JPET Fast Forward. Published on December 30, 2004 as DOI: 10.1124/jpet.104.079475

TITLE: Proximal tubular toxicity of Ochratoxin A is amplified by simultaneous inhibition of the extracellular regulated kinases 1/2.

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RUNNING TITLE: ERK1/2 inhibition aggravates ochratoxin A induced renal toxicity.

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Text pages:	29
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Abbreviations:

ERK1/2 - extracellular regulated kinase (isoforms) 1 and 2,

JNK – c-jun amino terminale kinase

MAPK - mitogen activated protein kinases,

MEK - mitogen activated/extracellular-signal regulated kinase kinase,

NRK-52E cells – normal rat kidney epithelial cells,

OK cells - opossum kidney cells,

OTA – ochratoxin A,

P38 – extracellular regulated protein kinase 38

Section recommendations: Gastrointestinal, Hepatic, Pulmonary, & Renal

Cellular & Molecular

Toxicology

ABSTRACT:

Ochratoxin A (OTA) is a mycotoxin involved in the development of chronic nephropathies and a known carcinogen. As we could show previously OTA activates MAPKs (ERK1/2, JNK, p38) in proximal tubular cells (OK, NRK-52E). ERK1/2 or JNK, p38 are thought to mediate opposite action on apoptosis, fibrosis and inflammation. As we have already shown, OTA activates the latter processes. Here, we investigated the effect of OTA in absence or presence of the ERK1/2 inhibitor U0126, to test whether OTA then will exert increased toxicity. In the presence of ERK1/2 inhibition OTA decreased cell number and protein to a significantly larger extent as compared to OTA alone. The same was true for epithelial tightness, apoptosis (caspase-3 activity) and necrosis (LDH release). Furthermore, simultaneous inhibition of ERK1/2 amplified the effect of OTA on markers of inflammation (NFκB activity), fibrosis (collagen secretion) and epithelial mesenchymal transition (α smooth muscle actin). OTA induces phenomena typical for chronic interstitial nephropathy and activates ERK1/2, JNK and p38 in proximal tubular cells. Inhibition of ERK1/2 aggravates the effects of OTA or even induces toxicity at normally non toxic concentrations. This is highly likely due to activation of JNK and p38. Our data indicate a new mechanistic explanation for the toxic actions induced by OTA and they are notable with respect to a possible co exposition of the kidney to OTA and naturally occurring ERK1/2 inhibitors. Finally, our data give rise to an attractive hypothesis on the coincidence of increased OTA exposition and urinary tract tumors in humans.

INTRODUCTION:

Ochratoxin A (OTA) is a secondary fungal metabolite that is found in a variety of animal feed and human food (Walker, 2002). OTA enters the food chain by cereals and its products, coffee, beer, wine, poultry and pork. Due to its extreme stability and its almost ubiquitous incidence as food contaminant, complete avoidance of dietary intake of OTA is impossible (Gekle et al., 1998).

OTA was shown to be a nephrotoxic substance since substantial time (Krogh et al., 1974) with an elimination half-life of around 840 h in humans (Schlatter et al., 1996). Numerous studies showed the occurrence in human blood serum and human kidney. 50 – 100 % of the human samples were positive for OTA (reviewed in (Petzinger and Ziegler, 2000)). There is evidence that OTA is involved in the pathogenesis of Balkan endemic nephropathy (Vrabcheva et al., 2004). The toxicological profile of OTA includes teratogenesis, nephrotoxicity and immunotoxicity (Petzinger and Ziegler, 2000). Moreover, OTA was classified as being carcinogenic in animals (Rodriguez-Barbero, 1989) and as a possible carcinogen in humans (1993).

Virtually all progressive renal diseases are characterized by cell death, inflammation, fibrosis (Klahr and Morrissey, 2002) and epithelial to mesenchymal transition (Liu, 2004). Balkan endemic nephropathy, which is qualified by the WHO as "..progressive and very gradually developing renal failure with insidious onset…The last stage shows marked fibrosis …" is characterized by tubular degeneration, interstitial fibrosis and impaired renal function (Vrabcheva et al., 2004). This is in line with numerous animal studies, showing development of renal disease accompanied by proximal tubular atrophy and cortical interstitial fibrosis and inflammation after exposition to OTA (Aukema et al., 2004).

Damage of proximal tubular cells is thought to be a motor driving tubulointerstitial disease (Remuzzi and Bertani, 1998). Therefore, we recently investigated the effect of OTA on selected parameters of chronic renal cortical disease in proximal tubular cell lines (Sauvant, accepted 07-2004) (Sauvant, accepted 08-2004). In the mentioned studies, we could show that OTA induces loss of cell number, necrosis and apoptosis, fibrosis, inflammation and epithelial to mesenchymal transition in proximal tubular cells. Moreover, we obtained clear evidence that in proximal tubular cells, OTA exposition leads to activation of the antagonistic MAPK ERK1/2 on the one hand and p38 and JNK on the other hand (Sauvant, accepted 08-2004). In general, ERK1/2 is supposed to act pro-mitotic, whereas JNK and p38 are kinases supporting apoptosis, fibrosis and inflammation (Tian et al., 2001). There is evidence that it is the balance between ERK1/2 on the one hand and JNK and p38 on the other hand that determines cellular fate, which was also shown for renal proximal tubular cells (Arany et al., 2004). Therefore, we hypothesized that increased apoptosis or necrosis, fibrosis, inflammation and epithelial to mesenchymal transition in proximal tubular after OTA exposure is likely due to activation of JNK or p38, shifting balance away from ERK1/2 influence on to influence of JNK or p38.

In the following study, we tested whether selective inhibition of OTA induced activation of ERK1/2 will shift balance of MAPK to the effects of p38 and JNK and thus will lead to increased toxicity of OTA with respect to the above mentioned typical parameters of chronic renal cortical disease.

METHODS:

Cell culture. OK cells were obtained from Dr. Biber, Dept. of Physiology, University of Zürich. Cells were maintained in culture at 37°C in a humidified 5% CO_2, 95% air atmosphere. The growth medium was minimal essential medium (MEM), pH 7.4, supplemented with Earl's salts, non-essential amino acids, 10 % (v/v) fetal calf serum (Biochrom KG, Berlin, Germany) and 26 mmol/l $NaHCO_3$. NRK-52E cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. NRK-52E cells were cultured in DMEM medium, enriched with 26 mmol/l $NaHCO_3$ and 5 % (v/v) fetal calf serum. NRK-52E cells were cultured on petri dishes or permeable supports (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). The cells were maintained at pH 7.4 and 37°C, and gassed with 95% O_2 / 5% CO_2. All studies were performed between passage 60 and 100. The seeding density was $0.4 \cdot 10^6$ cm^{-2}. The medium was changed every third day and the cells were used for experiments at day 10 after seeding. All experiments were performed with cells that were serum starved for 24 hours before the experiments. Cells were seeded on 6well or 24well plates, petri dishes or permeable supports as appropriate.

pMAPK-ELISA. Quantification of MAPK-phosphorylation was done by ELISA according to (Krug et al., 2002). Cells were seeded in 96-well plates (Maxisorp, Nunc) and serum-starved for 24 h prior to the experiment. After stimulation as indicated in the text, the cells were fixed with 4% formaldehyde in PBS and washed three times with PBS containing 0.1 %Triton X-100. Cells were blocked with 10% fetal calf serum in PBS/Triton for 1 h and incubated overnight with the primary antibody (1:1000). After three washes cells were incubated with the secondary antibody (peroxidase-conjugated mouse anti-rabbit antibody, dilution 1:10000) in PBS/Triton with 5% BSA for 1 h at room temperature and washed three times with PBS/Triton for 5 min and twice with PBS. Subsequently, the cells were incubated with 50 µl of a

solution containing 0.4 mg/ml o-phenylenediamine, 11.8 mg/ml Na_2HPO_4, 7.3 mg/ml citric acid and 0.015% H_2O_2 15 min at room temperature in the dark. The resulting signal was detected at 490 nm with a multiwell multilabel counter (Victor^2, Wallac, Turku, Finland). Finally, protein content in the wells was determined with Trypan Blue solution. Primary antibody against p-ERK1/2, p-p38 and p-JNK were from cell signaling technology®, Beverly, USA.

