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Regulation of the patulin- and zearalenone-induced oxidative stress processes in the fission yeast cells

PhD Thesis

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Introduction

The mycotoxins produced by fungi are of increasing importance because of their potential worldwide occurrence in food and feedstuffs. Mycotoxins are of both economic and food safety significance and their maximum daily intake is strictly regulated both nationally and internationally. Recognition of the intracellular molecular effects and identification of the target elements of mycotoxins could provide basic knowledge of use in food and feedstuff detoxification, thereby increasing the success of the development of therapy and promoting resistant crop breeding.

Every living creature on Earth strives to maintain an inner balance, homeostasis, by adaptation to the changes that occur in its internal and external environment, and every organism or (in the case of unicellular organisms) every cell is therefore in dynamic equilibrium. If the changes that take place exceed the optimum range, the dynamic equilibrium is upset and stress effects develop. As concerns their origin, the stress effects can be internal (mutation, intracellular parasites, etc.) or external (heavy metals, toxins, temperature, oxygenization, pH, availability of nutrients, changes in osmotic pressure, pathogens, UV irradiation, etc.) stresses. Within certain limits, organisms can accommodate to these stress effects. This accommodation, known as adaptation, involves coordinated molecular mechanisms.

Schizosaccharomyces pombe, a eukaryotic, haploid fission yeast, is one of the most common model organisms. Its responses to external stressors (e.g. mycotoxins) appear quickly and can readily be studied. Some of the 300 known mycotoxins can cause an oxidative stress status leading to cytotoxic processes. An understanding of oxidative stress processes is of special importance because a number of human diseases (e.g. Alzheimer disease, Parkinson disease, sclerosis multiplex, arthritis, inflammatory bowel disease, cardiovascular diseases, and arteriosclerosis) may begin with such processes.

Changes in the environmental conditions are perceived by the appropriate receptors of the fission yeast, which then activate different signalling pathways. Antioxidants (e.g. peptides, vitamins, flavonoids and carotenoids, such as glutathione (GSH), ascorbic acid, tocopherol, β-carotene, etc.) and antioxidant enzymes (e.g. catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), etc.) are activated by these signalling pathways via alterations in the gene expression profile of the cells, thereby assisting survival and adaptation. There are two different types of stress responses: a general (or core) environmental stress response and a specific environmental stress response. The core environmental stress response is the same in most cases. Thus, the same defence mechanism should be activated by different stress stimuli, because they generate the same signal, which activates the same transcription factor and in this way forms a general stress response. In this case, the expression of genes involved in the carbohydrate metabolism, the scavenging of reactive oxygen species (ROS), protein folding, the mitochondrial function and metabolite transport are activated. The specific environmental

stress response activates genes which have specific functions during the adaptation process. Different levels of adaptation are described: when only damage (e.g. mutations) is prevented, when the adaptation to the altered environment is perfect, and when the cells become resistant to further and stronger stress effects.

We chose *S. pombe* as model organism because complex methods are available for investigation of the oxidative stress responses and regulation of the fission yeast. From many aspects, *S. pombe* seemed to be a good model organism because its cell division and cell cycle regulation is very similar to that in higher eukaryotes, many genes display significant similarities to human disease genes, and the activation profile of the stress-activated signalling pathways is identical with that of the human signal transduction pathways.

Patulin (PAT) and zearalenone (ZEA), the two mycotoxins investigated by our research group, have different target elements, but the literature data indicate that the oxidative generating effect predominates in their mode of action, although identification of the exact mechanism demands further investigations. Some information was available on the two mycotoxins, but the experiments were carried out in different cell and tissue cultures, and the mode of action of toxins may therefore be different. However, from examinations of the toxic mechanisms on a well-known model organism, our results should be comparable.

Aims

One of the programmes at the Department of General and Environmental Microbiology, University of Pécs, comprises investigations of the mode of action of mycotoxins, and especially their oxidative stress-generating effects on the fission yeast *S. pombe*. Part of this project involves determination of the non-estrogen-specific cytotoxicity, oxidative stress processes and their regulation and the target elements of ZEA on the fission yeast, which does not have any estrogen receptors or analogous sequences.

The aims of our study were to answer the following questions:

1.1. Does ZEA exert a cytotoxic effect on *S. pombe* cells? To what extent does the toxin inhibit the growth of *S. pombe* cells? What degree of cell death is induced by the toxin?

