RESEARCH ARTICLE

The food contaminant and nephrotoxin ochratoxin A enhances Wnt1 inducible signaling protein 1 and tumor necrosis factor-α expression in human primary proximal tubule cells

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Scope: The underlying molecular mechanisms of nanomolar ochratoxin A (OTA) concentrations, especially those on pathophysiological relevant gene expression in target tissue and underlying signaling mechanisms are unknown.
Methods and results: qPCR arrays showed that 14 days exposure of human primary proximal tubule cells to 10 nM OTA influences the expression of genes that are related to inflammation, malignant transformation, and epithelial-to-mesenchymal transition. Wnt1 inducible signaling protein 1 (WISP1), an oncogenic, and profibrotic growth factor, turned out to be the gene with the strongest upregulation. Its expression, and that of TNF-α, an important inflammatory mediator, was further investigated in human renal cells and in primary human lung fibroblasts. OTA-induced upregulation of WISP1 and TNF-α occurs only in renal cells. Inhibition of ERK1/2 activation reverses the effect of OTA on WISP1 and TNF-α expression. Wnt or other signaling pathways were not involved. Upregulation of WISP1 and TNF-α occured independently of each other.
Conclusion: Long-term exposure of human kidney cells with OTA concentrations expectable in renal tissue due to average dietary intake leads in an ERK1/2-dependent manner to pathogenetic alterations of gene expression, notably WISP1 and TNF-α. Renal long-term risk by OTA is actually not excludable and argues for low but rational safety levels.

Keywords:
Gene expression / Mycotoxin / Ochratoxin A / Proximal tubule / Tubulointerstitial nephritis

1 Introduction

OTA is a mycotoxin produced by several fungi and is found in food and feed. It causes fibrotic alterations of the kidney in pigs [1, 2] and is suspected to be involved in the etiology of Balkan endemic nephropathy that is characterized by similar renal alterations [3]. Although improved methods are available to avoid fungal infection or to decontaminate feed and foodstuff OTA is still traceable in food and a contamination with OTA is hard to prevent. Therefore, OTA is detectable in human blood with an average concentration of around 1 nM [4–7]. In blood samples of patients with chronic interstitial nephritis of unknown origin in Tunisia the OTA serum concentration averaged even to 18 ng/mL (45 nM), whereas in the healthy control group the average concentration was 8.3 nM [8]. OTA at these low concentration ranges exerts distinct effects in human primary cells in culture [9, 10] that are sometimes in contrast to the effects of higher OTA concentrations (reviewed in [11]). The main target of OTA is the

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Abbreviations: EMT, epithelial-to-mesenchymal transition; **ERK1/2**, extracellular signal-regulated kinase; **HEK cells**, human embryonic kidney cells; **JNK**, c-Jun N-terminal kinase; **MEK**, mitogen activated protein kinase/ERK kinase; **MSK1**, mitogen- and stress-activated protein kinase 1; **NHLF**, normal human lung fibroblasts; **OTA**, ochratoxin A; **PKA**, protein kinase A; **PKC**, protein kinase C; **qPCR**, quantitative PCR; **RPTEC**, renal proximal tubule epithelial cells; **TNF**, tumor necrosis factor; **WISP1**, Wnt1 inducible signaling pathway protein 1

kidney. It has been suggested that OTA may contribute to the etiology of chronic interstitial nephritis in exposed patients [12], although the underlying mechanisms remain unclear. Cells in the kidney exposed and therefore threatened to a great extend by xenobiotics are those in the proximal tubule which handle the secretion of organic anions and cations including OTA [13]. An irritation of epithelial proximal tubule cells by OTA may result in altered cellular signaling and the release of cytokines that on the one hand leads to increased epithelial-to-mesenchymal transition (EMT) and on the other hand to activation of fibroblasts with altered extracellular matrix formation. These actions activate inflammatory events that in turn negatively affect proximal tubule cells leading into a vicious circle and ultimately to tubulointerstitial nephritis [14]. This hypothetic scenario is supported by findings in a cell culture model using proximal tubule cell lines that demonstrate that OTA is able to induce effects characteristic for interstitial nephritis like inflammatory events, EMT, and fibrosis [15].

Besides that, OTA causes apoptosis and can interact with cellular signaling cascades such as c-Jun N-terminal kinase (JNK) or calcium that suggests that OTA in low concentrations occurring due to normal diet may exert subtle alterations by working as a signal modulator and in this role disturbs cellular function [11, 16, 17]. However, the molecular mechanisms leading to tubulointerstitial nephritis imbedding proximal tubule cells are still in part under debate [18].

A new player in the pathogenesis of tubulointerstitial fibrosis is WNT1 inducible signaling pathway protein 1 (WISP1). WISP1 is a secreted oncogenic, promitogenic, profibrotic, and prohypertrophic protein [19, 20] identified in 1998 by Pennica et al. [21] that belongs to the connective tissue growth factor family [22]. WISP1 mRNA is expressed in a variety of adult human organs such as kidney, lung, and heart [21] and is upregulated in colon cancer cells, in hepatocellular and lung carcinoma and in fibrotic tissue [21, 23–25]. WISP1 gene is a target of the so-called canonical Wnt/beta-catenin pathway. The WISP1 promotor can also be activated independently of Wnt signaling [26]. This second Wnt1/beta-catenin-independent activation pathway includes extracellular signal-regulated kinases (ERK1/2) and cAMP response element-binding protein (CREB), which may be also activated by TNF-α [19, 27]. TNF-α is a potent proinflammatory cytokine. It participates in a large variety of biological actions and also may play a role in the context of OTA-induced tubulointerstitial nephritis.