Caspase-3 activity assay. Caspase-3 activity was measured according to the manufacturer's instructions (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications (Schwerdt et al., 1999): cells were washed once with PBS buffer (4°C) and incubated with 150 µl cell lysis buffer for 10 min on ice, harvested, and centrifuged at 16000g for 10 min at 4°C. 60 µl of the supernatant was incubated with 50 µmol/l DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; end-concentration) for 60 min at 37°C, and fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Victor^2, Wallac, Turku, Finland). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. As control, cell extracts were incubated as described above but in the presence of caspase-3 inhibitor zDEVD-CHO. No activity could be found under these conditions. Protein content was determined with bicinchoninic acid assay (Pierce) using bovine serum albumin as standard.

Determination of collagen secretion. In order to determine whether OTA induces collagen I, III or IV secretion in our cells we performed enzyme-linked immunosorbent assay (ELISA) as described in (Gekle et al., 2003). Media and collagen standards (Sigma, Deisenhofen, Germany) were incubated for 24 h in 96-well Nunc-Immuno Maxisorb plates (Nalge Nunc International, Naperville, IL) followed by washing and blocking with 2% bovine serum albumin in phosphate buffered saline. Subsequently the wells were incubated with rabbit antibody against collagen I,

III or collagen IV (1:1000; Biotrend, Köln, Germany) for 1h at room temperature. After three washes with 0.05% Tween in phosphate buffered saline, HRP-conjugated secondary antibody (1:5000; Biotrend, Köln, Germany) was applied for 1 h at room temperature. After three washes with 0.05% Tween in phosphate buffered saline the wells were incubated with o-phenylenediamine (Sigma, Deisenhofen, Germany) and the reaction was stopped after 15 min with 1 N H_2SO_4. The absorbance at 490 nm was determined using a multiwell-multilabel reader.

RT-PCR. RNA from cells was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). In brief, RT-PCR was performed according to Superscript One-Step RT-PCR system protocol (Invitrogen, Carlsbad, USA). cDNA was generated at 55°C for 15 min and then the samples were denatured at 94°C for 2 min. PCR amplification was performed in 35 cycles of 94°C for 15 s, then 55°C for 30 s and 72°C for 30 s. For α-SMA, the primers were 5′ – gat cac cat cgg gaa tga acg – 3′ (sense) and 5′ – ctt aga agc att tgc ggt gga – 3′ (antisense), resulting in an 389 bp RT – PCR – product, according to (Li et al., 2002).

Determination of epithelial tightness. For measurement of epithelial tightness, cells confluently grown on filter membranes (6well, Falcon, USA) were incubated with 100mg/l FITC marked inulin at 4°C for up to 55 min at the basolateral side. At 5, 25, 55 min 200 µl of the apical buffer (PBS) were analyzed for FITC fluorescence using a multiwell and multilabel counter (Victor^2, Wallac, Turku, Finland). The expired volume was immediately replaced by fresh buffer in order to avoid hydrostatic effects.

SEAP-Assay detection of NFκB. Cell signaling was assessed by the Mercury™ Pathway Profiling reporter gene assay system from Clontech Inc. using secretory alkaline phosphatase (SEAP) as reporter and pEGFP-C1 (Clontech Inc, San Fransisco, CA) as transfection efficiency control. SEAP-activity in the media was determined with the AttoPhos® System from Promega (Mannheim, Germany) and the cellular EGFP content was determined using a microplate

spectrofluorometer (Victor^2, Wallac, Turku, Finland). SEAP activity is always expressed as activity corrected for transfection efficiency, as determined by EGFP, and normalized to cell protein.

Other methods. Cell number and cell volume was determined using a Coulter Counter. Protein content as measured by BCA protein assay (Pierce, Rockford, USA) (see the results section or the figure legends for additional details on experimental protocols). LDH release was measured according to (Sauvant et al., 1998) at room temperature in a photometer.

Data analysis. Data are presented as means ± SEM respectively. The *n* value is given in the text or in the figures and stands for the number of supports used for the respective experiments (wells, filters, petri dishes). For all experiments, *n* equals the number of culture plates or filters used to perform the measurements. All experiments were performed with cells from at least three different passages. Statistical significance was determined by unpaired Student's *t*-test or ANOVA as appropriate. Differences were considered statistically significant when $P < 0.05$.

Materials. U0126 (1,4-Diamino-2,3-dicyano-1,4*bis*(2-aminophenylthio)-butadiene) was obtained from Alexis. If not stated otherwise, chemicals were from Sigma.

RESULTS:

In a former study we could show that half maximal effect of OTA occurred after 24 h in OK cells or after 48 h in NRK-52E cells (Sauvant, accepted 08-2004). Therefore, we chose these incubation periods for all subsequent experiments. As already mentioned, we found that OTA activates ERK1/2 in OK cells (Sauvant, accepted 08-2004) after 24 hours. To proof whether the inhibitor used (U0126) truly inhibits ERK1/2 activation, we performed ELISAs measuring the amount of activated (phosphorylated) ERK1/2 (p-ERK1/2) in OK cells. As suspected, OTA dose dependently activated ERK1/2 after 24 h (Fig. 1A). Inhibition of ERK1/2 activation alone (by 25µM U0126 given for 24 h) decreased ERK1/2 activity down to 60 % of untreated control and when U0126 was present neither 100 nM nor 1000 nM OTA activated ERK1/2 after 24 h (Fig. 1A). Thus, 25 µM U0126 inhibited the amount of p-ERK1/2 present and moreover completely abolished OTA induced ERK1/2 activation. Because we could also show before (Sauvant, accepted 08-2004) that OTA activates p38 and JNK in proximal tubular cells, we tested whether this activation will persist when ERK1/2 activation is abolished. As indicated in Fig. 1B, 25 µM U0126 alone had no effect on the amount of phosphorylated p38 (p-p38) and OTA induced activation of p38 is not at all affected by U0126. JNK activation is inhibited down to 50 % by 25 µM U0126 alone (Fig. 1C), but OTA still activates JNK in the presence of U0126 in a dose dependent manner. Moreover, relative increase of p-JNK induced by OTA incubation is in the same range under control conditions (1.4fold by 100 nM OTA and 2.4fold by 1000 nM) as compared to ERK1/2 inhibition (1.7fold by 100nM OTA in presence of U0126 and 2.8fold by 1000 nM OTA in presence of U0126). Thus, OTA induced activation of p38 and JNK was not affected by ERK1/2 inhibition in our setup. Therefore our setup was suitable to test the hypothesis that in case of ERK1/2 inhibition, OTA induced toxicity mediated by p38 or JNK should increase.

