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Research Article

Potential of deoxynivalenol to induce transcription factors in human hepatoma cells

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To assess the hepatotoxicity of deoxynivalenol (DON), human hepatoma cells (Hep-G2) were used as an *in vitro* model. After exposing Hep-G2 cells to low (1 μM) and high dose (10 μM), gene expression profiles were analysed by microarray. More than 5% of genes were up-regulated, most of them being involved in transcriptional regulation. By real-time RT-PCR, elevated expression of transcription factors, commonly induced by activation of MAPK-pathway, was demonstrated for Hep-G2 cells on mRNA and protein level. Further studies, involving U937 human monocytes, showed that effects of DON treatment on mRNA and protein level were concentration-dependent and cell-specific. An inverse relation was noticed for the level of DON induced expression of transcription factors (JUN, FOS, EGR1 and ATF3) and the susceptibility of the cell lines towards the mycotoxin. This is the first report giving evidence that on a molecular level the mild hepatotoxic effects of DON are probably caused by the induction of transcription factors which are known to be associated with injury-induced liver regeneration processes. With ATF3, a novel downstream target gene was identified in DON-related cell signalling suggesting a potential linkage between molecular action and biological effects like reduction of glycogen storage in liver tissue.

Keywords: Deoxynivalenol / Hep-G2 / MAPK / Mycotoxin / Transcription factors

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1 Introduction

The presence of mycotoxins in cereals is a common problem in agriculture and nutrition, particularly deoxynivalenol (DON) is frequently detected at high concentrations worldwide in food crops or related products [1 – 4]. DON is produced by several species of *Fusarium* fungi which primarily infect wheat, barley and maize crops [5, 6]. DON is only partly eliminated during milling and processing procedures [7, 8] and toxin levels are not significantly altered during baking and cooking [9]. A large collaboration study [10] revealed that 57% of the cereal based foods in the European Union were contaminated with DON. Recently published data from the United Kingdom highlighted the frequency of DON intake by the population. The majority of adults in this study appeared to be exposed to DON, as concluded from urine testing [11].

Numerous studies addressed DON toxicity in animals [12], with swine representing the most susceptible species [13, 14]. In particular, inflammation and organ failure related to DON toxicity have been evaluated in different experimental *in vivo* models [15 – 18]. In addition, there exist several *in vitro* studies documenting DON effects on human epithelial and immune cells [19 – 23]. Although the liver is the primary organ involved in xenobiotic metabolism, and a major target organ of many chemicals, drugs or other pathogens [24], our present knowledge on liver toxicity of DON is scarce. Recently, it has been shown that feeding of DON contaminated wheat elicits histopathological changes in porcine liver [25]. On cellular level, loss of bound ribosomes from ER was observed [26]. Furthermore, DON influences several basic metabolic parameters in rat liver cells and human primary hepatocytes [27, 28].

The trichothecene DON is supposed to disrupt normal cell function by inhibition of protein synthesis [29] *via*

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Abbreviations: DON, deoxynivalenol; **ERK,** extracellular signal-related kinase; **GO,** gene ontology; **IEG,** immediately early gene; **JNK,** c-Jun NH_2-terminal kinase; **MAPK,** mitogen activated protein kinase; **WST-1,** 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

binding to the ribosome and by activating cellular kinases involved in signal transduction. However, the cellular mode of action underlying these effects is not yet fully understood. Little is known about cell type-specific toxic mechanisms especially of the mitogen activated protein kinases (MAPKs) pathway and alteration of downstream transcription factor expression in 'nonimmune cells'. Most of the previous investigations focussed on immunomodulatory effects of DON indicating that early alterations in cell signalling, particularly MAPKs, are critical to trichothecene toxicity [30 – 32]. MAPK-dependent reactions, also known as ‚ribotoxic stress response', have been demonstrated for translation inhibitors such as ricin, anisomycin, T-2 toxin and DON [33, 34]. MAPKs are activated in response to a variety of stimuli, transduce signals from the cell membrane to the nucleus and phosphorylate nuclear transcription factors. Induction of the serine – threonine kinase pathway include the three MAPK subfamilies namely c-Jun NH_2-terminal kinases (JNKs), extracellular signal-related kinases (ERKs) and p38 MAPK. All of these factors enhance the activation of transcription [35] and mediate the regulation of cell signalling under various aspects [36]. As for DON, they markedly induce phosphorylation of p38, JNK and ERK as well as cytokine expression and apoptosis [17, 37 – 39].

Generally, in immune competent cells, trichothecene-induced MAPK-activity drives activation of immediately early genes (IEG) that promote processes such as cytokine production and cyclooxygenase 2 expression and apoptosis [32, 37, 40]. Two of the best characterized IEGs are the transcription factors JUN and FOS which belong to the AP-1 (activator protein 1) family, regulating a wide range of cellular processes including cell proliferation, cell life or death, survival and differentiation [41, 42]. In murine lymphoid tissue, DON activates components of AP-1 [33, 43]. Another IEG, the early growth response 1 protein (EGR1) has been investigated in DON-treated epithelial intestine 407 cells modulating their IL-8 expression [22]. Transcription factors like JUN and EGR1 are often induced in the same cluster as ATF3 [44], an IEG belonging to the ATF/CREB family of transcription factors which are induced by a variety of stress signals in different cell types.

Based on these data the present study aimed to evaluate early DON effects on mRNA and protein levels in human cell lines as an *in vitro* model to assay the hepatotoxic potential. In order to get a first impression, DON-induced alterations of gene expression were screened by microarray. Selected results were confirmed by real-time RT-PCR and Western blot. Besides, the expected results that DON activates members of the MAPK family also in hepatocytes, differentially induced expression of IEGs like EGR1, JUN and FOS was shown to depend on incubation time and toxin concentration. Comparing the effects on two different cell lines, it was observed that the level of DON induced expression of transcription factors and the susceptibility of the cell lines towards the toxin was inversely related. Finally, with ATF3 a novel downstream gene was identified in DON-related cell signalling suggesting a potential linkage between molecular action and biological effects such as hepatic metabolism and homeostasis [45, 46].

