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Citrinin-induced fluidization of the plasma membrane of the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

Citrinin (CTN) is a toxic fungal metabolite that is a hazardous contaminant of foods and feeds. In the present study, its acute toxicity and effects on the plasma membrane of *Schizosaccharomyces pombe* were investigated. The minimum inhibitory concentration of CTN against the yeast cells proved to be 500 μM. Treatment with 0, 250, 500 or 1000 μM CTN for 60 min resulted in a 0%, 2%, 21% or 100% decrease, respectively, in the survival rate of the cell population. Treatment of cells with 0, 100, 500 or 1000 μM CTN for 20 min induced decrease in the phase-transition temperature of the 5-doxylstearic acid-labeled plasma membrane to 16.51, 16.04, 14.18 or 13.98 °C, respectively as measured by electron paramagnetic resonance spectroscopy. This perturbation was accompanied by the efflux of essential K^+ from the cells. The existence of an interaction between CTN and glutathione was detected for the first time by spectrofluorometry. Our observations may suggest a direct interaction of CTN with the free sulfhydryl groups of the integral proteins of the plasma membrane, leading to dose-dependent membrane fluidization. The change in fluidity disturbed the ionic homeostasis, contributing to the death of the cells, which is a novel aspect of CTN cytotoxicity.

1. Introduction

Citrinin (CTN), a secondary product of the fungal metabolism of the genera *Penicillium* (*P.citrinum*, *P. expansum*, *P. verrucosum* and *P.camamberti*), *Aspergillus* (*A.terreus* and *A.niveus*) and *Monascus* (*M.ruber* and *M.purpureus*), often occurs in naturally contaminated commodities, such as corn, wheat, rye, barley, apples and other foods. Besides its hepato- and nephrotoxicity, it is embryocidal and fetotoxic. It is cytotoxic in a dose- and time-dependent manner to single-cell pro- and eukaryotes and tissue cultures (for reviews, see Bennett and Klich, 2003; Xu et al., 2006; Flajs and Peraica, 2009; Shi and Pan, 2012; EFSA, 2012).

Results relating to acute toxicity to renal cortical and liver mitochondrial swelling suggested that CTN interfered with the mitochondrial membrane fluidity by decreasing the transmembrane potential (Chagas et al., 1992a, 1992b, 1995). Genesan et al. (1979) demonstrated that the membrane of liposomes carrying a net positive charge suffered a high degree of damage upon CTN treatment, leading to the leakage out of 45% of trapped Ca^{2+}. In contrast with amphotericin B (which forms addition complexes with the sterols of the plasma membrane), 500 μM CTN did not induce the leakage of intracellular phenol–sulfuric acid-positive substances and materials adsorbing at 260 nm from the cells of the budding yeast *Saccharomyces cerevisiae*, which may suggest the non-occurrence of disorganization of the plasma membrane in CTN-treated cells (Haraguchi et al., 1987). In a suspension of renal proximal tubules, CTN caused a 2-fold increase in the lipid peroxidation of the membranes; however, deferoxamine (which prevents ion-mediated lipid peroxidation) did not afford protection from CTN-induced cell death (Aleo et al., 1991). These partly controversial data suggested the interaction of CTN with the plasma membranes, but a direct *in vivo* CTN–membrane interaction such as that in the case of nucleophilic patulin (PAT) has not yet been demonstrated. PAT induces the dose-dependent fluidization of

Abbreviations: $2A'_{zz}$, hyperfine splitting constant; 5-SASL, 5-doxylstearic acid; CTN, citrinin; EPR, electron paramagnetic resonance; GSH, glutathione; MIC, minimum inhibitory concentration; OD, optical density; PAT, patulin; ROS, reactive oxygen species; *S*, order parameter; SM, minimal liquid medium; *S. pombe*, *Schizosaccharomyces pombe*.

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the plasma membrane via modification of the membrane-bound protein structure and function (Fliege and Metzler, 1999, 2000a, 2000b; Horvath et al., 2010, 2012). However, no information is available concerning interactions between CTN and molecules containing sulfhydryl (–SH) groups [e.g. glutathione (GSH)].