Therefore, this study was undertaken to detect alterations of gene expression as well as modulation of signaling pathways in human proximal tubule cells in primary culture after exposure to nanomolar OTA concentrations for a prolonged time period (up to 14 days). These conditions resemble the situation during natural exposure as best as possible. Two genes, WISP1, a profibrotic gene with the strongest upregulation in PCR-arrays, and TNF-α as an important proinflammatory factor, were studied in further detail in renal proximal tubule epithelial cells (RPTEC) and human embryonic kidney (HEK293) cells and compared to the effect of OTA on primary human fibroblast cells (NHLF). Finally, the involvement of different signaling pathways in OTA-induced expression of WISP1 and TNF-α was explored in further detail.

2 Materials and methods

2.1 Cell culture

Renal proximal tubule epithelial cells (RPTEC) and normal human lung fibroblasts (NHLF) in primary culture were purchased from ATCC and Cambrex (East Rutherford, NJ, USA). RPTEC and NHLF were cultivated as described in [28]. Under these conditions, cells maintain their proximal tubule or fibrotic characteristics at least up to passage ten. Human embryonic kidney cells (HEK293) were purchased from ATCC. Cells were cultivated as described in [29]. Cells were kept serum-free 24 h prior to experiments. OTA exposure was under serum-free conditions and media were replaced against fresh media (with or without OTA) after 7 days if cells were exposed for 14 days. Under these conditions OTA concentration in media did not change significantly.

2.2 Gene array

RNA was isolated by phenol-chloroform extraction, reverse transcription was done using a commercial kit from SABiosciences (Qiagen). Arrays were performed using the following RT^2 ProfilerTM PCR arrays from SABiosciences (Qiagen, Hilden, Germany) according to their instructions: Human Cell Cycle (PAHS-020), Human Signal Transduction PathwayFinder (PAHS-014), Human Oncogenes & Tumor Suppressor Genes (PAHS-502), and Human Epithelial to Mesenchymal Transition (PAHS-090).

For a detailed description of these arrays see also http://www.sabiosciences.com/ArrayList.php?pline=PCRArray.

2.3 qPCR

RNA from RPTEC was isolated as described above. RNA from NHLF and HEK293 cells was isolated by a kit from Invitrogen (Life Technologies, Darmstadt, Germany). Reverse transcription was done using a commercial kit from Invitrogen according to their instructions. Fold change of gene expression was calculated by the $2^{\Delta\Delta ct}$-method normalized to 18S and/or GAPDH. If in untreated control cells a threshold was not reached after 45 cycles, but in treated cells the threshold was arrived at least 10 cycles before, $2^{\Delta\Delta ct}$ calculation was done using the difference of 10 cycles. This leads to an underestimation of the real increase.

Primer against human WISP1 and TNF-α were from SABiosciences. Other primers were synthesized by Eurofins MWG GmbH, Ebersberg, Germany. Annealing temperatures

Table 1. Primer sequences and annealing temperatures

Gene name	Accession number	Forward (5′-3′)	Backward (5′-3′)	Annealing temperature
18S rRNA	X03205	GCATATGCTTGTCTCAAAGA	CCAAAGGAACCATAACTGAT	55°C
GAPDH	NM_002046.3	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG	58°C
SERPINE1	NM_000602.3	CAGACCAAGAGCCTCTCC	ATCACTTGGCCCATGAAAAG	55°C
CDKN2A	NM_000077.4	CCCCCACTACCGTAAATGTCCAT	CTGCCATTTGCTAGCAGTGTGACT	58°C
FN1	NM_212482.1	CCATAAAGGGCAACCAAGAG	AAACCAATTCTTGGAGCAGG	55°C

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SERPINE1, serpin peptidase inhibitor member 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; FN1, fibronectin 1.

and sequences are given in Table 1. Sequencing of qPCR products was done by Eurofins.

2.4 Western blot

Cell lysates were matched for protein content, separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The bound primary antibody was visualized using horseradish peroxidase-conjugated secondary IgG and detection system (Thermo Scientific, Rockland, IL, USA). After detection of phosphorylated proteins, membranes were stripped and probed for the unphosphorylated protein.

2.5 ELISA

ELISA against TNF-α was purchased by PeproTech (Hamburg, Germany). It was carried out according to the manufactures' instructions. TNF-α content was calculated on the basis of a provided rhTNF protein standard and was normalized to cellular protein content.

2.6 Materials

Unless stated otherwise all materials were from Sigma, Munich, Germany. Protease inhibitors were obtained from Calbiochem (Bad Soden, Germany). Antibodies against ERK1/2 and their phosphorylated form as well as β-catenin, β-actin, and horseradish peroxidase-coupled second antibodies were from Cell Signaling (Danvers, MA, USA).

2.7 Statistics

All measurements are given as mean values ± SEM. The significance of difference in PCR-Arrays was determined by the unpaired Student's *t*-test. Significant differences in qPCR, Western Blot, and ELISA experiments were determined by nonparametric tests generated in GraphPad Prism (Wilcoxon signed rank test if the samples were paired, Mann–Whitney test if the samples were unpaired). $p \leq 0.05$ was considered to be statistically significant.