Activation of ERK1/2 is known to induce cellular growth and mitosis whereas JNK and p38 are thought to exert opposite effects. Therefore we investigated the effect of OTA with or without inhibition of ERK1/2 on cell number (Fig. 2A) and total cellular protein (Fig. 2B). 100 nM OTA had no significant effect on cell number and did only induce a slight reduction in total protein content, whereas in the presence of U0126 both, cell number and total cell protein, were markedly decreased as compared to control or OTA alone. Incubation of OK cells for 24 h with 1000 nM OTA alone reduced both cell number and protein. Again, additional inhibition of ERK1/2 activation led to a further reduction of both parameters.

Substantial loss of cells should lead to leakage of epithelia. Therefore, we measured diffusion of FITC labeled inulin through OK cell epithelia incubated with 100 nM or 1000 nM OTA in absence or presence of 25 µM U0126. As presented in figure 3 incubation with both, 100 nM or 1000 nM OTA for 24 h increased inulin diffusion which is a measure for paracellular epithelial leakage. Transepithelial diffusion of inulin induced by 1000 nM OTA further increased if U0126 was present, whereas the effect of 100 nM OTA was unaffected by U0126. 25 µM U0126 had no effect on epithelial tightness. Thus, OTA induced detectable epithelial leakage when given alone and this effect was enhanced when ERK1/2 activation was inhibited.

Cytosolic LDH is released into the medium if integrity of the cell membrane is deteriorated in cells suffering from necrotic cell death. To determine whether the decrease in cell number described above is due to necrosis, we measured the effect of OTA on LDH release in absence or presence of ERK1/2 inhibiting U0126 (Fig. 4A). OTA led to a dose dependent increase of LDH release after 24 h in OK cells. Inhibition of ERK1/2 activation amplifies LDH release induced by both, 100 nM or 1000 nM OTA. Inhibition of ERK1/2 alone did not affect LDH release. Thus, necrotic cell death in OK cells induced by OTA is aggravated if activation of ERK1/2 is prevented simultaneously.

OTA is known to induce apoptosis in renal cells (Gekle et al., 2000) (Sauvant, accepted 08-2004). As ERK1/2 is known to antagonize apoptosis (Johnson and Lapadat), we investigated the effect of OTA incubation with or without simultaneous inhibition of ERK1/2. We assayed apoptosis measuring activation of caspase-3 (Fig. 4B). No increase in caspase-3 activity was detected after incubating the cells for 24 h with U0126 or 100 nM OTA alone. However, when 100 nM OTA and U0126 were applied together, caspase-3 activity increased around threefold. Incubation for 24 h with 1000 nM OTA already led to a tremendous activation of caspase-3 (up to 400 %), which is even doubled by inhibition of ERK1/2. We additionally performed DNA fragmentation experiments, which show the same pattern as presented for caspase-3 activation (data not shown). Therefore, the effects described for caspase-3 activation are reflected by apoptotic endpoint, which is regulated DNA fragmentation and cellular degradation. Thus, apoptotic cell death in OK cells induced by OTA is strongly enhanced if activation of ERK1/2 is inhibited.

OTA is thought to be associated with generation of renal cortical fibrosis (Pfohl-Leszkowicz et al., 2002). We could show previously that OTA induces fibrosis in proximal tubular cells (Sauvant, accepted 07-2004) (Sauvant, accepted 08-2004). Thus, we investigated the effect of ERK1/2 inhibition on OTA induced secretion of collagen I, III and IV by specific ELISA technique (Table 1). U0126 for 24 h did not change secretion of collagen I, III or IV. 100 nM OTA did not induce secretion of any collagen tested, whereas inhibition of ERK1/2 together with 100 nM OTA stimulated secretion of collagen I and III, as compared to OTA alone. 1000 nM OTA stimulated secretion of collagen I, III and IV and inhibition of ERK1/2 together with 1000 nM OTA further increased secretion of collagen I, III, IV as compared to 1000 nM OTA alone. Thus, OTA induced secretion of extracellular matrix in OK cells is significantly aggravated if ERK1/2 is inhibited together with OTA incubation.

Inflammation is thought to be a hallmark in development of chronic renal disease and additionally is strongly associated with renal fibrosis. As OTA induced fibrosis was amplified by ERK1/2 inhibition, it seemed reasonable to investigate whether this is also the case for inflammation. NFκB is an important central inductor of inflammation (Lentsch and Ward, 2000). We could show previously that NFκB is activated in OK cells by OTA using a specific SEAP assay (Sauvant, accepted 08-2004). As indicated in figure 5A, inhibition of ERK1/2 by U0126 did not increase NFκB activation in the presence of 100 nM OTA, whereas together with 1000 nM OTA the same maneuver activated NFκB. Inhibition of the ERK1/2 had no affect on NFκB activity in OK cells after 24 h. Thus, OTA induced inflammation is amplified by ERK1/2 inhibition in proximal tubular cells.

Transformation of tubular epithelial cells into a myofibroblast like phenotype is described to occur in renal disease (Yang and Liu, 2001), which leads to loss of epithelial polarity. Together with cellular transformation, expression of α-smooth muscle actin (αSMA) takes place which is not present in intact renal tubular epithelial cells (Masszi et al., 2003). For reasons indicated in (Sauvant, accepted 08-2004) RT-PCR experiments were performed using RNA from NRK-52E (renal cortical epithelial cells from rat) cells extracted after 48h incubation. Figure 5B shows that incubation of NRK-52E with 100 nM OTA alone did not induce αSMA, whereas simultaneous inhibition of ERK1/2 clearly induced αSMA. αSMA induced by 1000 nM OTA was not further increased by inhibition of ERK1/2. Inhibition of ERK1/2 alone was without effect. Thus, OTA induced epithelial – mesenchymal transition can be significantly aggravated if ERK1/2 is inhibited.

DISCUSSION:

We presented experiments testing the hypothesis that inhibition of the ERK1/2 pathway increases OTA induced cell death, increased inflammation, fibrosis and epithelial to mesenchymal transition. Most of the experiments were performed using OK cells, which are an accepted and well established model system representing proximal tubular cells. Hence, rat proximal tubular NRK-52E cells behaved similarly to OK cells (with only half maximal action takes place at 48 h as compared to 24 h) and were thus used for RT-PCR experiments (Sauvant, accepted 08-2004). Moreover, we could additionally show that NRK-52E cells also behaved similarly to OK cells with respect to MAPK activation pattern in presence of OTA and / or U0126 (data not shown), which is additional evidence that both cell lines behave similarly to OTA exposure.

100 nM OTA alone is sufficient to decrease epithelial tightness that inulin (5000 Da) passes the OK cell epithelium paracellularly. 100 nM OTA did not reduce cell number, but clearly induced epithelial leakage. Thus, the mechanism involved must be independent from a decline in cell number. This is in agreement with data from (Horvath et al., 2002) showing impaired cell – cell contact after non cytotoxic doses of OTA. Additionally, recent data indicate opposite influence of ERK1/2 and p38 on renal paracellular permeability (Kiely et al., 2003), which is in agreement with the above described activation of MAPKs by OTA. Inhibition of ERK1/2 together with 100 nM OTA did not further increase epithelial leakage, whereas it dramatically decreased total cell number. This may be due to spreading and flattening of the remaining cells leading to maintenance of epithelial tightness. However, inhibition of ERK1/2 together with 1000 nM OTA almost doubles epithelial leakage as compared to 1000 nM OTA alone, which may result from greater toxicity (see below) abolishing the rescue effects discussed

above. In summary, if ERK1/2 is inhibited simultaneously the effects of OTA on cell number, cell protein and epithelial tightness are clearly amplified.