1.2. Does an adaptation process exist against the toxin? If it does, what signalling pathway regulates the phenomenon?

1.3. What degree of oxidative stress is induced by the toxin in the cells? How do the intracellular amounts of the different ROS superoxide redical (O_2^{-}•), hydrogen peroxide (H_2O_2) and hydroxil radical (•OH) change?

1.4. How do the cells respond to oxidative stress? How do the cells regulate the specific activity of the different antioxidant enzymes and the intracellular concentration of GSH?

1.5 Is there a direct interaction between ZEA and GSH?

1.6. Does the oxidative stress generated by ZEA result in DNA damage? If yes, do the cells strive to repair it via cell cycle arrest? If the repair mechanism is inefficient, is apoptotic cell death induced?

1.7. Does ZEA influence the sterol composition of the membrane because of its structural similarity with ergosterol?

The mechanism of action of the mycotoxin PAT was earlier investigated by our research group. The main target element of PAT and its membrane-perturbing effect were known from former investigations. The main processes of the PAT oxidative stress-generating effect (e.g. the rates of action of the different ROS and the changes in the specific activities of the antioxidant enzymes) have also been investigated in detail by our research group.

Further questions have emerged:

2.1. What signal transduction pathways are responsible for the intracellular regulation of these processes?

2.2. Does oxidative DNA damage occur? If yes, is the extent of the damage sufficient to initiate apoptotic cell death? Are the alterations in the apoptotic cell detectable morphologically?

2.3. Are the photoluminescent and polarization measurements that we use appropriate to reveal a direct interaction between GSH and PAT, known to react with SH groups?

Materials and methods

The *S. pombe* ura4$^-$D18 h$^-$, heterothallic, uracil auxotrophic strain was used in all of our experiments, with the exception of the examination of signal transduction pathways (MAPK), where the parental strain (leu 1-32 ura4-D18 his 7-366 ade6-M210, h$^+$) and its deletion mutants Δ*wis1* (wis1::ura4 leu 1-32 ura4-D18, h$^-$), Δ*sty1* (sty1::ura4 leu 1-32 ura4-D18 ade6-704, h$^+$), Δ*atf1* (atf1::ura4 leu 1-32 ura4-D18, h$^+$) and Δ*pap1* (pap1::ura4 leu 1-32 ura 4-D18, h$^+$) were used.

Mid-log phase cell cultures were applied in all of our experiments because of the identical physiological status. Cells were collected and washed centrifugally at 1017g (3000 rpm) for 5 min. The concentrations of the PAT and ZEA stock solutions were 405.5 and 300 mM, respectively, in acetonitrile and ethanol, respectively. In all of the experiments, the final concentration was 0.8%.

Determination of minimal inhibitory concentration (MIC), growth inhibition, survival rates, adaptation and toxin uptake

The growth inhibition of ZEA was measured at 0, 100, 500 and 1000 μM at 30 °C with 10^6 cells ml^{-1} via the observation of optical density (OD) changes at 595 nm. The MICs of *S. pombe* and the MAPK deletion mutant strains against ZEA and PAT were determined by a standard microdilution method (NCCLS M27-A). Survival rates and adaptation after ZEA treatment were determined by streaking.

For determination of the ZEA uptake, after centrifugation of the treated cells and cell wall-free protoplasts (10^7 cells ml^{-1} in 0.6 M KCl at pH 7.0), the ZEA concentrations of the

supernatants were measured at OD_{269nm}. The concentration of the toxin taken up by cells or protoplasts was determined by means of a calibration curve.

Detection of ROS

To determine the intracellular amounts of H_2O_2 and $O_2^{-}\bullet$ radicals, cells were stained with dihydrorhodamine 123 (DHR 123) and dihydroethidium, respectively and a BD FACSCalibur flow cytometer was used. The working concentration of the dyes was 10 μM. After a 0, 15, 30, 45 or 60-min ZEA treatment in SM medium, the signal of 10,000 cells was detected. The excitation wavelength for both dyes was 488 nm, while the emission wavelength was 585 nm for dihydroethidium and 530 nm for DHR 123. The intracellular concentration of the $^{\bullet}OH$ radical and the oxidoreduction balance of the cells (via investigation of the Cr(VI) reduction capacity) were measured by EPR spectroscopy.

Detection of antioxidant enzyme activities and GSH level

The intracellular activities of GR, GST, GPx, G6PD, CAT, Cu/ZnSOD and MnSOD, the specific intracellular concentrations of GSH and oxidized glutathione (GSSG) and total protein contents were determined by colorimetric assays.