3 Results

3.1 Altered gene expression after prolonged nanomolar OTA exposure

RPTEC cells were incubated with 10 nM OTA for 14 days and gene expression was determined initially by at least two independent microarrays. Figure 1 shows the set of genes, of which the expression was up- or downregulated and that are related to inflammation, EMT/fibrosis and malignant transformation. The expression of selected genes related to Wnt signaling, fibrotic, and inflammatory processes was further confirmed by qPCR as specified in Table 2. One gene related to EMT and malignant transformation was markedly

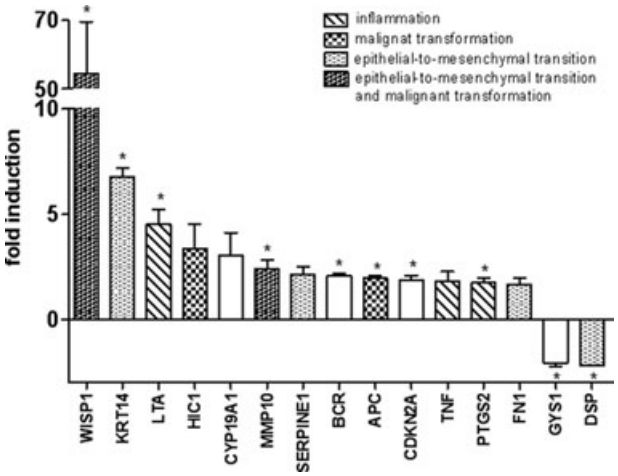

Figure 1. Changes of gene expression in RPTEC after 14-day exposure to 10 nM OTA. Results of at least two independent arrays are shown. * indicates significant difference to untreated cells. Abbreviations: WISP-1, WNT1 inducible signaling pathway protein 1; KRT14, keratin 14; LTA, lymphotoxin α (TNF superfamily, member 1); HIC1, hypermethylated in cancer; CYP19A, cytochrome P450, family 19, subfamily A, polypeptide 1; MMP10, matrix metallopeptidase 10; SERPINE1, serpin peptidase inhibitor member 1; BCR, breakpoint cluster region; CDKN2A, cyclin-dependent kinase inhibitor 2A; APC, adenomatous polyposis coli; TNF, tumor necrosis factor; PTGS2, prostaglandin-endoperoxide synthase 2; FN1, fibronectin 1; GYS1, glycogen synthase 1; DSP, desmoplakin.

Table 2. Altered gene expression in RPTEC after 14-day exposure to 10 nM OTA determined by PCR array or qPCR. Values are given in fold induction or reduction

Gene	PCR-array	qPCR
WISP1	54.4[b)]	37.8[e)]
KRT14	6.8[a)]	n.d.
LTA	4.5[b)]	n.d.
HIC	3.4[b)]	n.d.
CYP19A1	3.0[b)]	n.d.
MMP10	2.4[b)]	n.d.
SERPINE1	2.2[a)]	1.8[c)]
BCR	2.1[b)]	n.d.
CDKN2A	1.8[d)]	3.1[c)]
APC	2.0[b)]	1.6[c)]
TNF-α	1.8[b)]	5.8[d)]
PTGS2	1.7[b)]	1.3[c)]
FN1	1.8[b)]	2.1[c)]
GYS1	−2.1[b)]	n.d.
DSP	−2.2[a)]	n.d.

WISP-1, WNT1 inducible signaling pathway protein 1; KRT14, keratin 14; LTA, lymphotoxin α (TNF superfamily, member 1); HIC1, hypermethylated in cancer; CYP19A, cytochrome P450, family 19, subfamily A, polypeptide 1; MMP10, matrix metallopeptidase 10; SERPINE1, serpin peptidase inhibitor member 1; BCR, breakpoint cluster region; CDKN2A, cyclin-dependent kinase inhibitor 2A; APC, adenomatous polyposis coli; TNF, tumor necrosis factor; PTGS2, prostaglandin-endoperoxide synthase 2; FN1, fibronectin 1; GYS1, glycogen synthase 1; DSP, desmoplakin; n.d., not determined.
a) $n = 2$.
b) $n = 5$.
c) $n = 6$.
d) $n = 7$.
e) $n = 8$.

upregulated (54-fold): Wnt-inducible signaling pathway protein 1, WISP1. Other genes related to EMT that were also upregulated include keratin 14 (KRT 14, 6.8-fold), matrix metalloproteinase-10 (MMP10, 2.4-fold), serine peptidase inhibitor E1 (SERPINE1, 2.2-fold), and fibronectin (FN1, 1.8-fold). Desmoplakin (DSP) expression (related to EMT) was downregulated 2.2-fold. Lymphotoxin alpha (LTA), TNF-α, and prostaglandin-endoperoxide synthase 2 (PTGS2), genes related to inflammatory events, were upregulated (4.5-, 1.8-, and 1.7-fold, respectively). Two other genes related to malignant transformation, hypermethylated in cancer 1 (HIC1), and adenomatous polyposis coli (APC), were also upregulated 3.4-fold and twofold, respectively. Other genes, not related to the above-mentioned groups, that were regulated include cytochrome P450 19A1 (CYP19A1), breakpoint cluster region (BCR), cyclin-dependent kinase inhibitor 2A (CDKN2A), and glycogen synthase 1 (GYS1) (3-, 2.1-, 1.8-, and −2.1-fold, respectively). In the subsequent study, we focused on the effects of OTA on WISP1 as the gene with the most prominent upregulation as well as on TNF-α that has the potential to activate WISP1 signaling and is related to inflammatory events.

3.2 Expression of WISP1 in different cells

To validate the above-described dependence of WISP1 expression on OTA exposure, we used qPCR. The identity of the qPCR products was confirmed by sequencing. WISP1 showed a nearly 38-fold upregulation in RPTEC after 2 weeks exposure to 10 nM OTA. After 2 days, WISP1 gene expression was not significantly altered, whereas after 7 days a fivefold elevation was detectable (Fig. 2A). In contrast, one nanomolar OTA did not change WISP1 expression after 14 days. In HEK293 cells, a human kidney cell line, an elevation of WISP1 expression was detectable already after 6 h and increased markedly within 1 day (Fig. 2B). Longer exposure did not further increase WISP1 expression. This shows that HEK293 cells react much faster and stronger than RPTEC. The strong upregulation of WISP1 expression in HEK293 cells after 24-h exposure to 10 nM OTA is also shown in a gel loaded with the qPCR products (Fig. 2C). One nanomolar OTA elevates WISP expression to a clearly lesser extent (roughly eightfold). In contrast to renal cells, NHLF did not react with an increase of WISP1 gene expression after 14 days exposure to 10 nM OTA (Fig. 2A). Unfortunately, determination of WISP1 protein in media or cell cytosol failed due to insufficient antibody quality. We tested three different antibodies from different suppliers and no concordant results could be observed, even with recombinant human WISP1 as positive control.