The CTN-induced overall accumulation of reactive oxygen species (ROS) has been proved in acute tests of Swiss albino mouse skin (Kumar et al., 2011) and human hepatocarcinoma HepG2 cells (Chen and Chan, 2009), resulting in a number of ROS-induced modifications of the cell processes, including as altered concentration of GSH, an elevated level of lipid peroxidation and apoptosis. ROS can cause oxidative damage both in polyunsaturated fatty acids and, to a lower extent, in monounsaturated fatty acids in biological membranes through a chain reaction process known as lipid peroxidation (Kunimoto et al., 1981; Krasowska et al., 2002). In this way, ROS may induce a disturbance of the plasma membrane fine structures, with alterations in its integrity, fluidity and permeability (Greenberg et al., 2008). Treatment of cells of the yeast *Yarrowia lipolytica* with linolenic acid hydroperoxide, a product of C18:3 peroxidation, increased the membrane fluidity in a concentration dependent-manner, as measured via fluorescence generalized polarization (Thanh et al., 2007). In contrast with this finding, the exposure of rats to oral CTN treatment (20 mg kg^{-1} b.w. for 2 d) did not increase the concentration of malondialdehyde, a parameter of lipid peroxidation in the kidney and liver (Flajs and Peraica, 2009). As regards GSH, which is one of the main targets of PAT, both increased and decreased levels have been reported in CTN-treated cells (Johannessen et al., 2006; Kumar et al., 2011).

CTN may also exert effects on the plasma membrane through the time-dependent, irreversible inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, thereby disturbing the synthesis of the ergosterol/cholesterol/testosterone system and leading to hypocholesterolemia (Endo and Kuroda, 1976; Tanzawa et al., 1977; Liu et al., 2013).

The aims of the present study were to investigate the effects of CTN on the plasma membrane, to determine the consequences of alterations in the barrier function of the membrane through examinations of the eukaryotic, haploid, petite-negative, ergosterol-producing well-characterized strain of the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) in electron paramagnetic resonance (EPR) spectroscopy experiments, and to investigate the possibility of molecular interaction between CTN and GSH.

2. Materials and methods

2.1. Strain, culturing conditions, determination of minimum inhibitory concentration (MIC) and survival rate

The uracil auxotrophic (ura4-D18) heterothallic (h^-) *S. pombe* strain was used in all experiments (Grimm et al., 1988; Horvath et al., 2010; Papp et al., 2012). Minimal liquid medium (SM) containing glucose 1%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.05%, $MgSO_4$ 0.01%, and Wickerham vitamin solution 0.001% (Spencer and Spencer, 1996) with 100 mg l^{-1} uracil was used for cultivation at pH 4.5. Overnight preculturing of the cells in the SM medium resulted in an exponential-phase culture, which was washed twice with 0.9 M NaCl by centrifugation (1017 g, 5 min). This cell suspension was used to prepare mid-log-phase cultures (15 h) in a 3.33-Hz incubator shaker at 30 °C, with a starting cell number of 10^6 cells ml^{-1} (optical density OD_{595nm} = 0.05). The generation time was monitored according to Iwahashi et al. (2006). The stock solution of CTN (250 mM) was prepared in acetonitrile according to Murillo et al. (2008). The MIC of *S. pombe* was determined by the standard microdilution method of NCCLS M27-A (NCCLS, 2002; Papp et al., 2012). Rates of cell survival were estimated according to Lee et al. (1995). All experiments were repeated three times.