GSH-ZEA interaction

For fluorescence measurements, stock solutions of ZEA and PAT in phosphate buffer (pH=8.0) were prepared in different concentrations. During the measurements, the GSH concentration was kept constant while the toxin concentration was varied. The photoluminescent spectra were recorded on a Fluorolog τ3 spectrofluorometer (Jobin-Yvon/SPEX, Longjumeau, France). The intensity of the photoluminescent spectra of GSH were measured at an excitation wavelength of 315 nm both in the absence and in the presence of ZEA or PAT.

The results were validated with an intensity-independent polarization measurement where the degree of polarization of GSH was detected at an excitation wavelength of 315 nm in the absence and presence of ZEA or PAT.

Cell cycle analysis by flow cytometry, nucleus staining, apoptosis and necrosis determination

For measurement of the cell cycle arrest, 10^7 cells ml^{-1} were treated with 500 μM ZEA for 60 min. The cells were prepared according to the ForsburgLab protocols. The percentages of the cells in the different stages of the cell cycle were determined by counting 10^4 cells with a Becton Dickinson FACSCalibur flow cytometer. For the nucleus staining, a suspension of 10^8 cells ml^{-1} from a mid-log-phase culture were treated with 500 or 1000 μM ZEA for 60 min, and 5 μl suspensions were then dispersed on slides, and stained with 10 μg ml^{-1} DAPI (λ_{abs}: 358 nm, λ_{em}: 461 nm). The nucleus morphology was observed with a Nikon Eclipse 80i fluorescent microscope with an UV filter. On average, 500 nuclei from each sample were counted.

For the determination of apoptosis and necrosis, protoplasts were treated with 500 or 1000 μM ZEA, and the cells were then stained with Annexin V and propidium iodide and detected by means of flow cytometry.

Determination of sterol compositions

The sterol components were extracted from 500 μM ZEA-treated early stationary-phase cells according to the appropriate protocol, and the peaks of the sterol structures were identified by GC-MS analysis.

Results and discussion

Characterization of the S. pombe strain

The MIC of ZEA on *S. pombe* cells was determined according to the NCCLS 2002 standard method. Complete inhibition was not detected; MIC_{50} was 500 μM and this concentration was used in further experiments.

In most cases, the estrogen-specific effects of ZEA have been described in the literature, but we have only a little information on the estrogen analogy-independent, harmful effects of ZEA. We therefore used *S. pombe* as a model system, which has been proven not to have any β-estrogen receptor analogue sequences or any sequences similar to that of the *Candida albicans* estrogen-binding protein. Examination of the MIC of the β-estradiol confirms the lack of receptors because it does not influence the growth of *S. pombe* cells.

The cytotoxic effects of ZEA on S. pombe

During the examination of the uptake of ZEA, the bioadsorbing capacity of the cell wall was demonstrated. 20% and 40% of the ZEA was taken up by the cell wall-free protoplasts and the vegetative cells, respectively. The kinetics of the uptake suggests that, instead of bioaccumulation (e.g. active transport), diffusion occurs, in parallel with the bioadsorption. Both curves exhibit saturation, which indicates that there is no active metabolism of ZEA within the cells.

The growth of the yeast cells was inhibited by ZEA in a dose-dependent manner up to 500 μM. 500 and 1000 μM ZEA treatment caused 83% growth inhibition, which was decreased by 38% 6 h later. This moderating phenomenon suggests an adaptation process against ZEA. After 60 min, the colony-forming ability was decreased by 20, 85 and 100% after 250, 500 and 1000 μM ZEA, respectively. Pretreatment of the cells with a subinhibitory concentration (250 μM) for 1 h resulted in a significantly increased survival rate in the presence of 500 or 1000 μM ZEA, which indicated an adaptation process.

The oxidative stress-generating effects of ZEA

Among the deletion mutant strains, Δ*pap1* proved to be the most sensitive to ZEA treatment. It may therefore be concluded that the transcription factor Pap1 is primarily responsible for the gene expression alterations necessary for the defence mechanism and adaptation. Another transcription factor, Atf1, activated by the MAPK cascade, is also ZEA-sensitive, but to a lesser extent.