3.3 Signaling pathways related to WISP1 gene expression

To identify signaling pathways that may mediate OTA-induced WISP1 expression, we first investigated the canonical Wnt signaling pathway involving β-catenin. Upon phosphorylation β-catenin is rapidly degraded [30] so that an increase of β-catenin indicates an upregulation of this pathway. β-Catenin protein level did not change after incubation with 10 nM OTA neither in RPTEC nor in HEK293 cells (Fig. 3). Furthermore, WISP1 expression was independent on p38 (p38 mitogen activated protein kinase), JNK (c-Jun N-terminal kinase), PKA (protein kinase A), PKC (protein kinase C), or MSK1 (mitogen- and stress-activated protein kinase 1) -dependent signaling pathways according to our pharmacological screen (Fig. 4). Therefore, Wnt signaling as well as several other signaling pathways seem not to play a role in OTA-induced WISP1 expression.

3.4 The role of ERK1/2

Because WISP1 expression has been reported to be at least in part dependent on ERK1/2 signaling pathways, we investigated the role of ERK1/2 in the activation of WISP1 expression by OTA. OTA-induced ERK1/2 phosphorylation could be detected in RPTEC in a time span ranging from

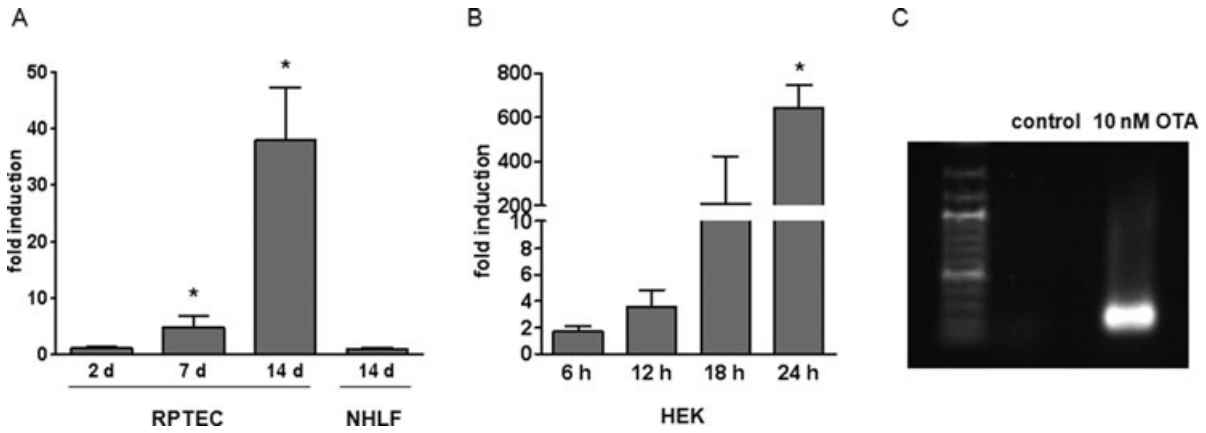

Figure 2. Time-dependent upregulation of WISP1 in RPTEC, NHLF (A), and HEK293 (B) cells. Cells were exposed to 10 nM OTA for the times indicated and gene expression was determined by qPCR. $n =$ at least 3. * indicates significant difference to untreated cells. In (C) a sample of the qPCR products of HEK293 cells exposed for 24 h to 10 nM OTA or control cells was applied onto an agarose gel.

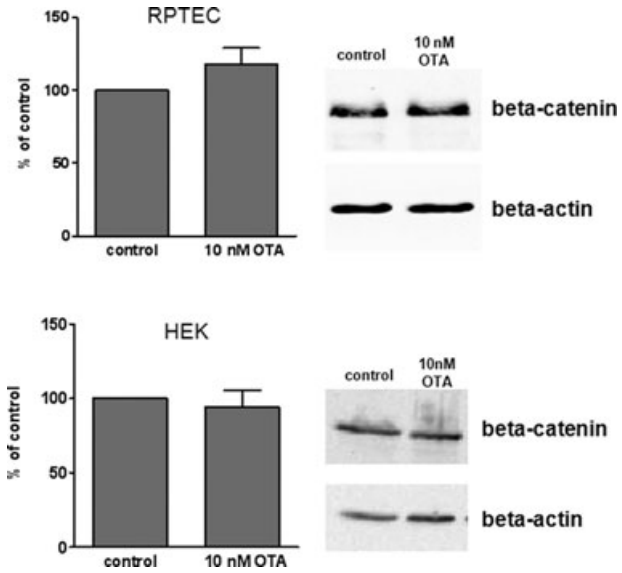

Figure 3. Western blot against beta-catenin in RPTEC and HEK293 cells after 48 or 24 h, respectively, exposure to 10 nM OTA. $n = 4$

