The role of the enzymatic antioxidant system in cylindrospermopsin-induced toxicity in human lymphocytes

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ABSTRACT

Cylindrospermopsin (CYN) is known to induce cytotoxic effects in eukaryotic cells although the exact mechanism underlying these alterations is not fully explained. Given that CYN was previously found to decrease the proliferation of human lymphocytes through DNA damage and cell cycle arrest followed by an increase in the apoptotic rate, the present study evaluated the possible involvement of reactive oxygen species (ROS) and oxidative stress in these cytopathic responses. The status of enzymatic antioxidants: superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) as well as level of lipid peroxidation (LO) under CYN influence in human lymphocytes were also studied. It was found that CYN exposure (0.01–1.0 µg/ml) induces a concentration-dependent increase in H_2O_2 content within a time as short as 0.5 h, reaching its maximum level after 3 and 6 h. The highest H_2O_2 content was accompanied by a significant decrease of SOD and CAT activity and an elevated level of GPx. Moreover, CYN treatment resulted in a detectable increase in LO. We conclude that ROS and the products of LO play an essential role in CYN-induced toxicity in human lymphocytes. Our study helps to elucidate the sequence of events caused by CYN in eukaryotic cells and explain the background for previously observed cytopathic responses.

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1. Introduction

The toxicity of cylindrospermopsin (CYN), a cyanobacterial metabolite of freshwater origin, has been extensively studied in recent decades. This sulfate ester of a tricyclic guanidine substituted with a hydroxymethyluracil is currently being identified worldwide; in Antarctica, Australia, South and North America as well as Europe (Poniedziałek et al., 2012a; Kleinteich et al., 2014; Rzymski and Poniedziałek, 2014). Over ten different species, members of the genera *Aphanizomenon*, *Anabaena*, *Cylindrospermopsis*, *Oscillatoria, Raphidopsis* and *Umezakia*, have been implicated in the production of CYN (Rzymski and Poniedziałek, 2014). Contrary to other metabolites produced by Cyanobacteria, CYN appears to be actively released from intact cells under specific environmental conditions, particularly phosphorus limitation (Bar-Yosef et al., 2010). It has been demonstrated that CYN can potentially serve as an allelochemical and supply inorganic phosphorus by inducing alkaline phosphatase secretion in sympatric phytoplankton (Bar-Yosef et al., 2010; Rzymski et al., 2014). This, at least partially, explains the relatively high share of extracellular form in the total CYN quota, constituting up to over 90% (Rücker et al., 2007; Bormans et al., 2014). The maximum identified concentrations of CYN in freshwater have exceeded 500 µg/L in Australia (Saker and Eaglesham, 1999) and 100 µg/L in Europe (Messineo et al., 2010) although the most common toxin levels are usually found in the range of 1–10 µg/L (Rücker et al., 2007; Falconer and Humpage, 2006).

The adverse effects of CYN have been broadly documented in both, *in vivo* and *in vitro* studies (Poniedziałek et al., 2012a). In rodents, CYN demonstrated the ability to affect different organs, including the adrenal glands (Hawkins et al., 1985), thymus, heart (Terao et al., 1994), and lungs (Oliveira et al., 2012), as well as act as a dermatitis-inducing agent (Torokne et al., 2001; Stewart et al., 2006). At cellular level, the toxin revealed a variety of cytopathic effects. These include: DNA damage (Štraser et al., 2013), increase in the frequency of micronuclei and nuclear buds (Bazin et al., 2010; Žegura et al., 2011), inhibition of protein synthesis (López-Alonso et al., 2013), cell cycle arrest (Štraser et al., 2013; Poniedziałek et al., 2014a), decreased mitotic indices (Poniedziałek et al., 2012b, 2014a,b) and inducement of apoptosis and necrosis (Gutiérrez-Praena et al., 2012a; López-Alonso et al., 2013; Poniedziałek et al., 2014a, 2015). There are also two documented cases of human poisoning that involve CYN. The first resulted from drinking water from a CYN-contaminated reservoir in Australia (Griffiths and Saker, 2003) while the second was due to the effect of exposure at a renal dialysis center in Brazil and led to the death

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of over 50 patients (Carmichael et al., 2001). Both cases revealed the high degree of hepatotoxicity and nephrotoxicity of this compound. Unfortunately, the real concentrations to which the individuals were exposed were not reported and remain unknown.

The provisional safety level of CYN in drinking water has been set at 1.0 μg/L (Humpage and Falconer, 2003), which is lower than the usual concentration found worldwide in surface waters. CYN demonstrates high stability in visible and UV light, under anoxic conditions and over a wide range of pH and temperature changes (Chiswell et al., 1999; Wörmer et al., 2010; Klitzke and Fastner, 2012). Taking all these circumstances into account, the risk of human exposure can be potentially much higher than for any other known cyanotoxin (Rzymski et al., 2011). This, on the other hand, highlights the continuous necessity for the systematic monitoring of CYN in water bodies. However, in many countries due to the lack of a legal framework to regulate its presence in drinking and recreational waters (e.g., in Europe), CYN identification is still regarded more as a subject for scientific investigation than part of thorough monitoring programme (Rzymski and Poniedziałek, 2014).