Loss of cell number induced by a toxic stimulus can be due to necrosis or apoptosis. As both necrosis (Benesic et al., 2000) and apoptosis (Gekle et al., 2000) (Horvath et al., 2002) is described to be induced by OTA in renal epithelial cells, we investigated the effect of OTA and ERK1/2 inhibition on both parameters. OTA induced necrosis and simultaneous inhibition of ERK1/2 amplified necrosis. Apoptosis is only slightly induced by 100 nM OTA but additional inhibition of ERK1/2 clearly increases apoptosis. Induction of apoptosis by 1000 nM OTA to 400 % was even further increased up to 800 % by simultaneous inhibition of ERK1/2. In summary, the effects of OTA on both, necrosis or apoptosis are clearly amplified if ERK1/2 is inhibited.

Thus, the decrease in cell number is due to apoptosis and necrosis. As already mentioned, there are numerous publications indicating an anti apoptotic role for ERK1/2 and pro apoptotic activity for JNK and p38. Thus, it is reasonable that after activation of ERK1/2, JNK and p38 by OTA, inhibition of ERK1/2 increases apoptosis. As the same pattern was found for necrosis, it seems obvious that necrosis in proximal tubular OK cells also is under antagonistic control of ERK1/2 and JNK, p38. Although only little information on MAPK and necrosis in the kidney is available, in vivo data on reperfusion induced cell necrosis in rat kidney (di Mari et al., 1999) also indicate that ERK1/2 improves cell survival, whereas JNK has the opposite effect.

OTA is supposed to be involved in the development of renal disease of the chronic interstitial type in endemic areas, namely Balkan endemic nephropathy (Pfohl-Leszkowicz et al., 2002). In general, chronic renal interstitial disease is characterized by inflammation, fibrosis and epithelial to mesenchymal transition (Klahr and Morrissey, 2002) (Yang and Liu, 2001). OTA induced the pro inflammatory mediator NFκB and additional inhibition of ERK1/2 (together

with 1000 nM OTA) further increased NFκB induction. ERK1/2 is supposed to act anti inflammatory, whereas JNK and p38 are pro inflammatory (Tian et al., 2001). As OTA was shown to induce all of the three MAPK pathways, increased induction of NFκB by OTA together with ERK1/2 inhibition as compared to OTA alone most likely is due to a changed ERK1/2 to JNK, p38 ratio.

As NFκB is also known as an inductor of renal fibrosis, it was not surprising that OTA also enhances secretion of collagenes (I, III, IV) in OK cells (Sauvant, accepted 08-2004). In general, ERK1/2 is supposed to act anti – fibrotic, while JNK and p38 are pro fibrotic kinases (Tian et al., 2001). It has already been shown for the kidney that ERK1/2 acts anti fibrotic (Hung et al., 2001). Thus, amplification of collagen induction by ERK1/2 inhibition together with OTA as compared to OTA alone can be again explained by a changed ERK1/2 to JNK, p38 ratio, leading to increased influence of JNK and p38 and therefore increased fibrosis. Not too much is known about the implication of JNK or p38 on renal inflammatory disease. Recently induction of JNK and p38 has been shown in proximal tubular cells in a rat model of crescentic glomerulonephritis (Stambe et al., 2003). The authors conclude that inhibition of JNK and / or p38 may be useful in therapy of inflammatory kidney disease. This may also the case in OTA induced kidney disease.

Myofibroblast activation is a key event in progression of chronic renal disease and myofibroblasts can derive from epithelial cells by epithelial to mesenchymal transition (Yang and Liu, 2001). Epithelial to mesenchymal transition (assayed by αSMA induction) was induced by 1000 nM OTA alone and by 100 nM OTA and simultaneous inhibition of ERK1/2. αSMA was recently shown to be induced by JNK and p38 in rat renal cells (Wang et al., 2002). Virtually nothing is known about the role of ERK1/2 in renal αSMA induction. Therefore from our data we propose, that proximal tubular epithelial to mesenchymal transition depends on the

balance of ERK1/2 (inhibitor of transition) on the one hand and JNK and p38 (activator of transition) on the other hand.

We show that, in vitro, OTA can exert changes in renal proximal tubular cell function also described in chronic renal disease, which gives additional evidence supporting the epidemiological data indicating that these diseases are induced by OTA exposition. Even more interesting, we could show for the first time that inhibition of ERK1/2 was sufficient to exert these effects at OTA concentrations which alone are not effective. Thus, our data indicate that agents inhibiting ERK1/2 will sensitize the cells with respect to OTA toxicity, reducing threshold concentrations. For example, anthocyanidins are substances present in fruit, vegetable and wine, some of them were recently shown to inhibit ERK1/2 (Hou et al., 2003) and thus may amplify nephrotoxic effects of OTA at normally non toxic concentrations. As OTA is found in plasma and urine of almost any individual (Petzinger and Ziegler, 2000), this may also be of importance in humans.

MAPKs are activated in pathologic situations of the kidneys, e.g. ischemia / reperfusion (di Mari et al., 1999) or experimental glomerulonephritis (Stambe et al., 2003). According to this and our data, differential activation of MAPK might also serve as mechanistic explanation for the renal toxic action described for OTA, which represents a completely new approach in explanation of OTA toxicity. The importance of MAPK activation in OTA toxicity is underlined by preliminary data from our lab showing that reduction in cell number induced by 100 nM OTA and ERK1/2 inhibition (as compared to 100 nM OTA alone) is totally reversible by inhibition of JNK and p38. As activation of JNK or p38 was not affected by inhibition of ERK1/2 we conclude that integration of both signal strands happens downstream of the mentioned MAPKs. Thus, we hypothesize that renal toxicity of OTA is mediated to a substantial amount by disturbance of MAPK balance, which is indicated in figure 6.

Finally, our data give rise to a very attractive hypothesis on the increased incidence of renal tumors and urinary tract tumors associated with Balkan endemic nephropathy (Pfohl-Leszkowicz et al., 2002). According to our data, constant exposition of the kidney should lead to clonal selection of cells showing higher ERK1/2 activity and decreased activity of JNK and p38, thereby leading to cells with increased mitogenic potential and reduced apoptosis. Moreover, OTA was shown to decrease cell – cell interaction (see above data and (Horvath et al., 2002)), which additionally will lead to impaired cell cycle arrest and possibly anoikis (Evan and Vousden, 2001). ERK1/2 activation was shown to be increased in a number of tumors from e.g. breast (Santen et al., 2002), lung (Yoshida et al., 2002) and kidney (Oka et al., 1995). Moreover, constitutive activation of the ERK1/2 activator MEK, was shown to induce an invasive phenotype in canine kidney epithelial cells (Montesano et al., 1999), which may happen also after OTA induced ERK1/2 activation. In summary, chronic exposition of renal cells to OTA can lead to a number of phenomena which are necessary to induce cancer. This mode of action might help to explain increased incidence of tumors after OTA exposition. Future studies will have to investigate this in more detail.

ACKNOWLEDGEMENTS:

Thanks to Dr. Jinhua Li (Baylor College of Medicine, Houston, Texas, USA) for e-mail help with αSMA RT-PCR. Special thanks to Dr. Gerald Schwerdt (Physiologisches Institut, Universitaet Wuerzburg, Germany) for help with apoptosis detection.