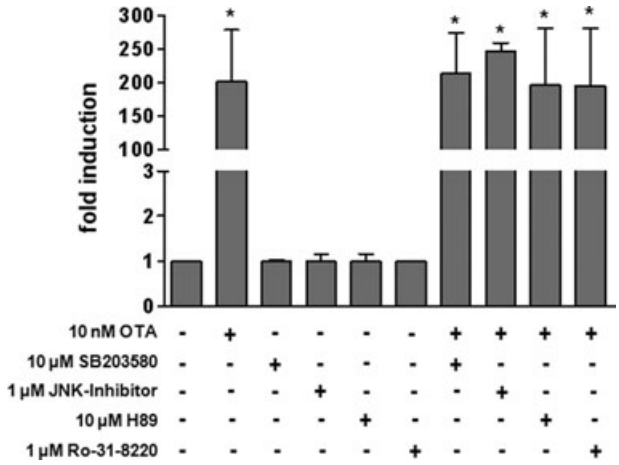

Figure 4. Effect of several signaling pathway inhibitors on OTA-induced WISP1 expression in HEK293 cells. Cells were exposed for 24 h to the inhibitors indicated and WISP1 expression was determined by qPCR. $n = 3$. * indicates significant difference to untreated cells. SB203580, inhibitor of p38; H89, inhibitor of PKA and MSK1; Ro-31-8220, inhibitor of PKC and MSK1.

3 h to 14 days exposure to OTA. The strongest effect (nearly twofold) of ERK1/2 phosphorylation was observed after 48 h (Fig. 5A). Therefore, we concentrated on 48-h exposure. Inhibition of the ERK kinase MEK1/2 by U0126 prevented the OTA-induced ERK1/2 phosphorylation. In HEK293 cells, the elevation of phosphorylated ERK1/2 was observed already after 30 min OTA exposure (3.5-fold) and could also be inhibited by U0126 (Fig. 5A). This again shows that HEK293 cells react faster to OTA exposure when compared to RPTEC.

More important, the OTA-induced expression of WISP1 was clearly abrogated after 24 h in HEK293 cells when ERK1/2 phosphorylation was inhibited (Fig. 5B). These results demonstrate that the regulation of WISP1 expression by OTA is mediated by MEK/ERK signaling.

3.5 Expression of TNF-α

OTA-dependent TNF-α expression was validated by qPCR. The identity of the qPCR product was additionally confirmed by sequencing. In NHLF cells no significant increase of TNF-α expression was detectable after 14-day exposure to 10 nM OTA (Fig. 6A). In contrast, TNF-α was upregulated time dependently in RPTEC as well as in HEK293 cells. In RPTEC (Fig. 6A), an augmentation of TNF-α expression appeared after 2 days (3.6-fold, $p = 0.06$) and further increased significantly within 7 days (4.2-fold) and 2 weeks (roughly sixfold). Also the amount of TNF-α released into the medium was increased already after 48 h (Fig. 6C). One nanomolar OTA had no effect on TNF-α expression. In HEK293 cells,

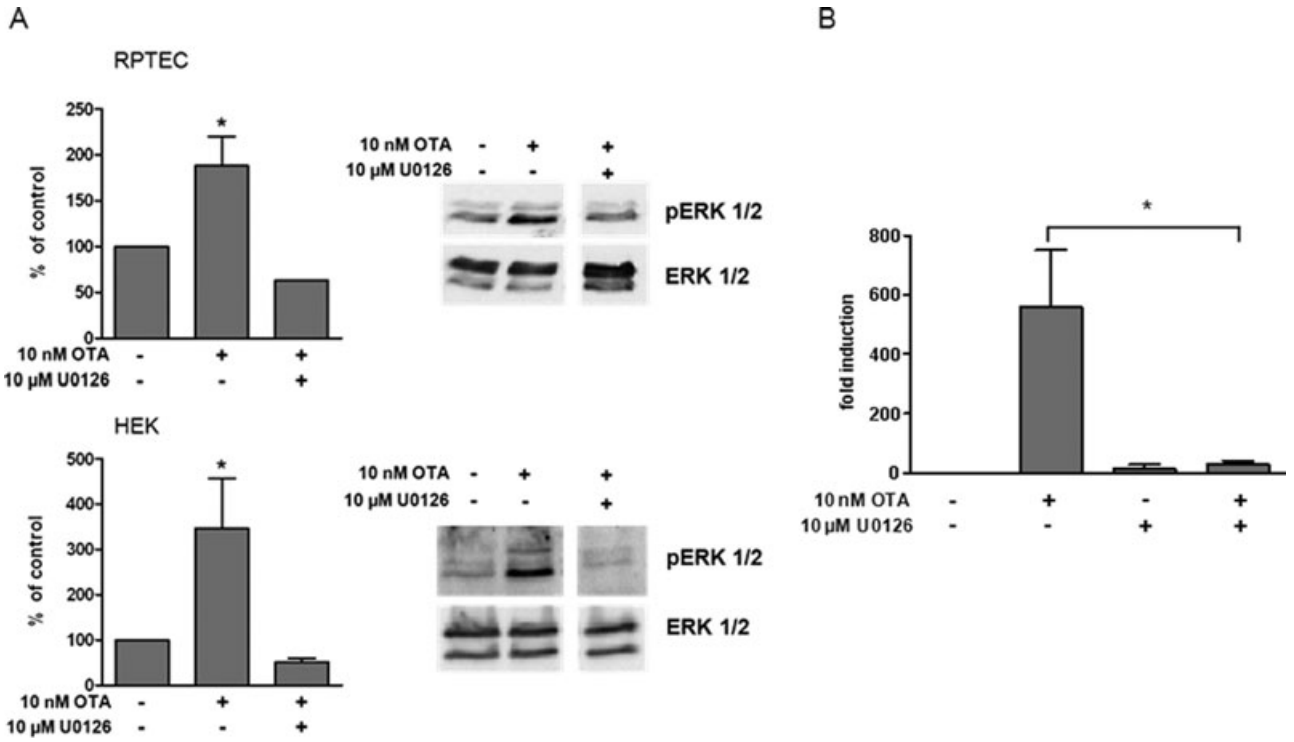

Figure 5. (A) ERK1/2 phosphorylation in RPTEC or HEK293 cells after 48 h (RPTEC) or 30 min (HEK293) exposure to 10 nM OTA and Western blot showing unphosphorylated and phosphorylated ERK1/2. $n =$ at least 6. (B) ERK1/2-dependent WISP1 expression determined by qPCR in HEK293 cells incubated in the presence of the MEK1/2 inhibitor U0126 for 24 h, $n = 6$. * indicates significant difference to untreated cells.

a 13-fold elevation of TNF-α mRNA was detectable already after 24-h exposure to 10 nM OTA that again shows the faster response of these cells to OTA (Fig. 6B). One nanomolar OTA led to a lower increase (roughly threefold).

