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Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells

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ABSTRACT

The newly emerging cyanotoxin cylindrospermopsin (CYN) is showing genotoxic effects in a range of test systems. However, the knowledge on the mechanisms involved is limited. To get insight into the cellular responses to CYN a toxicogenomic analysis of selected genes commonly affected by genotoxic stress was performed on HepG2 cells exposed to a non-cytotoxic but genotoxic concentration of CYN (0.5 μg/ml for 12 and 24 h). CYN increased expression of the immediate-early response genes from the *FOS* and *JUN* gene families and there was strong evidence for the involvement of P53 and NF-κB signaling. Strong up-regulation of the growth arrest and DNA damage inducible genes (*GADD45A* and *GADD45B*), cyclin-dependent kinase inhibitors (*CDKN1A* and *CDKN2B*), checkpoint kinase 1 (*CHEK1*), and genes involved in DNA damage repair (*XPC*, *ERCC4* and others) indicated cell-cycle arrest and induction of nucleotide excision and double strand break repair. Up-regulation of metabolic enzyme genes provided evidence for the involvement of phase I (*CYP1A1*, *CYP1B1*, *ALDH1A2* and *CES2*) and phase II (*UGT1A6*, *UGT1A1*, *NAT1* and *GSTM3*) enzymes in the detoxification response and potential activation of CYN. The obtained transcriptional patterns after exposure of HepG2 cells to CYN provide valuable new information on the cellular response to CYN.

1. Introduction

The cyanobacterial toxin cylindrospermopsin (CYN), a potent protein synthesis inhibitor (Froscio et al., 2003; Liang et al., 1999; Runnegar et al., 2002), is increasingly being found in freshwater bodies infested by cyanobacterial blooms worldwide, and was reported to be implicated in human intoxications and animal mortality (Carmichael et al., 2001; Froscio et al., 2001; Hawkins et al., 1985). Evidence for its genotoxic activity and carcinogenic potential is accumulating and was summarized in the review by (Žegura et al. (2011b)). At present, CYN is being included in the revision of the WHO "Guidelines for Drinking-water Quality, chemical hazards in drinking-water" and the US Environmental Protection Agency (EPA) has classified it on the list of compounds with highest priority for hazard characterization (EPA, 2010).

The main target of CYN is the liver, but other organs can also be affected, suggesting it is a general cytotoxin (Falconer et al., 1999; Hawkins et al., 1985; Humpage and Falconer, 2003; Terao et al., 1994). Structurally it is a stable tricyclic alkaloid (415 Da) with several potential sites for reactivity that may form protein and DNA adducts. The later have been identified *in vivo* (Shaw et al., 2000). It contains uracil that could potentially interact with adenine groups in RNA and DNA. This suggests that CYN is a potential genotoxic carcinogen, acting through DNA synthesis interference and induction of mutations (Falconer and Humpage, 2001). CYN was shown to be genotoxic in a range of test systems *in vitro* (Bazin et al., 2010b; Humpage et al., 2000, 2005; Štraser et al., 2011; Žegura et al., 2011a) and *in vivo* (Bazin et al., 2010a; Shaw et al., 2000; Shen et al., 2002) and several studies demonstrated that CYN needs to be activated by cytochrome P450 (CYP450) enzymes to become genotoxic (Bazin et al., 2010b; Humpage et al., 2005; Štraser et al., 2011; Žegura et al., 2011a). Moreover, preliminary data has shown it could have tumor-initiating activity (Falconer and Humpage, 2001) and although there has been an indication of CYN carcinogenicity for humans (Falconer, unpublished data), the studies on CYN carcinogenic activity are scarce and the mechanisms involved are not well understood.

Toxicogenomic approaches represent a promising tool to elucidate mechanisms of toxicity and are becoming increasingly used for hazard identification and risk assessment of genotoxic and

Abbreviations: AP-1, activator protein; BER, base excision repair; CDK, cyclin-dependant kinase; CDKI, cyclin-dependant kinase inhibitor; CYN, cylindrospermopsin; CYP450, cytochrome P450; DSB, DNA double-strand break; ER, endoplasmic reticulum; GST, glutathione S-transferase; HPBLs, human peripheral blood lymphocytes; HRR, homologous recombination repair; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, none-homologous end joining; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; TGF-B, transforming growth factor B; TNFα, tumor necrosis factor α.

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carcinogenic properties of different substances (Ellinger-Ziegelbauer et al., 2009; Thybaud et al., 2007). It was shown that using genomic signatures it is also possible to identify carcinogens and identify cellular pathways affected by genotoxic and non-genotoxic carcinogens *in vivo* (Ellinger-Ziegelbauer et al., 2005). The aim of this study was to investigate CYN induced changes in the expression of selected genes from pathways, involved in (i) the immediate-early response and signaling, (ii) regulation of cell-cycle and cell-proliferation, (iii) DNA damage repair (iv) apoptosis and cell survival, and (v) metabolism and detoxification. The gene expression analysis was performed with the metabolically active human hepatoma HepG2 cells, exposed to CYN, using custom quantitative PCR (qPCR) arrays.