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FOOTNOTES:

This work was supported in part by the Deutsche Forschungsgemeinschaft DFG grant Ge 905/3-4. the Wilhelm-Sander-Stiftung (2002.038.1) and the Universitätsbund Würzburg (AZ 02-46).

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LEGENDS FOR FIGURES:

Figure 1: ELISA detecting the relative amount of activated (phosphorylated) MAPK per cell protein after incubation with OTA for 24 h. Data is given in % of control with control values set as 100 %.

(A) Relative amount of phosphorylated ERK1/2 (p-ERK1/2) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is 0,53 ± 0,10.

(B) Relative amount of phosphorylated p38 (p-p38) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is 0,23 ± 0,05.

(C) Relative amount of phosphorylated JNK (p-JNK) in OK cells after 24 h incubation period with OTA. Control value in ELISA O.D. per trypanblue O.D. is 0,25 ± 0,03.

n is given in the respective bar. * indicates significant difference from control, § indicates significant difference from the U0126 group and # indicates significant difference to the lower OTA concentration used in either case.

Figure 2: Effect of OTA and / or ERK1/2 inhibition (U0126) on OK cell number and total OK cell protein in absence or presence of U0126 after 24 h.

(A) Cell number after 24 h incubation with 100 nM or 1000 nM OTA in absence or presence of 25 µM U0126.

(B) Cell protein after 24 h incubation with 100 nM or 1000 nM OTA in absence or presence of 25 µM U0126.

n is given in the respective figure. * indicates significant difference from control and # indicates significant difference of OTA + U0126 from OTA alone.

Figure 3: Effect of OTA and / or ERK1/2 inhibition (U0126) on epithelial tightness after 24h. Epithelial tightness was determined by diffusion of fluorescence labeled inulin for up to 60 min through incubated OK cell epithelia. All data were normalized to % of control at t = 5 min. Increase of fluorescence in the apical compartment is given as increase in % of control at t = 5 per minute. Diffusion is given as pmol fluorescein per minute and per filter. n is 19 – 22 for every bar. * indicates significant difference from control and # indicates significant difference of OTA + U0126 from OTA alone.

Figure 4: Effect of OTA and / or ERK1/2 inhibition (U0126) on necrosis and apoptosis after 24h.

(A) Necrosis was determined by release of cytosolic lactate dehydrogenase (LDH) into cell culture medium. LDH release is given as % LDH activity in the supernatant in relation to total LDH activity of supernatant and cell lysat set as 100 %. Control values given in units enzymatic activity are $9{,}89 \cdot 10^{-5} \pm 2{,}17 \cdot 10^{-5}$ for supernatant and $261{,}9 \cdot 10^{-5} \pm 40{,}5 \cdot 10^{-5}$ for lysat. n = 5 for all bars.

(B) Caspase-3 activity was measured to determine apoptosis. Caspase-3 activity was measured in OK cells after 24 h exposure to OTA. n is 9 – 12 for every bar. Overall control value is 11219 ± 7035 in AFC counts per mg cell protein. Caspase activity was measured from 4 different passages with 3 dishes per group. Experiments were done over a range of around 20 cell passages. Resulting control is 100 % ± 16 % as indicated in the figure.

* indicates significant difference from control and # indicates significant difference of OTA + U0126 from OTA alone.

Figure 5: Effect of OTA and / or ERK1/2 inhibition (U0126) on parameters of inflammation and epithelial to mesenchymal transition.

(A) Effect of 24 h OTA incubation on NFκB activity in OK cells. NFκB activity was detected by SEAP assay as described in the methods section. Control value is 4,27 ± 0,27 in SEAP counts per µg cell protein. n is given in the respective bars. * indicates significant difference from control and # indicates significant difference to the lower OTA concentration used.

(B) Effect of OTA incubation on generation of αSMA after 48 h in NRK-52E cells. αSMA – mRNA was detected by multiplex RT – PCR using GAPDH as internal control. RT – PCR products were separated in agarose gels and were analyzed densidometrically. Control values (in AUC density counts) are 491 ± 166 for αSMA and 4983 ± 539 for GAPDH. The control ratio was set as 100 %. The graph represents data from 4 to 5 different sets of experiments, RNA extraction and subsequent RT – PCR (as indicated in the bars). * indicates significant difference from control.

Figure 6: Hypothetical ("ying – yang") model for explanation of MAPK mediated OTA toxicity in proximal tubular cells.

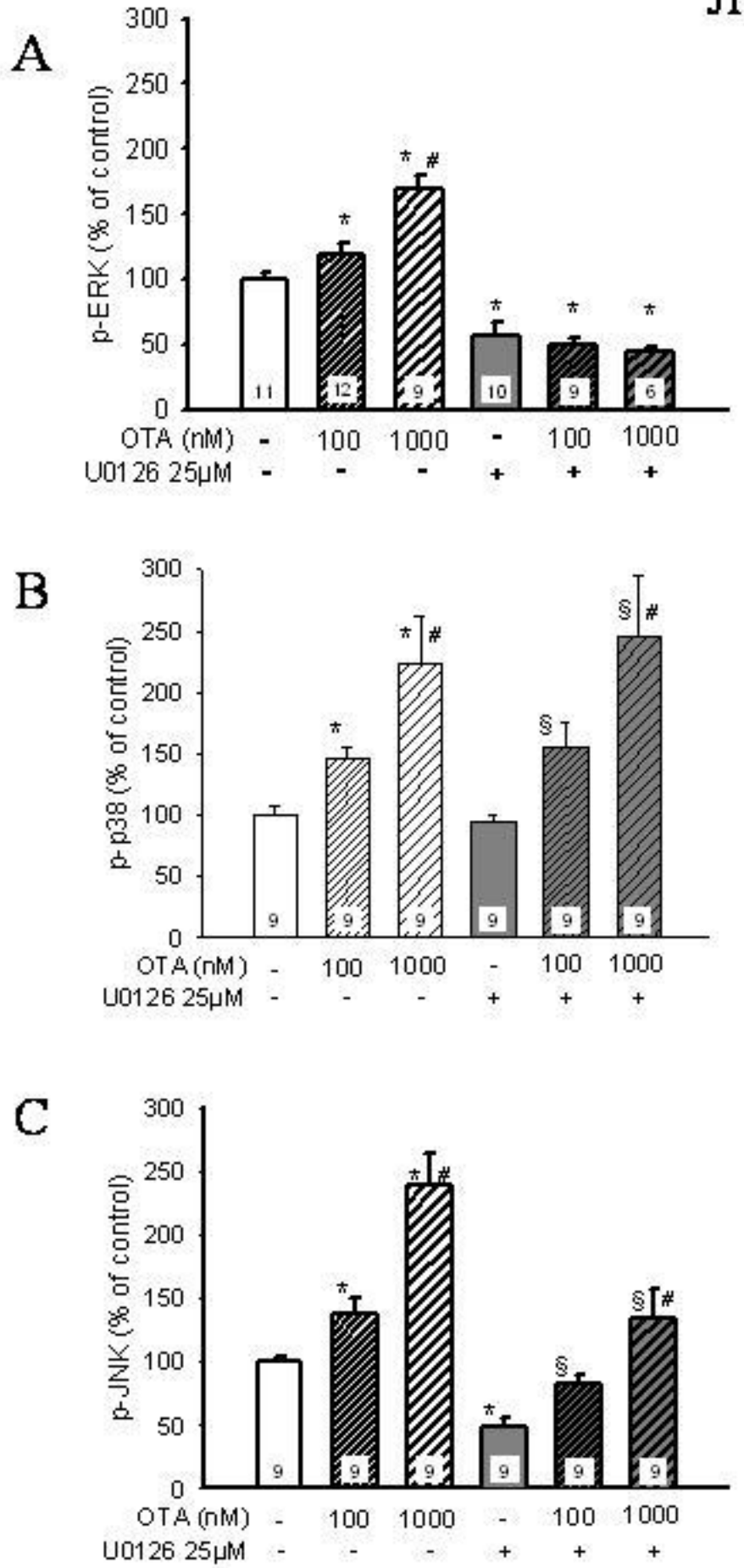
TABLES:

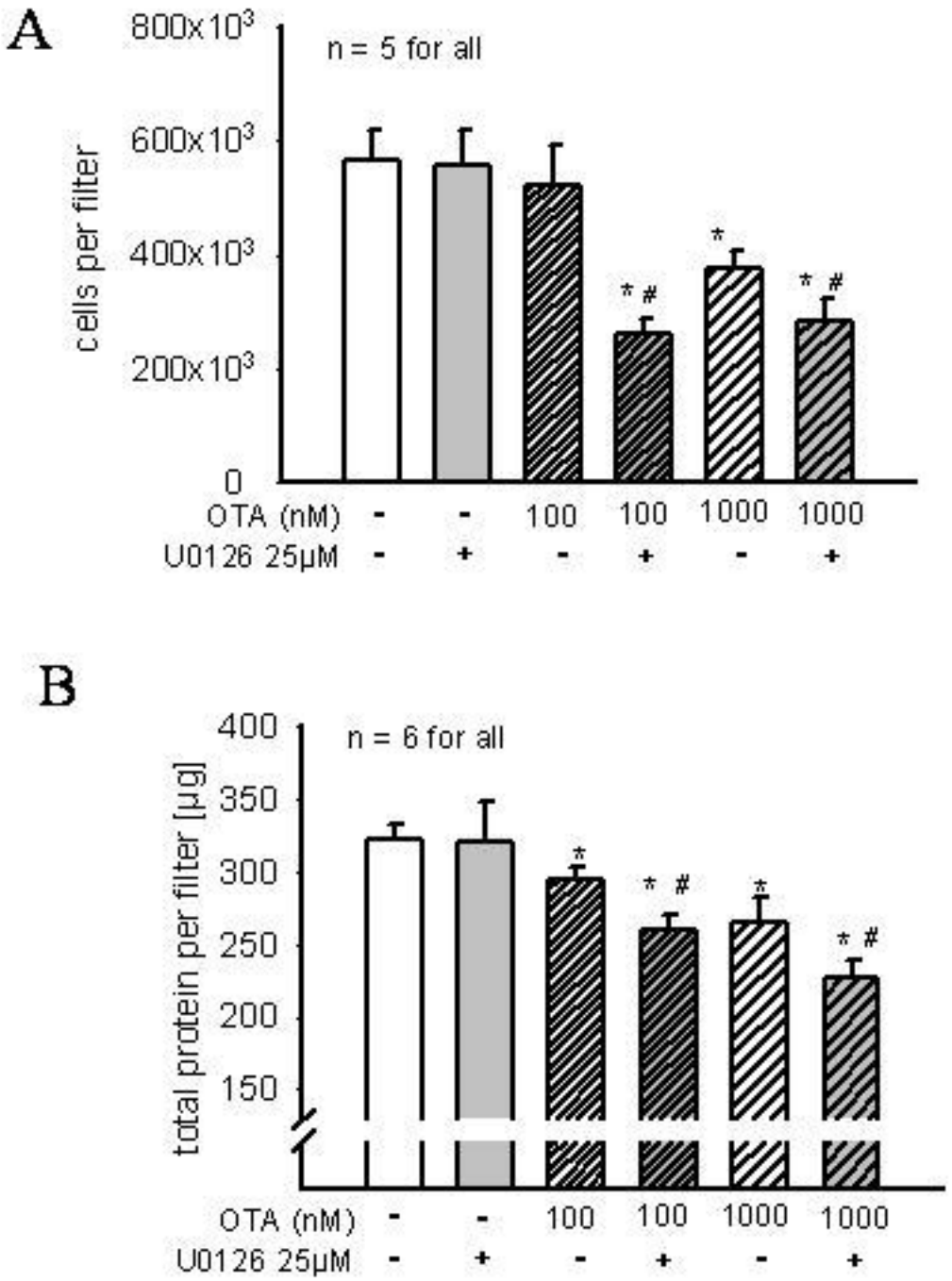
Tab. 1: Effect of 24 h incubation with OTA in absence or presence of U0126 on collagen secretion.

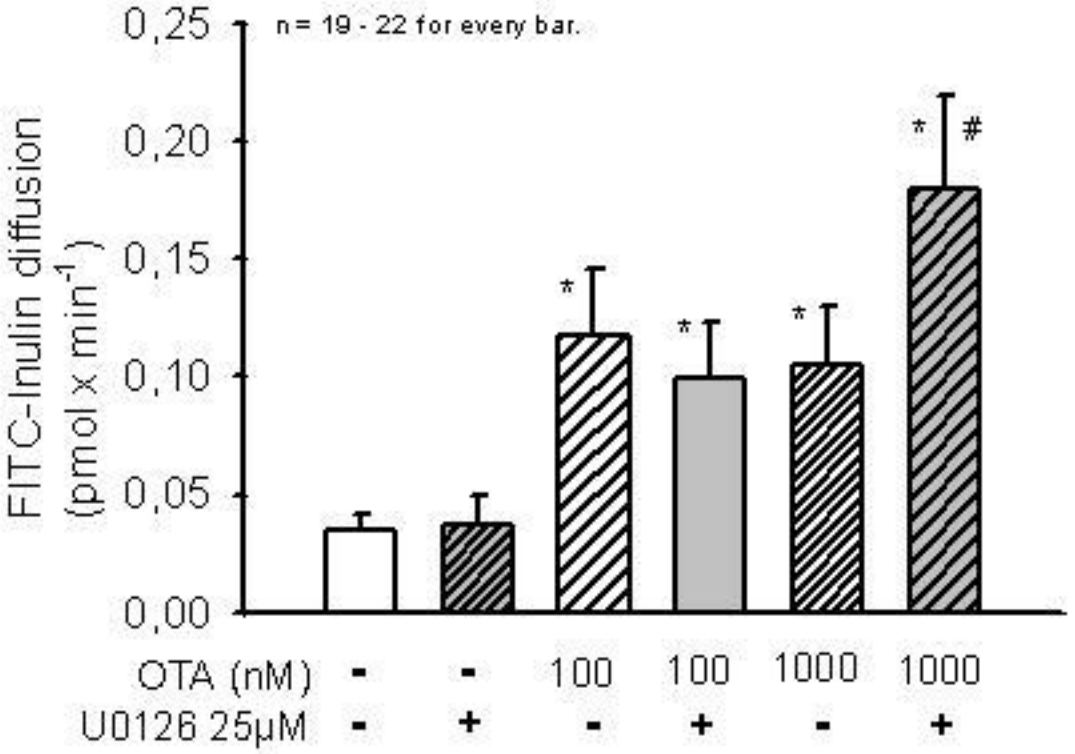
	control			**25 µM U0126**			**100 nM OTA**			**100 nM OTA + 25 µM U0126**			**1000 nM OTA**			**1000 nM OTA + 25 µM U0126**		
	mean	SEM	n	**mean**	SEM	n	**mean**	SEM	n	**mean**	SEM	n	**mean**	SEM	n	**mean**	SEM	n
Col. I	**100**	-	12	**147**	35.1	5	**79**	9.3	9	**127**$^{\#}$	22.4	9	**167***	23.5	9	**256***$^{\#}$	37	9
Col. III	**100**	-	13	**100**	4.6	5	**123**	16.5	9	**193***$^{\#}$	23.1	6	**224***	34.3	9	**364***$^{\#}$	59.2	7
Col. IV	**100**	-	13	**113**	11.2	5	**113**	10.7	9	**135**	22.9	9	**198***	18.7	9	**255***$^{\#}$	8.1	9