2 Materials and methods

2.1 Reagents

Antibodies against ATF3 (sc-188), EGR1 (sc-189) and c-FOS (sc-52) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p44/42 MAP kinase, phospho-p44/42 MAP kinase, p38 MAP kinase (5F11), SAPK/JNK, phospho-SAPK/JNK, c-JUN (60A8), phospho-c-JUN (Ser63), phospho-c-JUN (Ser73), β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-p38 MAP kinase (pT180/pY182) was obtained from BD Biosciences (San Diego, 125 CA, USA).

2.2 General cell culture conditions

Human cell lines were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and were used at maximum for 30 passages.

Hepatoma cell line Hep-G2 and monocytic cell line U937 were cultured in RPMI 1640 medium supplemented with 10% v/v FCS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were fed with fresh medium 12 h prior to the various treatments listed below. For cytotoxicity test, cells were plated at 2×10^4 cells per well in 96-well plates. Immunofluorescence was performed on chamber slides (Nunc, Wiesbaden, Germany) plated with 4×10^5 cells and incubated at 37°C for 24 h. For immunoblotting and RNA isolation 2.5×10^5 cells per well were plated on 24 multi-well culture dishes and incubated for 24 h.

2.3 Determination of DON-related cytotoxicity by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) test

Cytotoxicity data were obtained from at least three independent experiments. Results were expressed as percentages of the mean absorbance (OD) of treated cells *versus* negative controls (normal growth medium). Mean OD of the negative controls was set to 100% viability. For the determination of cell viability, the water-soluble dye WST-1 (Roche Diagnostics, Penzberg, Germany) was used. This assay which is similar to the MTT test is based on the cleavage of the tetrazolium salt WST-1 (slightly red) to formazan (dark red) by various mitochondrial enzymes. After incuba-

tion of the cells with varying concentrations of DON for 48 h, 10 µL of WST-1 reagent was added to each well and incubated at 37°C for variable time periods (0.5 – 2 h). Then, microtitre plates were thoroughly shaken for 1 min and absorbance was measured in a microplate reader at 450/655 nm.

2.4 Microarray

Total RNA was isolated using the E.Z.N.A. Total RNA kit (PEQLAB Biotechnologie GMBH, Erlangen, Germany). For the microarray analyses, Human Genome U133A 2.0 GeneChips (Affymetrix, High Wycombe, UK) with 22 277 probe sets including 14 500 genes were used. The generation of labelled cRNA, hybridization, washing, staining and scanning was done at the *Kompetenzzentrum für Fluoreszente Bioanalytik* (KFB; Regensburg, Germany) following the Affymetrix GeneChip® Expression Analysis Technical Manual. For identification of differentially expressed genes, experimental and baseline arrays were analysed by two-condition-experimental-design using GeneChip® Operating Software (GCOS) software. Herein increased/decreased, marginally increased/marginally decreased or no change calls were identified. Additionally, differences in signal intensities between perfectly matching and mismatching oligonucleotide probes on the arrays were compared to recorded alterations in gene expression based on One-Sided Wilcoxon's Signed Rank test. Alterations are depicted by GCOSv1.2 as signal $\log_2$ ratio. Significances (p-values) of each change were assigned.

2.5 Data analyses

All present and increased genes (p-value < 0.0005; $\log_2 \geq 2$) were selected for further analysis. For this purpose, the software tool BiNGO (Biological Network Gene Ontology plugin tool) [47] from the Cytoscape package [48] was used wherein over-representation of gene ontology (GO) categories (www.geneontology.org) in biological networks can be assayed. GOs consist of three hierarchically structured vocabularies that describe gene products in terms of their associated 'biological processes', 'molecular functions' and 'cellular components'. Gene products may be annotated to one or several nodes in each hierarchy. Significantly altered genes were assigned to above named categories. For hypergeometric statistical tests resulting in a significance level of $p < 0.05$, Benjamini and Hochberg's FDR multiple testing corrections were performed.

2.6 RNA isolation and reverse transcription for real-time RT-PCR

Total RNA was isolated from cells as described in Section 2.4, quantity and purity was measured spectrophotometrically. The RNA was reversely transcribed from 1 µg starting input using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) and subjected to real-time RT-PCR.

2.7 Real-time RT-PCR

All samples were assayed in quadruplicates. RT-PCR reactions for target genes in the different cell lines were performed on a LightCycler 480 instrument (Roche Diagnostics, Penzberg, Germany). Typical reactions contained 5 µL of 2 × LightCycler SYBR Green I Master (Roche Diagnostics, Penzberg, Germany), in a total volume of 10 µL. Cyclic amplification was preceded by incubating the reaction mixtures for 10 min at 95°C followed by 40 cycles of amplification. Steps and temperatures were as follows: denaturation 15 s at 95°C, annealing 10 s at 60°C and extension 10 s at 72°C, with single fluorescence acquisition at 72°C after each cycle. Finally a melting curve was generated. Expression levels of HMBS (hydroxymethylbilane synthase) and 28S rRNAs were used as housekeeping-genes. LightCycler experiments further included a calibrator sample measured in triplicates and a standard curve for the determination of amplification efficiency. Relative expression of target genes was calculated after normalization by the housekeeping-genes. Data were analysed by LC480 software (Roche Diagnostics, Penzberg, Germany). Finally, relative increase or decrease of RNA was expressed as $\log_2$ ratio calculated by comparing relative expression levels in controls to treatment groups.

2.8 Primer design

The RT-PCR primers described in Table 1 were designed using IDT SciTools PrimerQuest (http://eu.idtdna.com) or Primer3 software (http://frodo.wi. mit.edu/). Primers were located in the coding region. Primer sequences and additional information are depicted in Table 1.