Acute, 60-min ZEA treatment induced significant, 1.8- and 2-fold increases in the quantities of intracellular H_2O_2 and O_2^{-}•, respectively, as compared with the control samples. Nevertheless, the amount of •OH and the Cr(VI) reduction capacity of the cells did not change significantly *in vitro.*

In response to increased concentrations of H_2O_2 and O_2^{-}•, the specific activities of CAT and SOD (the H_2O_2 and O_2^{-}•-neutralizing enzymes) increased significantly. The concentration of the most important non-enzymatic antioxidant molecule, GSH, underwent a 3-fold decrease because of the increased ROS concentration.

The decreased concentrations of GSH and GSSG suggested an interaction between ZEA and GSH. The photoluminescent signal changes of GSH were therefore measured in the presence of different ZEA concentrations. The maximum in the fluorescence intensity increased with increasing toxin concentrations, which confirmed the existence of the interaction. On investigation of this phenomenon with an intensity-independent polarization measurement, however, the degree of polarization of GSH did not change in the presence or absence of ZEA, which confutes an interaction between ZEA and GSH.

In the absence of an interaction, the O_2^{-}• and H_2O_2-generated GSH-consuming processes are presumably responsible for the decreased GSH concentration. Many antioxidant enzymes carry out glutathione homeostasis, and their activity changes prove to be toxin-specific. The activity of GPx decreased during the H_2O_2 neutralizing process. The activities of GR, the GSSG back-reducing enzyme, the activity of GR decreased and this is presumably the reason for the decreased GSSG concentration. The specific activity of G6PD, an important enzyme of NADPH production and therefore the maintenance of GSH production, decreased, which could be the reason for the decreased GSH concentration. The explanation of the common decrease in concentration of GSH and GSSG could be the increased activity of GST, which is capable of conjugating xenobiotics to GSH and releasing them from the cell.

Cell-cycle arrest and its effects caused by ZEA

After ZEA treatment, the oxidative stress generated and the ROS molecules formed can cause oxidative DNA damage, which we examined through cell-cycle arrest. A 1-h treatment with 500 μM ZEA resulted in a significant decrease in the number of phase G1 cells, which were compensated in phase G2.

The probable cause of the arrest is the damage to the DNA, because the cell cycle stops in order to correct it. DNA damage can also be examined through fragmentation of the nucleus in *S. pombe.* To do this, we compared the morphological changes of the nuclei of treated and control cells. We experienced a slight, concentration-dependent increase in the number of cells with a fragmented nucleus. These nuclei with irregular chromatin point to DNA damage, which explains the cell-cycle arrest.

Apoptosis caused by oxidative DNA damage has been observed in numerous cell lines, but in the case of *S. pombe* we did not experience either apoptosis or necrosis, even for cells with nucleus fragmentation and cell-cycle arrest.

Changes in sterol composition caused by ZEA treatment

Although ZEA is not a steroid-like compound, it displays similarities in structure with β-estradiol, so we assume that it can induce changes in sterol biosynthesis and in the composition of various sterol intermediates. Relative to the control, the proportions of various intermediates in the early stationary phase cells and the amount of ergosterol changed

significantly. The sterols are the most important membrane lipids and changes in their composition can alter the membrane functions.

Defence mechanisms against cytotoxic effects of PAT

Oxidative stress-generating effects of PAT and the compensating responses to them through specific antioxidant enzymes and regulation of the amount of GSH are known from earlier research projects at the Department.

In the case of *S. pombe,* MAPK has a role in the regulation of the transcriptional responses to external oxidative stressors. Δ*pap1* proved to be the most sensitive of the deletion mutants, because 50 μM PAT induced a 95% inhibition in reproduction and 75 μM PAT caused a 100% inhibition, while at similar concentrations the parental strains survived almost totally. However, like Δ*pap1,*at a higher toxin concentration (600 μM), Δ*wis1*, Δ*sty1* and Δ*atf1* also appeared to be sensitive, although the extent of reproduction inhibition was lower (98%, 95% and 65%), whereas the inhibition in the case of the parental strains was only 10%.

Mutants that have alterations in any member of the MAPK cascade have problems in adaptation to the stress caused by PAT, but Δ*pap1* mutants are the most affected. In the absence of Pap1 transcription factor, *S. pombe* is unable to adapt to the oxidative stress generated by PAT.