We also tested the role of ERK1/2 on OTA-induced TNF-α expression in HEK293 cells. Inhibition of MEK1/2 by U0126 leads to a significant (70%) decrease of OTA-induced TNF-α gene expression after 24 h and therefore demonstrates that the effect of OTA on TNF-α is also, at least in part, mediated by MEK/ERK-including signaling pathways (Fig. 6D).

3.6 Influence of recombinant TNF-α on WISP1 expression and of recombinant WISP1 on TNF-α expression

It has been shown that WISP1 expression via ERK1/2 is inducible by TNF-α in cardiac fibroblasts [19]. We tested the hypothesis that the observed OTA-induced elevation of WISP1 expression is mediated by enhanced TNF-α secretion. In contrast to published results, no effect of recombinant TNF-α on WISP1 expression could be observed after 24 h in HEK293 (Fig. 7). On the other hand, the OTA-induced increased WISP1 expression may have an effect on TNF-α expression. Exposure for 24 h to recombinant human WISP1 did not influence TNF-α expression (Fig. 7). These results demonstrate that the OTA-induced elevated WISP1 and TNF-α expression rates are regulated independently of each other.

4 Discussion

The consumption of an average diet leads to a nearly unavoidable uptake of OTA. Thus, it can be detected in low concentrations in human blood (in average around 1 nM [6, 7]) and presumably in higher concentrations in renal tissue due to transport and concentration mechanisms [31]. During the last years it turned out that nanomolar OTA concentrations administered during a prolonged exposure time had distinct effects on human primary cells in culture like an increase of collagen III and fibronectin production or hypertrophy, these effects are sometimes in contrast to high, and therefore nonrelevant concentrations [9–11]. However, the underlying mechanisms of these alterations are not understood, but involve most probably alterations in gene expression. Therefore, we first studied changes in the expression of genes related to inflammation, EMT/fibrosis, and malignant transformation after prolonged OTA exposure and focused subsequently on the markedly upregulated and profibrotic gene, WISP1. Additionally, the influence of OTA on TNF-α expression was

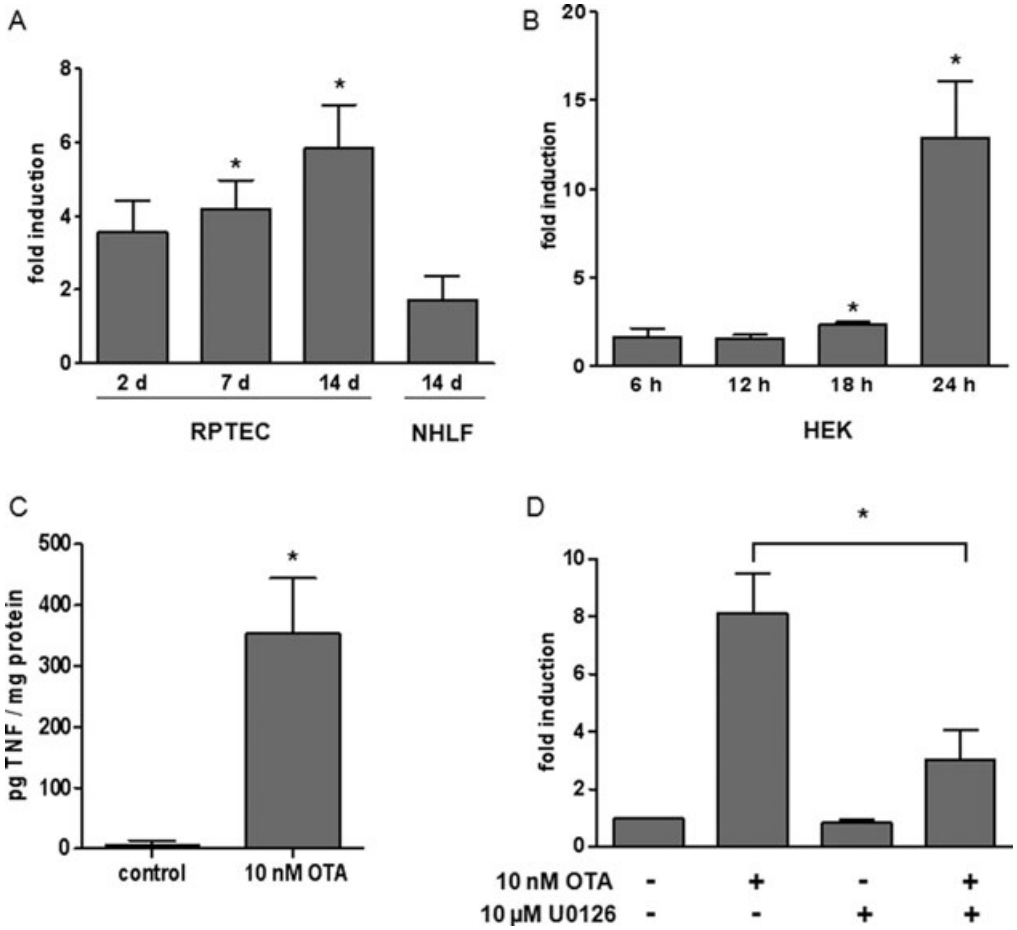

Figure 6. Time-dependent upregulation of TNF-α in RPTEC, NHLF (A), and HEK293 (B) cells. Cells were exposed to 10 nM OTA for the times indicated and gene expression was determined by qPCR. n = at least 3. (C) TNF-α content in media of RPTEC after exposure to 10 nM OTA for 48 h determined by ELISA. $n = 6$. (D) ERK1/2-dependent TNF-α expression determined by qPCR in HEK293 cells incubated in the presence of the MEK1/2 inhibitor U0126 for 24 h, $n = 6$. * indicates significant difference to untreated cells.

further investigated because TNF-α not only plays a role in inflammatory events and contributes to tubulointerstitial nephritis but also may be involved in the regulation of WISP1 [19].