2.9 Whole-cell protein preparation and Western blot analysis

Whole-cell extracts were prepared following the Cell Signalling protocol for Western blot analysis, and the amount of protein was quantified by the means of bicinchoninic acid (BCA) assay (Sigma, Deisenhofen, Germany) using BSA as standard. Equal amounts of whole-cell protein extracts were mixed with protein sample buffer and separated on a 10 – 15% SDS – PAGE gel. Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bredford, MA) according to standard protocols. The membrane was probed with the corresponding primary antibodies following Cell Signalling protocols. Usually, phosphorylated and unphosphorylated MAPK-kinases were analysed on the same membrane. For this purpose, membranes were reprobed by using stripping buffer (Pierce) following the

Table 1. Primer pairs applied in real-time RT-PCR for determination of transcription factor expression levels

Name	5′-3′Sequence	GenBank ID	Amplicon (Size)
ATF3	for: ttg cta acc tga cgc cct ttg rev: tct gtc gct gac agt gac tg	NM_001674	107
EGR1	for :agg aca gga gga gga gat gg rev: gga agt ggg cag aaa gga ttg	NM_001964	129
JUN	for: aaa cag agc atg acc ctg aac c rev: gat tat cag gcg ctc cag ct	NC_002228	139
FOS	for: tct gtg gct tcc ctt gat ctg a rev: tca tca aag ggc tcg gtc ttc a	NM_005252	168
28S rRNA	for: ccc act aat agg gaa cgt gag- rev: gcc aag cac ata cac caa atg tc	M27830	142
HMBS	Housekeeping-Gene, QuantiTect Primer Assays		

RestoreTM Western Blot Stripping Buffer protocol. Signal visualization by chemiluminescence was achieved by SuperSignal ELISA Femto Maximum Sensitivity Substrate (Perbio Science, Bonn, Germany) on KODAK Image Station 2000R.

2.10 Immunofluorescence cell staining

Hep-G2 cells were treated with indicated DON concentrations for 24 h (for JUN and phospho-JUN detection). Cell labelling protocol for immunofluorescence-microscopy was as follows: cells were fixed in 3% formaldehyde for 15 min and ice-cold methanol for 10 min, blocked with 5% inactivated goat serum for 60 min and incubated 1 h with specific primary antibodies (1:100) against JUN. Subsequently, cells were incubated with Alexa Fluor 488 labelled secondary antibodies (1:250). For all immunoreagents, PBS containing 1% BSA and 0.3% Triton X-100 was used as a diluent. Nuclei were counter-stained with DAPI and slides were examined with a BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan).

2.11 Statistics

Data were statistically processed on SPSS (SPSS, Chicago, USA) software. A multiway ANOVA was applied for analysis of mRNA expression using Tuckey tests for post hoc comparison of individual factors.

3 Results

3.1 Cytotoxicity of DON in Hep-G2 and U937 cells

In the presence of 0.05 – 50 μM DON, cell viability decreased in a concentration-dependent manner (Fig. 1). After 48 h exposure to DON, treatment of Hep-G2 cells with 1 μM DON resulted in at least 75% cell viability, whereas at high-DON concentrations (10 μM) only 15% of the cells showed metabolic activity. Hence, these DON concentrations were defined as low- and high-cytotoxic doses

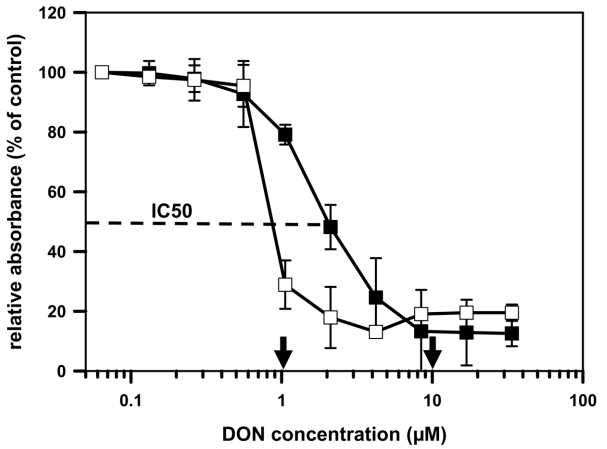

Figure 1. Dose-response curve on cell viability obtained after DON-treatment of different cell lines for 48 h: ■ = Hep-G2 cells, □ = U937 cells. IC_{50} values (dashed line) of Hep-G2 cells (1.89 μM) and U937 cells (0.95 μM) measured by the WST-1 bioassay are depicted. Arrows illustrate the low- and high-cytotoxic DON dose used for the further experiments. All assays were performed in triplicates.

and were used for all further experiments. Treatment of the more sensitive U937 cells with the low-DON dose resulted in cytotoxic effects which were within the dynamic range of the viability curves (Fig. 1). The calculated IC_{50} values for the cell lines under study were 1.89 μM (Hep-G2) and 0.95 μM (U937).

3.2 Gene expression analysis by microarray

In order to get a first impression of the early DON effects on cellular processes, changes in the gene expression profile of Hep-G2 cells were analysed by microarray after treatment with the indicated toxin concentrations for 3 h. After this time point, the DON exposure of the cells induced a statistically significant change in gene expression which was clearly distinct from the control. Furthermore, both treatment groups showed specific gene expression levels which clearly demonstrated a concentration-dependent