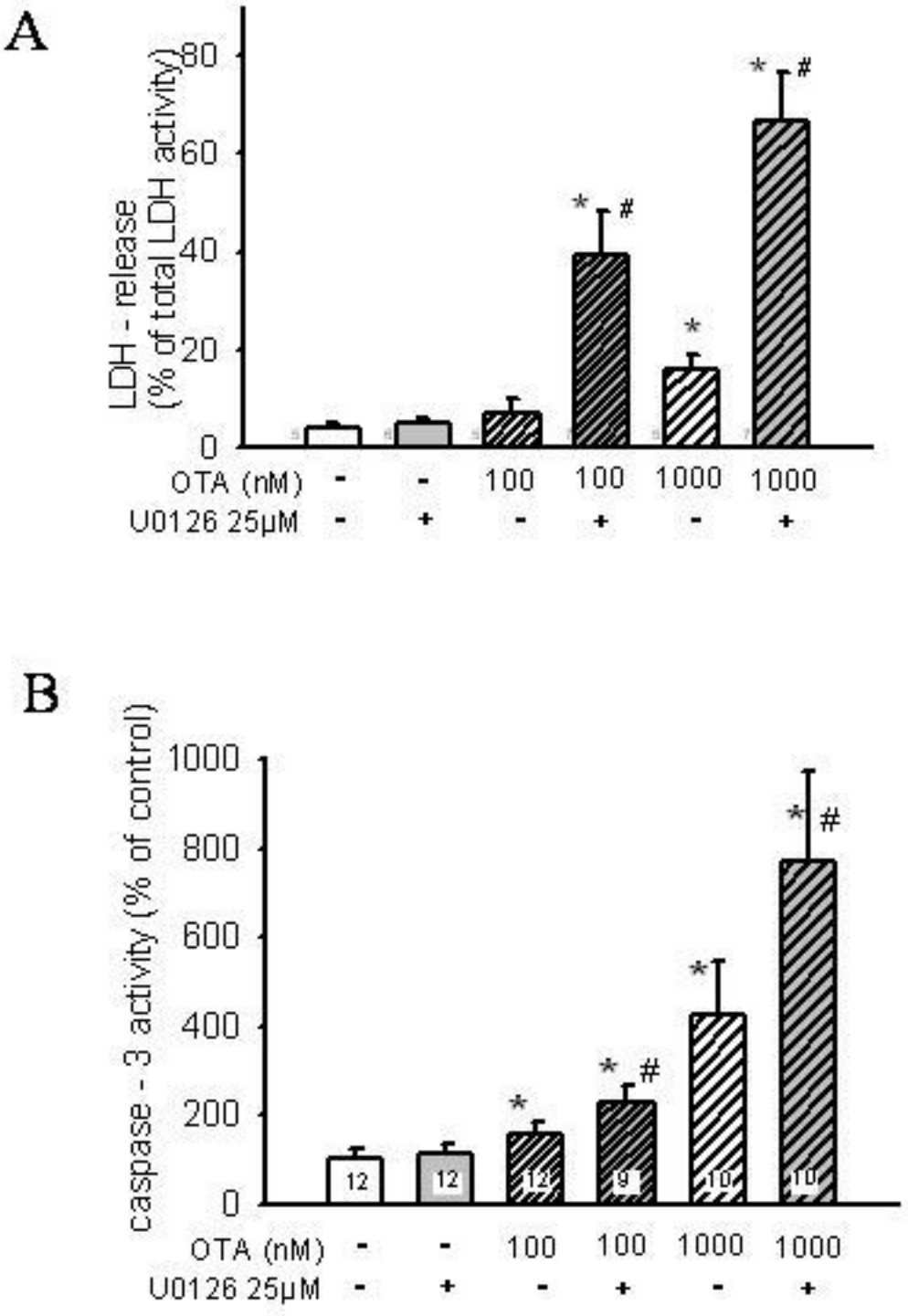
Collagen-ELISA detecting the amount of secreted collagens I, III, IV by OK cells after incubation with OTA. Collagen synthesis after 24 h OTA is shown. Collagen secretion was determined as described in the methods section and normalized to the respective control values (set as 100 %). Control values are as follows: collagen I: 1226 ± 41, collagen III: 66 ± 7; collagen IV: 41 ± 8, all given in ng of the respective collagen per mg cell protein. Significant difference from controls is indicated by asterisks and # indicates significant difference of OTA + U0126 from OTA alone.