Array data revealed the regulation of only a few genes in RPTEC after an exposure to 10 nM OTA for 14 days. Thus, low OTA concentrations administered over a prolonged period exert few but probably decisive alterations in gene expression. Higher OTA concentrations in contrast may lead to nonspecific alterations of a bundle of genes leading to nonspecific cell damage. For a subset of genes regulated by prolonged OTA exposure as identified by array analysis, the altered expression was verified by qPCR. Only genes, whose expression was changed at least by a factor of two either in the array or in qPCR were considered to be significantly affected. Array and qPCR-based data indicate that low OTA concentrations have the potential to interfere with processes related to inflammation (LTA, TNF-α, PTGS2), malignant transformation (HIC1, APC) and epithelial-to-mesenchymal transition (KRT14, PAI, FN1, DSP) or both (WISP1, MMP10).

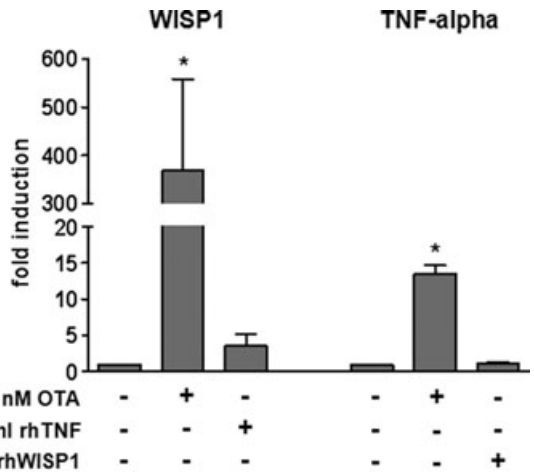

Figure 7. WISP1 and TNF-α expression in HEK293 cells incubated for 24 h with either recombinant human TNF-α or recombinant human WISP1. n = at least 3. * indicates significant difference to untreated cells.

Inflammation and epithelial-to-mesenchymal transition are prodromal profibrotic alterations.

It turned out that the expression of one gene was markedly elevated: WISP1. WISP1 is a secreted protein that has promitogenic, profibrotic, prohypertrophic, and oncogenic potential [19, 20]. Up to date, only few toxic or pharmacologic substances are known to modify the expression of WISP1 mRNA as for example indomethacin, methotrexate, bleomycin, or cadmium chloride [25, 32–34]. OTA therefore is a new member of this (to date) small family of molecules and this is the first time that WISP1-including mechanisms can be linked to OTA nephropathy.

In RPTEC, the elevation of WISP1 gene expression occurred after 7 days and was further increased after 14 days to OTA exposure. To compare the results obtained in RPTEC a renal cell line was used, HEK293 cells. Also in HEK293 cells WISP1 expression was strongly upregulated. This elevation was significant already after 12 h and became very prominent after 18 and 24 h. In fact, in control HEK293 cells virtually no WISP1 mRNA could be detected after 45 cycles whereas in OTA-treated cells the threshold was reached much earlier (30 cycles) so that the increase of WISP1 expression calculated here is even underestimated. The time difference in WISP1 expression between RPTEC and HEK293 cells is most probably based upon the difference between primary cells (RPTEC), which grow remarkably slow, and a cell line (HEK293), which possesses a much faster growth rate. In contrast, in fibroblast cells no elevated WISP expression was detectable after OTA exposure. This excludes unspecific, generalized effects of OTA.

WISP1 gene expression was described to be mediated by a Wnt1/beta-catenin involving pathway but also by ERK1/2-dependent mechanisms [19, 27]. We observed that the Wnt1/beta-catenin-dependent pathway seems not to play a predominant role in OTA-induced WISP1 expression: mRNA expression and protein abundance of (unphosphorylated) beta-catenin was not altered after OTA exposure. We also tested other pathways pharmacologically (JNK, p38, PKA, PKC, MSK1) but their inhibition did not influence OTA-induced WISP1 expression. Therefore, the pathway leading to OTA-induced WISP1 expression seems to involve ERK1/2 whose activation was observable already after 30 min (HEK)