GO term (category)		FDR-corrected *p*-value	
		DON (1 μM)	DON (10 μM)
GOterm			
	Biological Processes		
GO:43283	biopolymer metabolism	*8.15E-03*	*3.65E-10*
GO:16070	RNA metabolism	*1.25E-03*	*2.82E-09*
GO:6139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	*8.15E-03*	*3.67E-08*
GO:19219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	*1.25E-03*	*6.09E-10*
GO:32774	RNA biosynthesis	*7.54E-04*	*1.21E-10*
GO:6350	transcription	*1.25E-03*	*3.25E-10*
GO:45449	regulation of transcription	*1.09E-03*	*1.07E-09*
GO:6351	transcription, DNA-dependent	*7.54E-04*	*1.35E-08*
GO:6355	regulation of transcription, DNA-dependent	*7.54E-04*	*1.86E-10*
GO:6366	transcription from RNA polymerase II promoter	*8.15E-03*	*1.35E-08*
	Molecular Processes		
GO:5488	binding	*4.27E-03*	*5.45E-05*
GO:5515	protein binding	*1.17E-03*	*1.72E-06*
GO:46983	protein dimerization activity	*5.69E-04*	*1.17E-03*
GO:3676	nucleic acid binding	*5.69E-04*	*7.53E-10*
GO:3677	DNA binding	*6.83E-05*	*9.09E-15*
GO:43565	sequence-specific DNA binding	*7.26E-03*	*7.42E-04*
GO:8134	transcription factor binding	*1.23E-02*	*1.17E-03*
GO:3700	transcription factor activity	*1.05E-03*	*6.18E-13*
GO:3712	transcription cofactor activity	*5.12E-03*	*2.78E-03*
GO:3714	transcription corepressor activity	*1.70E-03*	*1.72E-03*
GO:30528	transcription regulator activity	*6.83E-05*	*6.18E-13*
GO:16564	transcriptional repressor activity	*8.94E-03*	*2.40E-04*
	Cellular Component		
GO:5622	intracellular	*2.76E-02*	*2.32E-05*
GO:44424	intracellular part	*9.66E-03*	*1.34E-06*
GO:43226	organelle	*6.93E-04*	*9.58E-06*
GO:43229	intracellular organelle	*6.93E-04*	*9.58E-06*
GO:43227	membrane-bound organelle	*1.87E-04*	*8.73E-08*
GO:43231	intracellular membrane-bound organelle	*1.87E-04*	*8.73E-08*
GO:5634	nucleus	*5.29E-07*	*1.61E-14*

Figure 2. Significantly over-represented gene categories (terms) in DON-treated Hep-G2 cells after 3 h exposure according to GO nomenclature. Only GO processes (www.geneontology.org) with a FDR *p*-value of <0.05, resulting from BiNGO analysis of microarray data, are shown.

gene modulation. In general, 52.5% of probe sets after low (1 μM) and 48.2% of probe sets after high-dose (10 μM) treatment were detected as present; an increased expression was noticed for 6.9 and 7.5%, respectively. In contrast, only 2.3% decrease in gene expression was observed after 1 μM DON treatment and 6.5% after 10 μM DON treatment.

The expression profile of low (high) dose revealed 256 (694) significantly (p-value < 0.001) up-regulated probe sets with a $\log_2$ ratio ≥ 1. Significantly (p-value < 1 – 0.001) decreased gene expression ($\log_2$ regulation ≤ – 1) could only be detected for 17 (86) genes. To detect enriched gene functions further data analysis was performed by BiNGO plugin of Cytoscape based on GO categories. Genes were only considered as biologically relevant if the signal $\log_2$ ratio was greater than 2 (4-fold change) and the p-value below 0.0005.

To extract the statistically over-represented categories and to correct for multiple testing, a hypergeometric test and the Benjamini Hochberg FDR correction were performed. Significantly enriched categories of the genes, up-regulated by low-dose or high-dose treatment, are summarized in Fig. 2. The hierarchical structure of GO results in the more specific category appearing in the deeper hierarchy. The genes involved in transcription for 'biological processes' and nucleic acid binding, transcription factor activity for 'molecular processes' were significantly up-regulated in hepatoma cells for both treatments. For 'cellular components' these genes were mainly located in the nucleus (Fig. 2). Similarly affected categories due to DON exposure could be identified for both treatments, in most of the cases the effect of the higher dose being more pronounced.

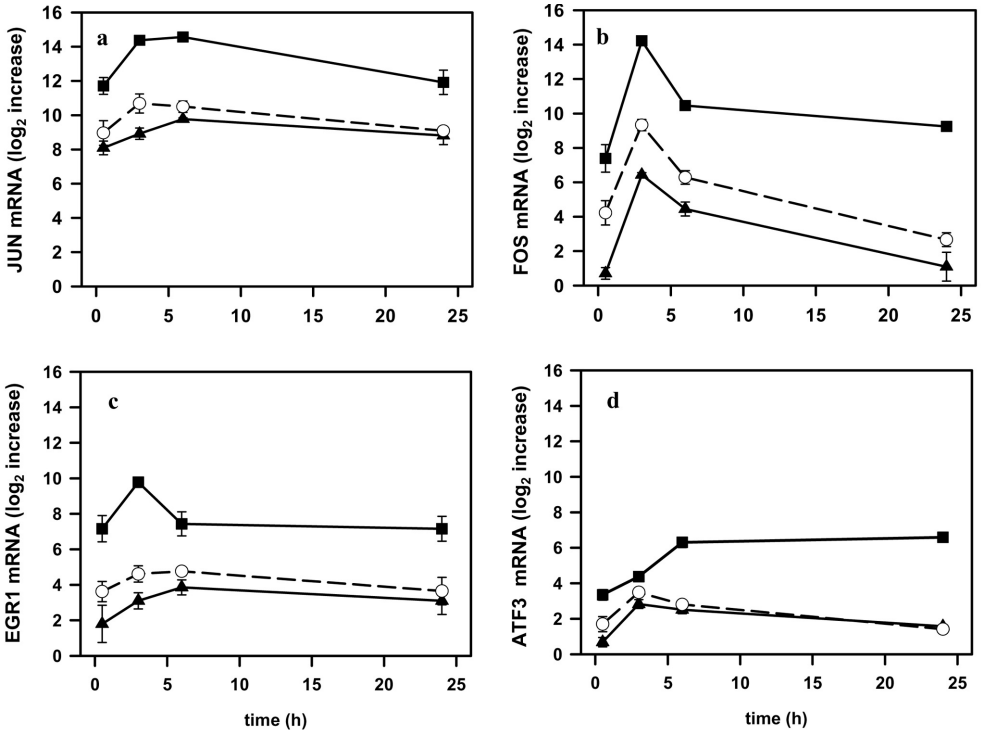

Figure 3. (a – d) Relative expression of transcription factor mRNA for JUN (a), FOS (b), EGR1 (c) and ATF3 (d) in Hep-G2 cells exposed to 1 μM DON (▲), 10 μM DON (■) or 0.1 μM anisomycin (O, dashed line). All assays were run in quadruplicates; values are means ± SD. Data points represent $\log_2$ regulation compared to untreated time-matched controls. Sampling times were at 0.5, 3, 6 and 24 h.