2.2. Measurement of interaction between GSH and CTN

To investigate the interaction of GSH with CTN, 0.1 mM CTN and 10 mM GSH stock solutions were prepared in phosphate buffer (pH 8.0). The CTN was then mixed with the GSH in the required concentration ratios, and photoluminescence signals were recorded as in previous work (Poór et al., 2012, 2013; Virág et al., 2010, 2012). The fluorescence of GSH observed at 425 nm after excitation at 315 nm was found to be responsible for the interaction of GSH with other bioactive molecules. Since CTN displays considerable exciting emission at 315 nm, the concentration dependence of CTN emission in buffer was first determined by using an excitation wavelength 315 nm. The emission spectra of CTN solutions up to 20 μM exhibited peaks with similar intensity to those of GSH emission at 405 nm. Thus, to investigate the GSH–CTN interaction, the GSH concentration was kept constant (0.1 mM) in mixtures with 0, 1, 3, 5, 7, 9, 11, 13, 15, 17 or 19 μM CTN. The steady-state photoluminescence spectra were recorded on a Fluorolog τ3 spectrofluorometer (Jobin–Yvon/SPEX, Longjumeau, France). For data collection, a photon-counting method with an integration time of 0.2 s was used. The excitation and emission bandwidths were set to 1 nm. Right-angle detection was used to eliminate the inner-filter effect. The measurements were performed at room temperature. An excitation wavelength of 315 nm was applied to obtain GSH fluorescence in the absence or in the presence of CTN. For converting data DataMax 2.20 software (Jobin–Yvon, Longjumeau, France) was used. HyperQuad program package (Protonic Software, Leeds, Great Britain, 2006) was applied for evaluating binding constants ($\log K$) from fluorescence emission spectra using a 1:1 stoichiometric model. The $\log K$ values were determined as described before on 425 nm wavelength by coulometric titration method (Poór et al., 2013).

2.3. EPR measurements

Mid-log-phase cells were transferred into the osmotically stabilized solution of Trichoderma lysing enzyme (1% (w/v) in 0.6 M KCl at pH 7.0) to obtain cell wall-free protoplasts of vegetative cells and incubated for 30 min at 30 °C with occasional shaking (Farkas et al., 2003). The lack of CTN-induced protoplast lysis was proved by treatment of a suspension of 10^8 protoplasts ml^{-1} with 1000 μM CTN for 240 min, after which the OD was measured at 595 nm.

For EPR measurements, samples containing 10^8 protoplasts ml^{-1} in 0.6 M KCl at pH 5.4 were treated for 20 min with the indicated CTN concentrations and the suspensions were then washed twice by centrifugation. A stock solution (5 mg ml^{-1}) of the probe molecule 5-(4′4-dimethyloxazolidine-N-oxyl)stearic acid, also known as 5-doxylstearic acid (5-SASL), was prepared in ethanol and stored at -18 °C until use. As a control, a 3-μl aliquot of 5-SASL, was added to 500 μl of protoplast suspension, and the mixture was gently shaken for 3 min at room temperature to facilitate spin probe incorporation. The suspension was next sedimented, then resuspended in 100-μl of 0.6 M KCl, placed in a 100-μl capillary tube and centrifuged again at 4 °C. After that, the supernatant was removed. Under these conditions, no isotropic triplet arising from unincorporated spin probe was detected (Pesti et al., 2000).

EPR spectra were recorded with an ESP 300E spectrometer (Bruker BioSpin, Germany) equipped with a diTC 2007 temperature regulator. The accuracy of temperature measurement was ±0.2 °C. The EPR spectra of the membrane-incorporated

Table 1
Analysis of the spectroscopic data. Cells were treated with different concentrations of CTN (0, 100, 500 and 1000 μM) and the membranes were spin-labeled with fatty acid spin probes. EPR spectra were taken at different temperatures in the range from 0 to 30 °C.

Citrinin concentration (μM)	Order parameter[a] (S) at 20 °C	Breakpoint[b] (°C)	RSS_{min} (broken line)	RSS (single straight line)	F-test[c] (P = 0.05)	t-Test[d] (P = 0.05)
0	0.61	16.5	0.395	0.726	5.456	1.1274
100	0.60	16.0	0.746	0.903	1.364	2.148
500	0.55	14.1	3.190	10.83	15.57	4.258
1000	0.54	13.9	0.238	12.51	335.6	12.96

[a] The order parameter was calculated as proposed by Gaffney (1976).
[b] Breakpoints were determined according to Jones and Molitoris (1984). The 95% confidence interval for the crossing points was determined graphically by using the actual *F*-value. The estimated value of the 95% confidence intervals around the breakpoints amounts to 0.2–0.3 °C.
[c] The *F*-test was calculated to compare the fit of a broken line and a single straight line by the residual sum of squares (RSS).
[d] The *t*-test with a degree of freedom of $[n - 4]$ was applied between the slopes of the broken lines to characterize the significance of the regression coefficients.