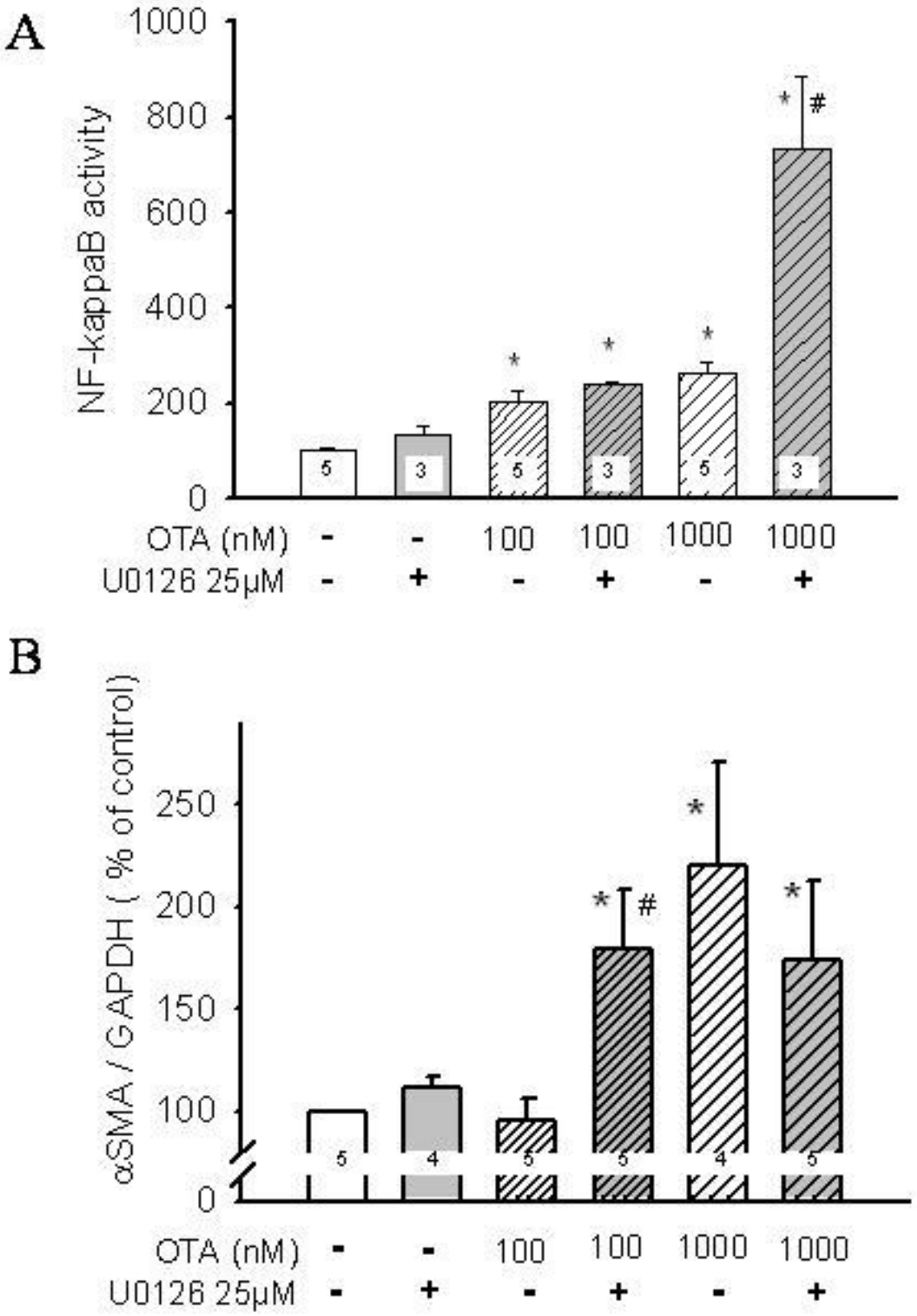
Fig.1

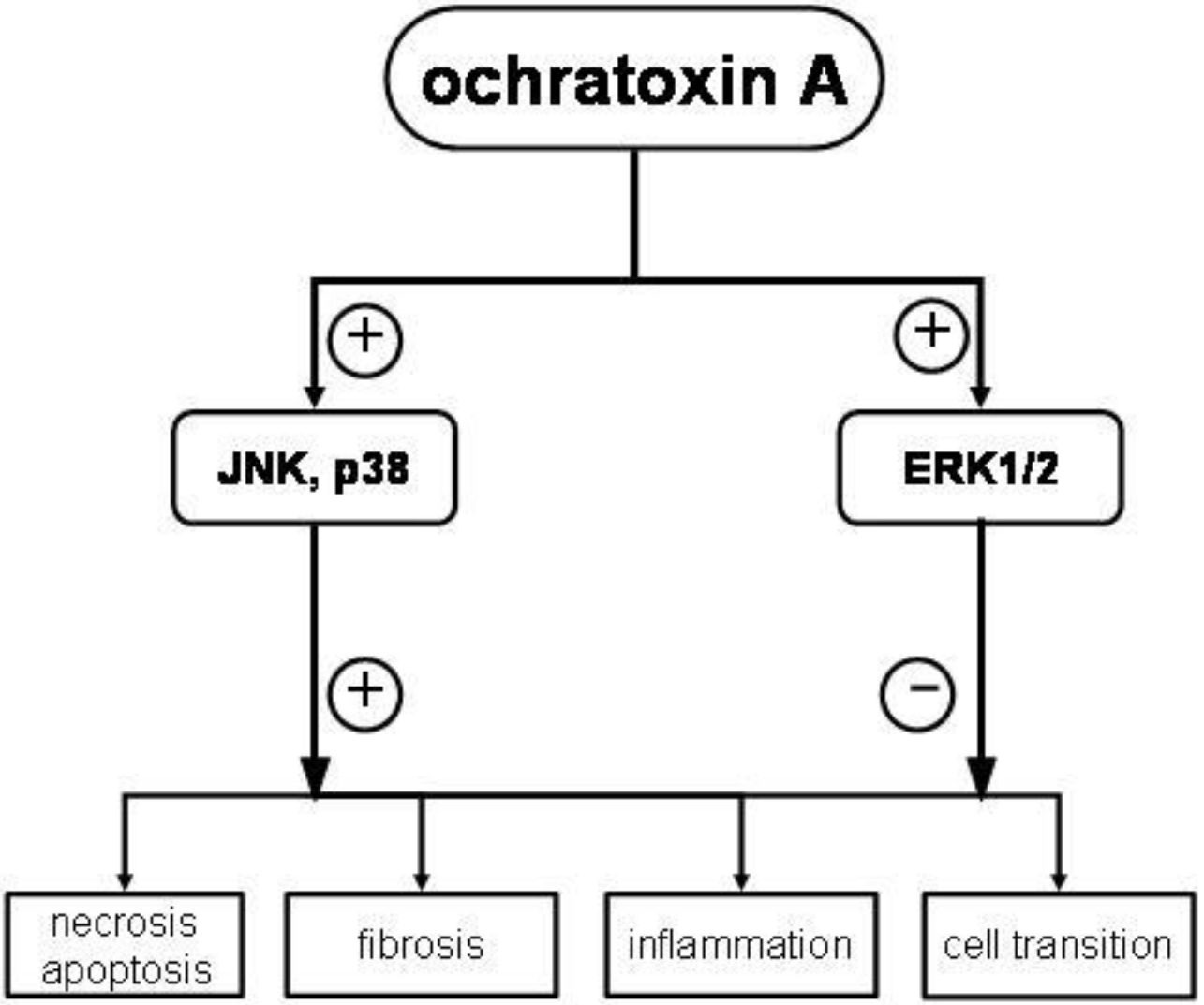

A
p-ERK (% of control)
300
250
200
150
100
50
0
11
12
9
10
9
6
OTA (nM) - 100 1000 - 100 1000
U0126 25μM - - - + + +
B
p-p38 (% of control)
9
OTA (nM) - 100 1000 - 100 1000
U0126 25μM - - - + + +
C
p-JNK (% of control)
9
OTA (nM) - 100 1000 - 100 1000
U0126 25μM - - - + + +


A
800x10³
600x10³
400x10³
200x10³
0
cells per filter
n = 5 for all
OTA (nM) - - 100 100 1000 1000
U0126 25µM - + - + - +
B
400
350
300
250
200
150
total protein per filter [µg]
n = 6 for all
OTA (nM) - - 100 100 1000 1000
U0126 25µM - + - + - +


n = 19 - 22 for every bar.
FITC-Inulin diffusion (pmol x min-1)
0,25
0,20
0,15
0,10
0,05
0,00
*
*
*
*
#
OTA (nM) - - 100 100 1000 1000
U0126 25µM - + - + - +


A
LDH - release (% of total LDH activity)
0
20
40
60
80
OTA (nM) - - 100 100 1000 1000
U0126 25µM - + - + - +
B
caspase - 3 activity (% of control)
0
200
400
600
800
1000
12
12
12
9
10
10
OTA (nM) - - 100 100 1000 1000
U0126 25µM - + - + - +


A
NF-kappaB activity
1000
800
600
400
200
0
5
3
5
3
5
3
*
*
*
*
#
OTA (nM) - - 100 100 1000 1000
U0126 25μM - + - + - +
B
αSMA / GAPDH (% of control)
250
200
150
100
0
5
4
5
5
4
5
*
*
*
#
OTA (nM) - - 100 100 1000 1000
U0126 25μM - + - + - +


ochratoxin A
+
+
JNK, p38
ERK1/2
+
−
necrosis
apoptosis
fibrosis
inflammation
cell transition