3.3 DON up-regulates transcription factor mRNA in Hep-G2 cells

To validate the microarray results on a quantitative level, different induced genes upon DON challenge were studied by using real-time RT-PCR. Time and dose-dependent effects of DON treatment on the relative expression of the four transcription factors JUN, FOS, EGR1 and ATF3 in human Hep-G2 cells were assayed. For this purpose, cells were exposed to high (10 μM) and low (1 μM) DON doses for up to 24 h. A low cytotoxic equivalence dose of anisomycin (0.1 μM) was included as internal control. Anisomycin is a well-known inhibitor of protein synthesis and potent inducer of transcription factor activity [31]. The results are shown in Figs. 3a – d. Compared to the control level, the most prominent increase in mRNA expression was detected for JUN with an approximately 9 – 12 $\log_2$ increase. This early increase in mRNA expression was observed already after 0.5 h of incubation with 1 and 10 μM DON. Peak levels of mRNA expression were reached for both toxin concentrations after 6 h. Up to 24 h, expression levels declined slightly but stayed clearly above those of untreated control cells. A similarly strong induction of mRNA was found for FOS and EGR1 in the high-dosed group where peak expression was reached after 3 h. In contrast to FOS, highest mRNA levels for EGR1 were detectable in the low-dose group after 6 h and remained constantly up-regulated up to 24 h. Moreover, a slight but constant increase in gene expression was found for ATF3. After reaching a maximum $\log_2$ regulation of 6, ATF3 transcripts remained up-regulated for 24 h in high-dose treated Hep-G2 cells. Low-dose treatment led to a maximum expression after 3 h and a subsequent decline of ATF3 expression to nearly background levels. Applying multiway ANOVA, significance of the alteration of expression was proved for all mRNAs under study ($p < 0.001$) as well as for each of the factors when assayed separately ($p < 0.01$) in comparison to negative controls. Equally, low-DON dose and anisomycin-treated groups responded significantly different from the high-toxic DON treatments ($p < 0.01$). Comparing anisomycin and low-dose DON treatment, only JUN expression was significantly ($p < 0.05$) influenced whereas no significant differences were detected for ATF3, EGR1 and FOS ($p > 0.05$).

3.4 DON activates MAPKs in Hep-G2 cells on protein level

To verify that the induction of transcription factors was accompanied by an activation of the MAPK pathway, phosphorylation of the main kinases was analysed by Western

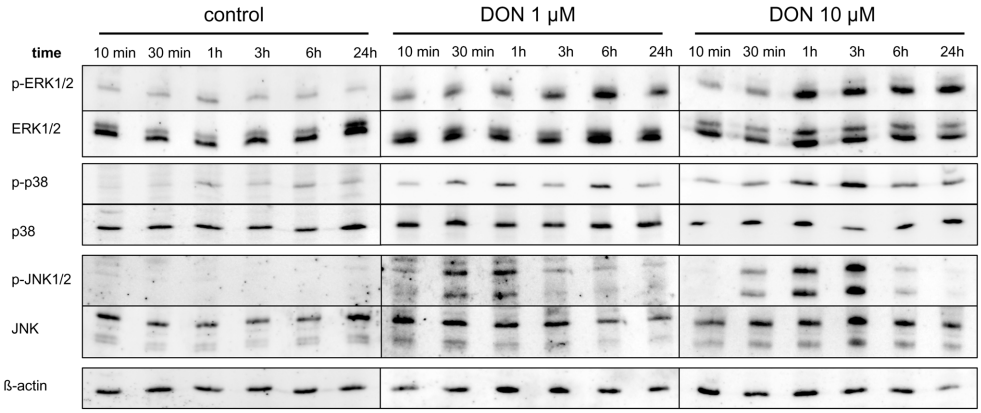

Figure 4. Concentration-dependent activation of ERK1/2, JNK1/2 and p38 MAPK in Hep-G2 cells by DON. Cells were treated with 1 or 10 µM for the indicated time periods. For control, cells were incubated with vehicle. Results are representative for three independent experiments.

blot. Strength and time-course of activation differed between the three factors under study. DON treatments resulted in rapid and strong activation of JNK1/2 and p38, and a long-term activation of the ERK1/2 was observed, particularly upon treatment with a high dose of DON (for details see Fig. 4). Both, JNK1/2 and p38 MAPK showed early peak activities 30 min after stimulation, whereas ERK1/2 activity was present for at least up to 24 h. The increase in activity of ERK1/2 did not result from an increase in expression of ERK1/2 protein, as determined by Western blot. At low-DON doses (1 µM), MAPK phosphorylation was also observed with similar kinetics but at lower induction levels. This suggests a dose and time-dependent activity.

3.5 Comparison of the DON-induced transcription factor mRNA expression in Hep-G2 and U937 cells

Both cell lines showed a clear dose-dependent effect on mRNA expression of the transcription factors assayed (Fig. 5). Overall, incubation with 10 µM DON resulted in higher induction of gene expression than low-dose experiments. Nevertheless, each cell line exhibited individual responses and reaction patterns, depending on toxin concentration and incubation time with DON. Due to the higher susceptibility of the U937 cells, no meaningful data could be collected after 24 h of high-dose treatment, total RNA amounts of the cells decreased to 30% compared to the negative control (data not shown).

Over the incubation time (3 and 24 h) and depending on the toxin concentrations (DON 1 and 10 µM), the modulation of transcription factor mRNA expression showed significant differences between the two cell lines ($p < 0.01$). Significance levels for individual genes under study with respect to treatment duration and applied toxin concentrations are indicated in Figs. 5a–h. Most prominent alterations were detected for JUN and FOS mRNA levels. Significant differences were observed for time-dependent JUN expression in the low- and high-dosed experiments comparing both cell lines. For FOS and ATF3 targets, both cell lines showed significant concentration-dependent expression at the 3 h measuring point. The expression of EGR1 in U937 was partly decreased and significantly different from that in Hep-G2 cells.

