Toxicon 111 (2016) 100–107

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Regulation of the antioxidant system in cells of the fission yeast *Schizosaccharomyces pombe* after combined treatment with patulin and citrinin

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ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form
10 December 2015
Accepted 29 December 2015
Available online 2 January 2016

Keywords:
Antioxidant enzymes
Citrinin
Fission yeast
Mycotoxin co-occurrence
Patulin
Reactive oxygen species

ABSTRACT

The effects of combined treatment with patulin (PAT) and citrinin (CTN) on *Schizosaccharomyces pombe* cells were investigated in acute toxicity tests. In comparison with the controls the exposure of fission yeast cells (10^7 cells ml^{-1}) to PAT + CTN (250 μM each) for 1 h at a survival rate of 66.6% significantly elevated the concentration of total reactive oxygen species (ROS) via increased levels of peroxides without affecting the concentrations of superoxides or the hydroxyl radical. This treatment induced a 3.08-fold increase in the specific concentration of glutathione and elevated specific activities of catalase and glutathione S-transferase, while at the same time the activity of glutathione reductase decreased. The pattern of the ROS was the same as that induced by CTN (Máté et al., 2014), while the presence of PAT in the PAT + CTN combination treatment modified the activities of the antioxidant system (Papp et al., 2012) in comparison with the individual PAT or CTN treatment, suggesting toxin-specific regulation of glutathione and the enzymes of the antioxidant system and the possibility that the transcription factor (pap1 and atf1) -regulated processes might be influenced directly by ROS.

1. Introduction

The undesirable substances, to which both humans and animals are exposed include the mycotoxins, a group of secondary metabolites of molds, and especially of the *Fusarium*, *Penicillium* and *Aspergillus* genera, which may cause both acute and chronic effects. The hazards of exposure to mycotoxin mixtures are giving rise to increasing concern as they can occur naturally in feed and foodstuffs: certain mold species produce more than one mycotoxin, and the raw materials are generally contaminated by more than one mycotoxin-producing fungal species. The co-occurrence and combined effects of mycotoxins demand a re-evaluation of food safety and risk assessments. It has been demonstrated that certain mycotoxins [including patulin (PAT), citrinin (CTN), zearalenone, ochratoxin A, etc.] can cause an unbalanced redox state of cells via the accumulation of reactive oxygen species (ROS), leading to harmful ROS-induced processes such as lipid peroxidation, cell cycle arrest, DNA damage, apoptosis, etc. Only a few studies have been published in which the interactions of two oxidative stress-inducing mycotoxins were investigated at the level of the antioxidant system (for reviews, see Bennett and Klich, 2003; Speijers and Speijers, 2004; Gajecki et al., 2007; Golli-Bennour and Bacha, 2011; Šegvić Klarić, 2012; Streit et al., 2012).

Martins et al. (2002) revealed the simultaneous occurrence of PAT and CTN in 351 samples of 7 different varieties of apples. *Penicillium expansum* isolates were also shown to co-produce PAT and CTN (Harwig et al., 1973; Ciegler et al., 1977; Andersen et al., 2004). Very few investigations have so far addressed the possible combined effects and cellular processes of PAT and CTN. Ciegler et al. (1977) characterized the teratogenic, embryotoxic effects of PAT and CTN on 4-day-old chicken embryos, but the intracellular

Abbreviations: CAT, catalase; CTN, citrinin; DCFDA, 2′,7′-dichlorofluorescein diacetate; DHR123, dihydrorhodamine 123; EPR, electron paramagnetic resonance; FIC, fractional inhibitory concentration; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; IR, interaction ratio; MIC, minimal inhibitory concentration; OD, optical density; $O_2^{\cdot -}$, superoxide anion radical; $^{\cdot}OH$, hydroxyl radical; PAT, patulin; PBN, N-*tert*-butyl-α-phenylnitrone; ROS, reactive oxygen species; SC, subinhibitory concentration; *S. pombe*, *Schizosaccharomyces pombe*; SOD, superoxide dismutase; SOD_{CuZn}, Cu Zn superoxide dismutase; SOD_{Mn}, Mn superoxide dismutase.