Our fluorescence experiments confirmed the earlier-reported interaction between PAT and GSH. In the presence of increasing toxin concentrations, the photoluminescent signal of GSH is intensified, demonstrating the interaction. This is confirmed by the intensity-independent polarization method, because the initial degree of polarization of GSH (3 ± 3%) increases significantly in the presence of PAT (17 ± 3%). Change in the degree of polarization indicates that the size and movement of the examined molecule has changed.

Signs of concentration-dependent oxidative DNA damage can be observed through changes in the morphology of the nucleus in treated cells. After a 90-min treatment with 50 μM PAT, the amount of cells with changed, fragmented nuclei increased 3.12-fold relative to the control, and the increase after 500 μM treatment was 4.87-fold. The morphological changes include longish nuclei and fibrillar and (at higher treatment concentration) damaged chromatin substance.

Summary

In all of our experiments the fission yeast *S. pombe* was used as a model organism, because it does not have estrogen receptors or analogous proteins. It is therefore suitable for investigations of non-estrogen-specific, cytotoxic, oxidative stress-generating effects, the unknown target molecules of ZEA and the intracellular regulation mechanisms of the cells.

1.1. ZEA induced concentration-dependent cell damage. The MIC_{50} was 500 μM, and its growth-inhibitory effect depended on the concentration applied. The cells took up approximately 40% of the toxin within 25 min. The cell wall had a bioadsorbing capacity because it bound 20% of the toxin, the remaining amount entering the cell via diffusion.

1.2. As far as we are aware, this is the first description of the adaptation phenomenon against ZEA. Cells pretreated with a subinhibitory concentration of ZEA showed an increased survival rate after ZEA treatment with a higher dosage.
The adaptation process is regulated primarily by the redox-sensitive transcription factor Pap1. In the absence of this transcription factor, the survival rate of the deletion mutant strain was much lower than that of the parental strain after treatment with a lower concentration of ZEA. The transcription factor Atf1 of the MAPK cascade was also partially affected in the regulation process.
1.3. 500 μM ZEA induced 1.8-fold and 2-fold increases in the intracellular amounts of H_2O_2 and O_2^-•, respectively, but the concentration of •OH and the Cr(VI) reduction capacity of the cells did not change significantly.
1.4. The cells regulated the activity of the antioxidant enzymes specifically via the known signalling pathways as a consequence of the increased ROS concentrations.
The concentration of the most important non-enzymatic antioxidant molecule GSH decreased significantly and the quantity of GSSG also decreased.
1.5. The reason for the depletion of GSH in the absence of a direct GSH-ZEA interaction is related to the ROS-induced GSH-consuming processes.
1.6. ZEA induced oxidative DNA damage, which was demonstrated by the occurrence of fragmented nuclei. When the cells repair the damage, the cell cycle is arrested in the G2/M phase. However, apoptotic cell death was not detected.
1.7. In our only chronic test quantitative changes in the sterol composition of the treated cells were observed; these may result in changes in the membrane functions.
Our novel results indicate that the non-estrogen-specific, oxidative stress-inducing ability of ZEA is at least as important as the estrogen-specific harmful effects.
The oxidative stress-generating and plasma membrane-fluidizing effects of PAT and the consequences of these effects were investigated earlier by our research group. These experiments demonstrated the ROS-generating effect of PAT and the regulation of the specific activity of the antioxidant enzymes.

2.1. Investigations of the signalling pathways of the regulation revealed that the primary regulatory element of the antioxidant defence mechanisms was the transcription factor Pap1. However, the MAPK cascade and its transcription factor Atf1 also played important roles in the regulation.
2.2. The oxidative stress generated by PAT affected the DNA of the cells, inducing aberration and fragmentation of the nuclei in a concentration-dependent manner. In spite of some typical structural markers of apoptosis on the treated cells, we were not able to identify either apoptotic or necrotic cell death.
2.3. It is known from the literature that PAT is capable of forming cross-links with the SH groups of the proteins, and the concentrations of GSH and GSSG decrease significantly after PAT treatment. The interaction between PAT and GSH was demonstrated through

photoluminescence and intensity-independent polarization measurements. We can therefore use these validated methods in our later experiments with other toxin-GSH interactions.

Publications related to the thesis:

Papp, G., Horváth, E., **Mike, N.**, Gazdag, Z., Belágyi, J., Gyöngyi, Z., Bánfalvi, G., Hornok, L., Pesti, M. (2012). Regulation of patulin-induced oxidative stress processes in the fission yeast *Schizosaccharomyces pombe*. Food and Chemical Toxicology, 50, 3792-3798. **IF: 2.999**

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