3.6 Transcription factor protein activation in Hep-G2 and U937 cells upon DON treatment

To determine treatment effects on level of target proteins, Western blot analyses were performed. Hep-G2 and U937 cells were incubated for 10 and 30 min as well as 3 and 24 h with either 1 or 10 µM of toxin, respectively. The β-actin protein served as the loading control and showed unaltered expression upon DON treatment. Occurrence of band patterns typical for the induction of activated protein increased in a time and concentration-dependent manner (Fig. 6). Transcription factor JUN was induced in both cell lines and at both treatment regimes. The highest levels of the transcription factor JUN was usually found after 3 h of DON exposure. Phosphorylated JUN was detected in Hep-G2 cells after 30 min of DON exposure and then consistently onward. To verify the activation of JUN, the translocation of the phosphorylated transcription factor to the nucleus was studied by immunofluorescence in Hep-G2 cells during DON-challenge. Translocation was shown after treatment with DON for 3 h and lasted for at least 24 h as shown in Fig. 7. In U937 cells, phosphorylated JUN was already detected after 10 min of treatment with the high-cytotoxic dose but could not be found after 24 h (Fig. 6). Similar results were obtained for the activation of EGR1 after low-dose treatment whereas in the high-dose experiments EGR1

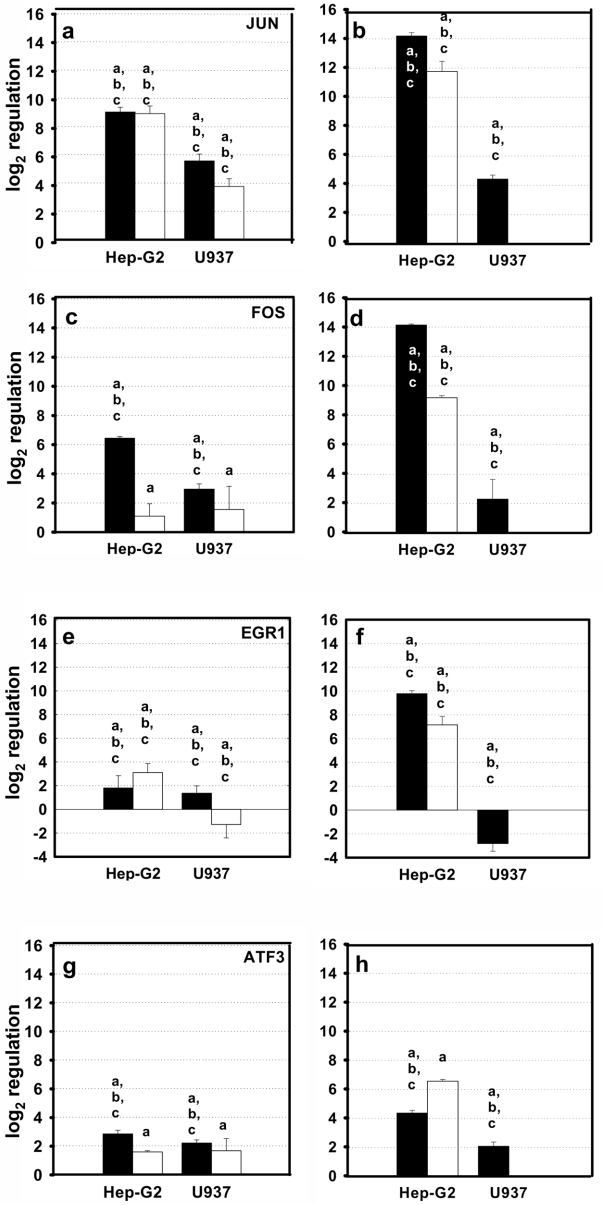

Figure 5. (a – h) Relative mRNA expression of transcription factors in human cell lines. Left row (graph a, c, e, g): cells were treated with 1 μM DON for 3 (black bars) and 24 h (white bars); right row (graph b, d, f, h): cells were treated with 10 μM DON for either 3 or 24 h. All samples were assayed in quadruplicates and data are depicted as means ± SD. Expression increases and decreases are significantly different in all experimental set-ups compared to untreated control cell with $p < 0.05$. Results of multiway ANOVA are indicated by characters: *a* = significant differences between cell lines and overall transcription factor expression ($p < 0.01$), *b* = significant differences between cell lines and specific transcription factor ($p < 0.01$); *c* = significant difference comparing each cell line to each transcription factor ($p > 0.01$).

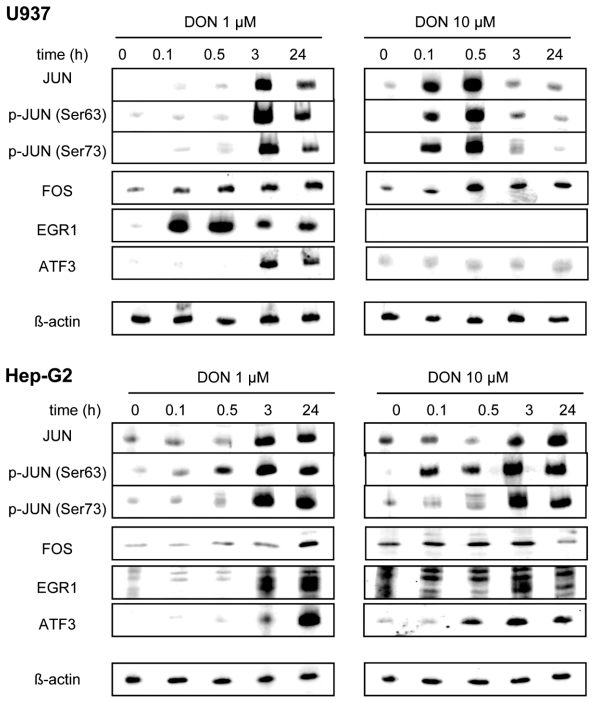

Figure 6. Western blot analyses of the DON-induced cell line-specific induction and phosphorylation of transcription factors. Cells were treated with DON (1 or 10 μM) for different time periods (0.1 – 24 h); Hep-G2 cells – upper panel; U937 cells – lower panel. Phosphorylation of JUN was determined by using antibodies specific for phosphorylated serine at position 63 or 73.

expression was not observed at all in the U937 cells. A constant and continuous expression of ATF3 was found in the DON-treated Hep-G2 cells but not in the U937 cells exposed to high-toxin concentrations. In addition, cell-related transcription factor expression of overall Western blot data was analysed. Comparison of cell response patterns after low- and high-dose treatment revealed a higher degree of transcription factor expression similarities in Hep-G2 cells (83%) than that observed in U937 (41.7% similarity). Finally, after Hep-G2 and U937 cells were treated with the high-DON dose, they showed an overall dissimilar expression pattern for all transcription factors under study. Remarkably, a considerable accordance in protein induction kinetics of 66.7% was found when comparing low dose-treated U937 with high dose-treated Hep-G2.