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http://dx.doi.org/10.1016/j.toxicon.2015.12.021
0041-0101/

mechanisms were not examined in detail. Combined treatment with PAT ($IC_{25} = 0.32$ μM) and CTN ($IC_{25} = 52.72$ μM) did not result in either additive or synergistic effects on the proliferation of a bovine macrophage cell line (Oh et al., 2013). However, by applying the porcine renal cell line LLC-PK1, Heussner et al. (2006) confirmed a potential for an interactive (synergistic) effect between CTN and ochratoxin A, and also such a possibility between other mycotoxin combinations (e.g. PAT and CTN). No other data concerning the combined effects of PAT and CTN on eukaryotic cells or tissues have been published to date.

The modes of action of PAT and CTN exhibit certain common aspects. A cell cycle arrest has been observed both in PAT-treated cells and in CTN-treated cells (Saxena et al., 2009; Kumar et al., 2011; Máté et al., 2014): (i) both mycotoxins are capable of inhibiting the polymerization of tubulin dimers (Pfeiffer et al., 1998), (ii) the DNA damage caused by the accumulated ROS blocks the ability of the cell cycle to prevent cellular damage, mutations or apoptosis. The induction of the accumulation of ROS such as the superoxide anion ($O_2^{\cdot -}$), peroxides (H_2O_2, hydroperoxides, etc.) and the hydroxyl radical ($^{\cdot}OH$) by PAT and CTN is a consequence of different processes. PAT inactivates the central antioxidant glutathione (GSH) of all living cells via the formation of a PAT-GSH adduct (Pfeiffer et al., 2005; Mike et al., 2013), leading to the accumulation of peroxides in rat hepatocytes after 1000 μM PAT treatment for 20 min (Barhoumi and Burghardt, 1996) or in human promyelocytic leukaemia (HL-60) cells (Liu et al., 2007; Wu et al., 2008). Exposure of the haploid eukaryotic petite negative fission yeast *Schizosaccharomyces pombe* (*S. pombe*) cells to 1000 μM PAT for 30 min induced the 2.44-fold and 2.6-fold accumulation of $O_2^{\cdot -}$ and H_2O_2 (but not $^{\cdot}OH$), respectively (Papp et al., 2012). In contrast with PAT, CTN did not inactivate GSH [in spite of the fact that CTN also participates in weak molecular interactions with free sulfhydryl groups (Blaskó et al., 2013)], but it did activate the GSH synthesis pathway in *Saccharomyces cerevisiae* cells exposed to 300 μg ml^{-1} CTN for 120 min (Iwahashi et al., 2007). Furthermore, CTN induces a dysfunction of the mitochondria by interfering with mitochondrial complex I (Chagas et al., 1992a,b; Riberio et al., 1997), leading to significantly elevated concentrations of peroxides without affecting the levels of $O_2^{\cdot -}$ and $^{\cdot}OH$ in cells of *S. pombe* exposed to 1000 μM CTN for 60 min (Máté et al., 2014). The oxidative stress generated by PAT or CTN induces an adaptation process via stress signalling pathways [e.g. mitogen-activated protein kinases, p38 kinase or c-Jun N-terminal kinase, etc.] which activate a stress-responsive transcription factor (Atf1) or a redox-sensitive transcription factor (Pap1) in *S. pombe* cells (Halliwell and Gutteridge, 2007; Liu et al., 2007; Papp et al., 2012; Máté et al., 2014).

Acute toxicity tests demonstrated the complex regulation of the levels of GSH and antioxidant enzymes such as the superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PD) and glutathione S-transferase (GST) after PAT or CTN exposure. In *S. pombe* cells exposed to PAT or CTN, the regulation of the elements of the antioxidant system exhibited toxin specificity (Papp et al., 2012; Máté et al., 2014).