2. Materials and methods

2.1. Chemicals

Cylindrospermopsin (CYN) was purchased from Enzo Life Sciences GmbH, Lausen, Switzerland. A 0.5 mg/ml stock solution of CYN was prepared in 50% methanol. William's medium E and glycogen were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, fetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Trypsin was from BD-Difco, Le Pont-De-Claix Cedex, France. TRIzol reagent and DNaseI amplification grade were from Invitrogen, Paisley, Scotland, UK. cDNA High Capacity Archive Kit, Power SYBR Green PCR Master Mix and Nuclease-free Water (not DEPC-treated), were from Applied Biosystems, New Jersey, USA. Custom human qPCR-arrays, StellARrays™, were from Lonza, Basel, Switzerland. All other chemical reagents were of the purest grade available. All solutions needed for RNA isolation, DNase digestion, transcription to cDNA and the master mix were prepared in RNase-free water (not DEPC-treated).

2.2. Cell culture and treatment protocol

The metabolically active human hepatoma cell line, HepG2, has retained inducibility and activities of several phase I and phase II xenobiotic metabolising enzymes and it has been shown that several classes of indirect acting genotoxic agents can be detected with this cell line (Knasmüller et al., 2004). HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cell line was tested for mycoplasma, and was confirmed to be negative. The cells were grown at 37 °C and 5% CO_2 in William's medium E, containing 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 10^6 cells/flask and incubated for 24 h at 37 °C and 5% CO_2. They were washed with 1× PBS, exposed to 0.5 μg/ml CYN and incubated for 12 and 24 h. In each experiment a vehicle control (0.05% methanol) was included. We previously showed that at the exposure conditions selected for this study CYN was not cytotoxic and induced DNA damage (Štraser et al., 2011).

2.3. Total RNA isolation

After the incubation, cells were washed twice with 1× PBS and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications. Glycogen (20 μg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at −20 °C to precipitate. Purified RNA was stored at −80 °C until analysis.

2.4. DNase digestion and RNA quality and quantity assessment

The isolated RNA was treated with modified DNase I, amplification grade, according to the manufacturer's protocol, to digest possible contaminating genomic DNA. The concentration and purity of total RNA were measured by spectroscopy using NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). RNA integrity was checked with agarose gel electrophoresis (Fig. 1) before gene expression analysis. Total RNA was isolated in 4 parallels for each time point, 3 parallels with purest RNA of highest quality were chosen for further analysis.

2.5. cDNA synthesis

The RNA was transcribed to cDNA using 1.5 μg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. The cDNA synthesis reactions were stored at −20 °C until further analysis.

2.6. Gene selection

A total of 190 genes representing key molecules from selected pathways and functional processes that are known to be activated in response to DNA damage and cell stress, and were expected to be expressed in HepG2 cells, were selected (summarized in supplementary data). The necessary information for selection of the genes was obtained from searching several databases, including NCBI gene, Pubmed and SwissProt, among others. 18S rRNA and *GAPDH* were selected as internal reference genes (normalizer genes). Other normalizer genes were selected from the test genes after the analysis (see Section 2.8).

2.7. Real-time quantitative PCR (qPCR) reaction

Gene expression was analyzed using custom human 384 well qPCR -arrays (StellARrays™) and qPCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). PCR reaction mixtures were prepared with Power SYBR Green master mix, nuclease-free water (non-DEPC treated) and the template c-DNA. Reagent set-up volumes were calculated according to the manufactures protocol. The conditions for the qPCR were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s following 60 °C for 1 min, and for the dissociation step 95 °C for 15 s, 60 for 15 s and 95 °C for 15 s.

2.8. Data analysis

For the evaluation of the amplification curves the threshold value for all arrays was set to 0.15. The data was analyzed based on the ΔΔCt method. For both time points 10 normalizer genes (reference genes) were selected using the GPR software, provided by the manufacturer. Statistical significance was calculated by the two tailed Student's *t*-test and the criteria for significance were $p < 0.05$ and absolute ΔΔCt (fold-change) >1.5 Independent experiments were repeated three times.

3. Results and discussion

The objective of this study was to determine which cellular pathways are affected by the cyanobacterial toxin CYN and thereby obtain insight into the mechanisms of CYN genotoxicity and potential carcinogenicity. The investigation was focused on changes in the expression of genes commonly affected by genotoxic stress (genes involved in the immediate-early response, signaling, cell cycle and proliferation, DNA damage repair, apoptosis and cell