4 Discussion

DON is frequently found in high concentrations in cereal-based products and represents one of the most abundant mycotoxin in food [10]. As a consequence, levels of human exposure can be high. For instance, in a recent study DON was detected in 98.7% of urine samples from adults in the United Kingdom [11]. In a recent study, pigs fed with low concentrations of DON showed toxin levels of up to 0.5 and 0.02 μM in bile and liver, respectively [15]. These levels

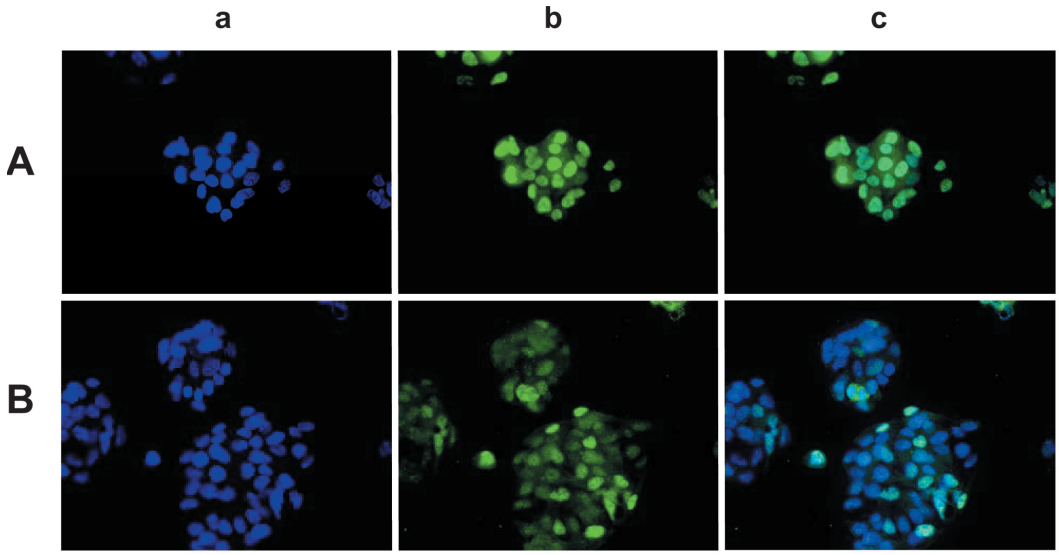

Figure 7. Effect of DON on JUN-translocation. Cells were challenged with 1 μM DON (row A) or vehicle (row B) for 24 h. Nuclei were counterstained with DAPI (column a); phospho-JUN was detected by antibody (phospho-c-JUN (Ser73); column b) and an Alexa488 labelled secondary antibody. Merge picture (column c) demonstrates that after DON treatment phosphorylated JUN is translocated to the nucleus (Ac) while in vehicle treated cells (Bc) only traces of JUN were found.

are clearly below the 1 μM dose used in this study, which therefore may reflect worst case intake scenario rather than mean exposure. The primary organ involved in metabolism of food-borne contaminants is the liver. Two recent studies showed that DON influences several basic metabolic parameters in rat liver cells and primary human hepatocytes. Main focus of these reports was laid on the final cell fate [27, 28]. The present study was predominately designed to evaluate the early DON effects in human hepatoma cells on mRNA and protein level. The human hepatoma cell line, Hep-G2, has been reported to retain many properties of primary liver cells, including metabolic activities [49, 50], and was therefore chosen as an *in vitro* model in the present study.

Knowledge on DON-induced modulation of signalling pathways is substantially derived from studies on murine macrophage lines and lymphoid tissues (for a review see Pestka and Smolinski [12]). So far, the cell transcriptional responses of liver cells to DON have not been studied despite recent reports on mild hepatotoxic effects of this mycotoxin [25, 28]. Therefore, gene expression of toxin-treated hepatoma cells was screened by microarray in a first orientating study.

The results obtained are in good accordance with previously published data on the gene expression profile in the spleen of DON-exposed mice identifying IEGs as potential targets of DON [43]. Comparison of low- and high-dose treatment results further indicate that primary activation of transcription tends to switch into negative regulation upon increased toxin concentration (details not shown) which may represent a negative feedback to the translational inhibitory effects of DON at higher concentrations (>3.3 μM) [31]. These inhibitory effects on transcriptional level became particularly obvious in the high dose 24 h treatment of U937 cells resulting in a reduction of total RNA levels down to 30% in comparison to vehicle controls. However, at inhibitory and subinhibitory DON concentrations, up-regulation of genes was the predominant effect. Data analysis by the software tool BiNGO revealed that the most prominent alterations in Hep-G2 cells upon DON treatment occur on the level of nuclear transcription factors (Fig. 2).

Under the microarray conditions the most prominent increase in mRNA expression was detected for the transcription factors JUN and FOS which, in liver, represent IEGs induced in regenerating cells [51]. Recently, it was reported that JUN also mediates hepatocyte survival during acute hepatitis [52]. Additionally, the microarrays also showed an increase of further genes, amongst others EGR1 and ATF3. Both factors are known to be induced in the liver by stress signals [46]. Therefore, further studies focussed on these transcription factors and the array data could be confirmed by means of real-time RT-PCR. In addition, analyses of whole cell extracts revealed that induction takes place not only on mRNA but also on protein level.