To acquire further knowledge on this field, in the present study we carried out quantitative measurements of the changes in the levels of $O_2^{\cdot -}$, peroxides and $^{\cdot}OH$ produced in PAT + CTN-treated cells. The levels of the ROS-scavenging reducing factor GSH and of the specific activities of the most important antioxidant enzymes (SODs, CAT, GPx, GR, G6PD and GST) were also determined to follow the whole cascade after PAT + CTN treatment and to compare these results with the data obtained through separate treatment with 500 μM PAT (Papp et al., 2012) or 1000 μM CTN (Máté et al., 2014) alone, mycotoxins known to induce opposite alterations in the GSH metabolism. The same *S. pombe* strain was used in these experiments so as to obtain results that could be compared with those from our previous studies (Horváth et al., 2010, 2012a,b; Papp et al., 2012; Máté et al., 2014).

2. Materials and methods

2.1. Strains, culture conditions, determination of growth inhibition, checkerboard titration, and survival rate at the cellular level

The *S. pombe* uracil auxotrophic (ura4-D18) heterothallic (h^-) deletion mutant used in all experiments (Grimm et al., 1988) was the same as that employed in earlier studies on the mechanisms of action of PAT (Horváth et al., 2010, 2012a,b; Papp et al., 2012), zearalenone (Mike et al., 2013) and CTN (Blaskó et al., 2013; Máté et al., 2014). Minimal liquid SM medium containing 1% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.01% $MgSO_4$ and 0.1% Wickerham vitamin solution (Spencer and Spencer, 1996) and supplemented with 100 mg l^{-1} uracil was used for cultivation at pH 4.5. The *S. pombe* strain was pre-cultured in the SM medium overnight at 30 °C, and the cells were harvested, washed by centrifugation (1017 g, 5 min) and used to prepare mid-log-phase cultures with a starting cell number of 10^6 cells ml^{-1} (optical density $OD_{595nm} = 0.05$). The *S. pombe* strain was then cultivated for 15 h in a shaking incubator (shaking frequency 3.33 Hz) at 30 °C in order to obtain mid-log-phase cultures (Horváth et al., 2010). Stock solutions of PAT (405.5 mM) and CTN (250 mM) were prepared and stored as described by Murillo et al. (2008). Mycotoxin combinations were prepared in equimolar concentrations from the stock solutions immediately prior to the experiments.

The inhibition of growth by 0, 62.5, 125 and 250 μM PAT, CTN or PAT + CTN was determined in growth-supporting SM medium at 30 °C with a starting concentration of 10^6 cells ml^{-1} in shaken cultures at pH 4.5. Cell proliferation was monitored spectrophotometrically (Hitachi U2910) via the OD at 595 nm.

Checkerboard titration was performed according to the CLSI M38-A2 (2008) method with some modifications. PAT and CTN were tested in series of 2-fold dilutions in liquid SM medium (pH 4.5) at concentrations ranging from 15.6 to 1000 μM. 5×10^3 cells ml^{-1} *S. pombe* were treated on microtitre plates for 48 h at 30 °C. The ODs of the cultures were determined with a Multiskan EX microtitre plate reader at 595 nm. Interaction was assessed algebraically by determining the interaction ratio (IR) value from the Abbott formula $IR = I_{measured}/I_{expected}$, where $I_{measured}$ was the observed percentage inhibition, and $I_{expected}$ was calculated according to the formula $I_{expected} = X + Y - (XY/100)$ (X = the PAT-generated percentage of inhibition, and Y = the CTN-generated percentage of inhibition when applied alone). IR values < 0.5 are regarded as antagonism, IR values > 0.5, but < 1.5, as indifference, and IR values > 1.5 as synergism. The IR between PAT and CTN was also calculated by using the fractional inhibitory concentration (FIC) index, using the equation for all of the wells of the microtitre plates that corresponded to a MIC: $FICI = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where C_A and C_B are the MICs of drug A and drug B in the combination and MIC_A and MIC_B are the MICs of drugs A and B alone (Nyilasi et al., 2014). FIC indices ≤0.5 were interpreted as synergism, those >0.5 to <4 as indifferent interactions, and those 4 ≤ as antagonism (Johnson et al., 2004).

The survival rates of *S. pombe* cells in the presence of 250 μM PAT, CTN or PAT + CTN were estimated in short-term (1 h) acute toxicity tests in SM medium at 30 °C, using a starting concentration of 10^7 cells ml^{-1} at pH 4.5, according to Lee et al. (1995).