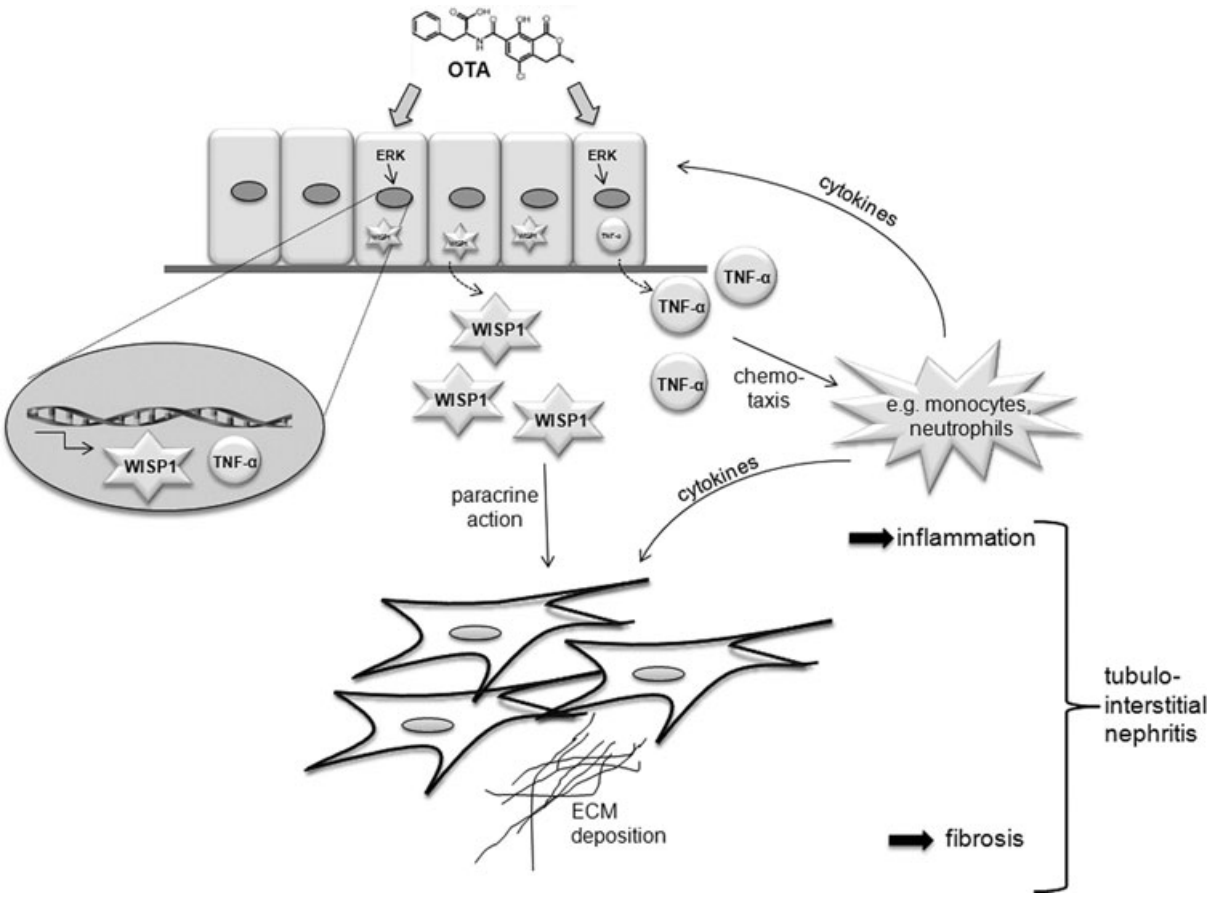

Figure 8. Scheme of OTA effect on proximal tubule cells and their subsequent interaction with fibroblasts. OTA leads to elevated formation of WISP1 and TNF-α that in a paracrine mode influence fibroblast cells and together with the activation of inflammatory cells by TNF-α lead to tubulointerstitial nephritis.

or 3 h (RPTEC). Altered ERK1/2 phosphorylation has been reported also in kidneys of mice fed with OTA [35] and in a canine distal tubule cell line in which it leads to morphological dedifferentiation [36] and stable transformation [37]. The possibility that OTA leads to an indirect elevation of WISP1 expression via OTA-induced TNF-α expression that in turn activates WISP1 by ERK1/2 (a mechanism described for TNF-α in cardiac fibroblasts [19]) seems unlikely because recombinant TNF-α exerted no effect on WISP1 expression under our experimental conditions.

WISP1 is a secreted protein. Therefore, it is tempting to speculate in how far WISP1 may exert autocrine or paracrine function. Autocrine, self-enhancing WISP1 expression involving Akt and beta-catenin was suggested for human vein smooth muscle cells [38]. The exponential-like time course of WISP1 expression increase supports an autocrine, self-enhancing mode. However, in contrast [38], we could show that OTA-induced WISP1 expression was independent of beta-catenin-including pathways. Acting in a paracrine manner, WISP1 secreted from proximal tubule cells could stimulate close-by interstitial fibroblasts thereby initiating and propagating tubulointerstitial fibrosis. Thus, a paracrine impact of proximal tubule cell-derived WISP1 on renal interstitial fibroblasts represents a pathomechanistic model: OTA exposure leads to augmented WISP1 expression in proximal tubule cells which alters interstitial fibroblast function followed by fibrotic renal changes ultimately leading to tubulointerstitial nephropathy (Fig. 8). In addition, enhanced TNF-α expression leads to the recruitment of inflammatory cells, thereby further stimulating proximal tubule cells and fibroblasts via cytokine secretion. Ultimately, a vicious circle is initiated, that may result in a continuous decline of renal function, even if the primary trigger (in this case OTA) is removed. Of course this model has to be investigated further, a.o. in coculture models. In future studies it is also important to determine the effect of an inhibition of WISP1 expression by siRNA on the impact of OTA on kidney cells.

In summary, we have shown that prolonged exposure of kidney cells to relevant nanomolar OTA concentrations, expectable in human kidney due to a normal diet, leads to a marked ERK1/2-dependent upregulation of WISP1 gene expression, which, accompanied by increased ERK1/2-dependent TNF-α expression, may be involved in the onset of tubulointerstitial nephritis caused by OTA. The involvement of a specific pathway in OTA-induced upregulation of two important genes only in renal but not in nonrenal cells is a further explanation for the tissue specificity of OTA. Because the uptake of OTA due to an average diet is almost unavoidable, a long-term risk by OTA on the kidney is not excludable and an argument for low safety values in food.

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