeloid leukemia cell line U937, JNK anti-sense oligonucleotides inhibit apoptosis (Seimiya et al., 1997), and in neuroblastoma cells it was found that Fas-mediated apoptosis requires JNK activation (Goillot et al., 1997). The fact that UV- and ceramide-induced apoptosis appear to be mediated by JNK-induced activation of the Fas pathway provides additional evidence for a JNK-stimulated apoptotic pathway (Brenner et al., 1997). The data of this study show that after long-term incubation, nanomolar OTA concentrations are able to activate JNK1 in MDCK-C7 cells but not in MDCK-C11 cells. Although the precise mechanisms underlying OTA-induced JNK stimulation in MDCK-C7 cells are unknown, this cell-specific activation of JNK was associated with the induction of apoptosis, suggesting a possible role of the JNK signaling pathway in OTA-induced MDCK-C7 cell apoptosis. Especially prolonged activation of JNK, as it has been induced by long-term incubation with OTA in this study, has been shown to be a powerful signal leading to apoptotic cell death (Butterfield et al., 1997). In addition, MDCK-C7 cells, which were highly sensitive in terms of JNK activation, responded very sensitively with respect to apoptosis. Using three different techniques, chromatin staining with DAPI, DNA ladder formation, and activation of caspase-3, we clearly showed that nanomolar concentrations of OTA are able to induce apoptosis in a cell type-specific way. However, although our data suggest that OTA has the ability to induce apoptosis via the activation of JNK, one has to keep in mind that the role of JNK in the apoptotic process is not straightforward. TNF-α, for example, is a potent activator of JNK, but in most cases it does not cause apoptosis unless cells are first treated with cycloheximide or actinomycin D. As apoptosis can be considered to be a form of stress, it is possible that JNK activation occurs in response to the stress of apoptosis and, thus, may even provide a protective signal (Whitmarsh and Davis, 1996; Nishina et al., 1997). However, besides inducing JNK-activation and apoptosis, OTA potentiated the action of the proapoptotic stimulus TNF-α in both MDCK-C7 and -C11 cells. As shown in this study, OTA exposure had a concentration-dependent additive effect on both the extent and the duration of TNF-α-induced JNK activation. Furthermore, the extent of TNF-α-induced DNA fragmentation and caspase-3 activation was potentiated by OTA, suggesting that OTA may also act as a stimulator of proapoptotic signals.

In conclusion, our data are the first to show that nanomolar concentrations of OTA can lead to JNK activation and apoptosis in renal tubular epithelial cells without causing general damage. This mechanism could explain at least some of the nephritogenic and teratogenic effects observed during long-term exposure to low doses of OTA.
Acknowledgments

We gratefully acknowledge the excellent technical assistance of Irene Mosser and Edna Nemati.

References


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