Activation of MAPKs due to phosphorylation is one main up-stream event triggering IEGs. These IEGs are known to act as transcription factors on distinct target genes, thus regulating cell differentiation, proliferation and apoptosis [53, 54]. Concerning cell activation pathways in general, it has been shown that depending on the cell type, toxicant exposure results in the disturbance of JNK, p38 MAPK and ERK signalling [31, 55 – 58]. Activation of MAPKs causes expression of IEGs which couple short-term signals to long-term cellular responses by acting as transcription factors and

regulating the expression of target genes [59, 60]. Earlier studies showed that DON is able to induce MAPK-pathways and IEGs *in vivo* as well as *in vitro via* a mechanism known as 'ribotoxic stress response' [40, 43, 61]. These studies, which were often confined to one cell type and/or one particular toxin concentration, were mostly carried out in lymphoid tissue and immune competent cells, and focused on the capacity of DON to modulate immune responses including cytokine induction and apoptosis [22, 32, 62 – 64]. In the present study, MAPK and transcription factor activation were shown both in Hep-G2 and U937 cells whereby the most striking result was the sustained activation and markedly elevated levels of phosphorylated ERK1/2, particularly upon treatment of Hep-G2 cells with high-DON doses (Fig. 5). These findings are in accordance with observations on murine cell lines [65, 66], murine lymphoid tissues [38, 40, 43] and more recently with human intestinal epithelial cells such as CaCo-2 and intestine 407 [22, 23]. In contrast, only a transient activation of the JNK1/2 as well as p38 MAP kinase cascade was observed both in Hep-G2 and U937 cells. Similar results have been described for human T-cells (Jurkat) and epithelial intestine 407 cells [17, 21]. These results are in good accordance with findings in Hep-G2 cells under DON exposure showing no caspase-3-activity as indicator for p38 activity and apoptosis below 50 μM toxin treatment [27, 28].

However, the JUN protein which represents the prototypic target of the JNK kinase, exhibits a prolonged phosphorylation and significant elevated expression upon DON treatment. Reactivity of the expressed protein with phosphospecific antibodies in immunoblots and verification of the translocation to the nucleus *via* immunofluorescence confirmed the activation of the transcription factor. Recently, Hasselblatt *et al*. [67] showed that, in humans, hepatocytes strongly express JUN in response to acute liver injuries suggesting a link between JUN expression and hepatic stress response. In murine macrophages and spleen, DON-induced activation of c-Jun has been shown by electrophoretic mobility shift assays [38, 66, 68] and increased c-Jun mRNA expression after DON exposition has been reported. In murine spleen an 85-fold increase of c-Jun mRNA expression was found by Kinser *et al*. [43] which corresponds to the levels detected in our study in human monocytes (U937). In contrast, DON treatment of hepatoma cells resulted in induction levels of >1000 emphasizing the characteristic response profile of this cell type. Similar results were obtained for FOS mRNA which was also significantly higher induced in Hep-G2 cells than in U937. A strong induction of FOS mRNA expression was seen as well by Kinser *et al*. [43] in murine spleen after feeding the mice with DON. According to Murphy *et al*. [69], FOS becomes stabilized by sustained ERK signalling, a cellular response which was particular pronounced in the Hep-G2 cells. Activation of the ERK pathway also mediates the production of EGR1 which results in the up-regulation of proinflammatory IL-8 [22]. Interestingly, an inverse relation was observed in our study between the level of DON-induced expression of transcription factors and the susceptibility of the cell lines towards the toxin. According to the results of the WST-1 test, Hep-G2 cells are less sensitive than U937 but were characterized by significantly higher expression of transcription factors (Fig. 5) suggesting that some of the target molecules are involved in cell repairing processes. Dichotomy between susceptibility and reactivity of two responder cell lines is underlined by results of the Western blot where the reaction pattern of the U937 cells tended to be more distinct from that of the Hep-G2 cells upon different treatments. The main focus was for the first time, to analyse the known actions of DON in sensitive immune competent cells (U937) compared to hepatoma cells.

One of the transcription factors under study was stress-inducible ATF3 – a member of the ATF/CREB family of basic leucine zipper type transcription factors – known to regulate several downstream activities related to cell proliferation [70], apoptosis [71] and survival [72]. Regulation of the ATF3 expression by the MAPK-pathway has been shown in previous studies [44, 51, 73]. Upon DON challenges, our data demonstrate that ATF3 mRNA expression depends on DON-treatment conditions and is influenced by cell type. In U937 cells, DON modestly activated ATF3 in comparison to Hep-G2 cells. Maximum activation occurred after 3 h. One can further speculate that ATF3 might be involved in the biological outcome of DON hepatotoxicity as this protein is known to be present during liver regeneration [46]. Transgenic mice over-expressing ATF3 in the liver had symptoms of liver dysfunction such as defects in glucose homeostasis and low levels of glycogen storage. It was further shown that Hep-G2 cells over-expressing ATF-3 reveal decreased promoter activity for phosphoenolpyruvate carboxykinase (PEPCK), which is known to be involved in reduction of gluconeogenesis [74]. Interestingly, *in vivo* feeding experiments with diets containing DON and ZON resulted in early acute pathological changes in liver cells, including glycogen reduction [25].

In summary, this study shows for the first time that on a molecular level the mild hepatotoxic effects of DON, shown in recent studies [25, 28], are probably caused by the induction of transcription factors which are known to be associated with injury-induced liver regeneration processes and stress response of the cell. Interestingly, cells treated with an equi-toxic dose of the type A trichothecene T2-toxin showed a markedly lower induction rate (results not shown) suggesting a DON-specific response of the cells under study. Particularly, the sustained activation of JUN and the prolonged expression of ATF3 may represent important modules of the cell response of hepatocytes to DON treatment. Recent studies demonstrating a high DON exposure risk of the population [11] and the remarkable high susceptibility of primary hepatocytes found in *in vitro* studies [27] underline the need for further research on hepatotoxicity of DON.

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