One of the main routes of postulated toxicity of CYN is through an increase in the production of reactive oxygen species (ROS). These include oxygen ions, free radicals (superoxide and hydroxyl radicals), and peroxides (hydrogen peroxide) and represent the products of normal oxygen consuming metabolic processes. Cell stress can, however, lead to greatly elevated ROS levels and, due to their highly reactive nature, modifications of proteins and lipids, a phenomenon known as oxidative stress (Halliwell and Gutteridge, 2007). An excess of ROS can eventually lead to cell necrosis or DNA damage and consequently, apoptosis (Roos and Kaina, 2006). A CYN-triggered increase in ROS has already been observed in different eukaryotic cells but the exact sequence of events that lead to oxidative stress requires further elucidation (Gutiérrez-Praena et al., 2011, 2012a, 2012b).

In our previous studies, CYN immunomodulatory potencies have been demonstrated using cells derived from healthy subjects. The toxin has been found to significantly alter the function of neutrophils (Poniedziałek et al., 2014b) and suppress the viability of lymphocytes (Poniedziałek et al., 2012b, 2014a). The main cytopathic response observed in the latter included a decrease in the proliferation rate due to cell-cycle arrest, apoptosis and increase in necrosis. In the present study, we aimed to investigate whether these effects were mediated by alterations in the ROS level of human-derived lymphocytes. In order to elucidate the exact routes through which CYN can potentially trigger oxidative stress in human lymphocytes, the activity of three main antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), as well as the level of lipid peroxidation, were studied. Our study extends the body of information on CYN-mediated toxicity in human lymphocytes and possibly, other eukaryotic cells.

2. Material and methods

2.1. Blood collection

Heparinized blood samples (6.0 ml) were collected in lithium heparin tubes from 5 healthy (screened by physical examination, medical history and initial blood tests), non-smoking and normal weighted (BMI 18.5–24.9) donors (age range 19–26 years old; 3 female, 2 male) at the Regional Center of Blood and Blood Treatment in Poznan, Poland, according to accepted safeguard standards and legal requirements.

2.2. Lymphocyte isolation and culturing

Lymphocytes were isolated from blood under sterile conditions by centrifugation (420g, 30 min) on Gradisol-L (Aqua-Med, Poland)

and washed twice in Eagle's medium (Biomed, Poland). The isolated cell suspension (1×10^6 cells/ml) was supplemented with 10% fetal bovine serum (Sigma Chemicals, USA) and antibiotic (gentamycine at 50 μg ml^{-1}, Sigma Chemicals, USA). The purity of the lymphocyte cultures was verified by counting under a light microscope, and by flow cytometry using CD3- and CD19-specific antibodies conjugated with fluorescein isothiocyanate and R-phycoerythrin, respectively (Loken et al., 2000), and exceeded 90%.

Lymphocyte cultures were established in a 96-well microplate (200 μL aliquots/well) and were incubated in a CO_2 incubator under controlled conditions (5% CO_2, temp. 37 °C, humidity 95%). Each culture was prepared in triplicate for further experimental procedures.

2.3. Experimental design

Isolated lymphocytes were stimulated with T-cell specific mitogen, phytohaemagglutinin-L (PHA-L, Roche Diagnostics, Sweden) at a concentration of 2.5 μg/ml. Immediately afterward, samples were exposed to purified CYN (>95%, HPLC; Alexis Biochemicals, USA) isolated from *C. raciborskii*, at three environmentally-relevant concentrations: 0.01, 0.1 and 1.0 μg/ml. In each experiment, a control consisting of lymphocytes stimulated with PHA-L and not treated with CYN was carried out. Two independent experimental sets were designed. In the first, lymphocytes were continuously exposed to CYN for 48 h and ROS production was analyzed at the following time intervals: 0.5, 1, 1.5, 3, 6, 24 and 48 h. This experiment was designed to evaluate whether CYN can trigger oxidative stress in human lymphocytes and to choose the two time intervals in which ROS production reached the highest level. These time intervals were then applied in the subsequent experiment, in which status of enzymatic antioxidants (SOD, GPx and CAT) and lipid peroxidation was investigated. Additionally, in order to ensure that any potential differences in enzymatic activity were not a result of altered lymphocyte densities, cell counts were performed after 3 h and 6 h of incubation in control and CYN-treated samples. Each experimental assay (ROS production, cell counts, SOD, GPx, CAT activities and lipid peroxidation) was performed on 5 independent lymphocyte cultures obtained from blood of 5 different healthy donors.

2.4. Analyses of intracellular ROS level

The intracellular ROS level was evaluated using an assay with dihydrorhodamine (DHR 123, Sigma, USA) which is most readily oxidized to rhodamine 123 (R 123), mainly in the presence of H_2O_2. At different time intervals (0.5–48 h), 10 μL of each sample was suspended in 500 μL of sterile phosphate buffered saline (PBS), gently vortexed and mixed with DHR 123 at a concentration of 0.1 mg/ml. All samples were then incubated in a CO_2 incubator under controlled conditions (5% CO_2, 37 °C, 95% humidity) for 5 min to allow the conversion of non-fluorescent DHR 123 to fluorescent R 123 in the presence of intracellular ROS. The samples were then measured using flow cytometry (CyFlow Space, Partec GmbH, Germany) with a 488 nm excitation wavelength. Based on signals collected by forward and side scatters, lymphocytes were fractioned by virtue of their size and internal granularity, respectively, and the data were plotted on two-dimensional histograms. For each sample 15,000 lymphocytes were acquired. R 123 fluorescence was measured at 515–548 nm, within the green band of the spectrum, with a logarithmic amplification of the signal. Fluorescence was given in Mean Fluorescence Intensity (MFI) units and for CYN-exposed samples it was recalculated as a percentage of the control.

2.5. Analyses of cell counts

Absolute cell counts were performed using flow cytometry. After 3 and 6 h of incubation, 10 μL of each sample was suspended