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Induction of proteotoxic stress by the mycotoxin patulin

Angel Guerra-Moreno, John Hanna*

Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States

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ABSTRACT

Patulin is a naturally occurring mycotoxin produced by a number of molds and may contaminate a wide variety of food products. In practice, patulin's main societal relevance concerns apple juice and its products. Multiple advisory bodies, including the U.S. Food and Drug Administration and the World Health Organization, recommend that producers monitor and limit patulin levels in apple juice products. The mechanism of patulin toxicity remains largely unknown. Here we show that patulin induces proteotoxic stress in the yeast *S. cerevisiae*. The transcription factor Rpn4 controls the abundance of the proteasome, the complex multisubunit protease that destroys proteins, including misfolded proteins. Rpn4 protein is strongly induced by patulin, and Rpn4 levels normalize over time, consistent with homeostatic regulation. A *rpn4Δ* mutant is highly sensitive to patulin, confirming the physiologic relevance of this response. Rpn4 is known to be regulated both transcriptionally and post-translationally. Patulin induces both pathways of regulation, but the post-transcriptional pathway predominates in controlling Rpn4 protein levels. These results indicate that proteotoxicity represents a major aspect of patulin toxicity. They not only have implications for patulin detoxification but in addition suggest the possibility of some potentially useful patulin applications.

1. Introduction

Patulin is a mycotoxin produced by many different molds. It was first isolated from *Penicillium patulum*, which was later renamed *Penicillium griseofulvum* (Bennett and Klich, 2003). Its main societal relevance is as a food contaminant, and its associated molds may grow on a wide variety of foods including fruits, grains, and cheeses (United States Food and Drug Administration, 2001). Foods that have rotted and have surface damage are at particularly high risk. In practice, however, safety concerns related to patulin are largely limited to apple juice and apple juice products (United States Food and Drug Administration, 2001). Significant contaminations of commercial apple juice products by patulin have been documented in various countries (Iwahashi et al., 2006), and several advisory bodies, including the U.S. Food and Drug Administration and the World Health Organization, recommend that producers control patulin levels so as to limit exposure to consumers (United States Food and Drug Administration, 2001; World Health Organization, 1990). These recommendations are particularly relevant to young children who may consume higher amounts of apple juice relative to body weight than adults.

Early attempts to develop patulin as an antibiotic were largely abandoned after recognition of patulin's toxicity (Bennett and Klich, 2003). The molecular basis for patulin's toxicity remains largely unknown. As a potent electrophile (Fig. 1A), patulin is capable of reacting with a wide range of macromolecules (Fliege and Metzler, 1999, 2000). DNA damage, for example, may contribute to patulin's toxicity (Schumacher et al., 2006).

Protein misfolding is generally toxic, and may harm cells through multiple mechanisms. A change in protein structure can lead to loss of normal protein function. However, protein misfolding may also cause gain-of-function toxicities by promoting promiscuous protein–protein interactions, which may lead to the formation of insoluble protein aggregates. Protein misfolding is thought to play a key role in a number of human neurodegenerative diseases, including Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Hipp et al., 2014).

The proteasome is a 2.5 MDa multisubunit complex capable of destroying proteins (Matyskiela and Martin, 2013). It houses three distinct proteolytic active sites within a central 700 kDa cylindrical chamber known as the core particle (CP). At either end of the CP is an approximately 900 kDa complex known as the regulatory particle (RP), which orchestrates the various aspects of proteasome function. Most substrates are targeted to the proteasome through covalent modification by the small protein ubiquitin. The RP recognizes the substrate via the ubiquitin tag, unfolds the substrate, removes the ubiquitin molecules, and directs the substrate into the CP for degradation (Finley et al., 2012).

The term proteotoxicity has been used to refer to conditions which

* Corresponding author.
E-mail address: jwhanna@partners.org (J. Hanna).

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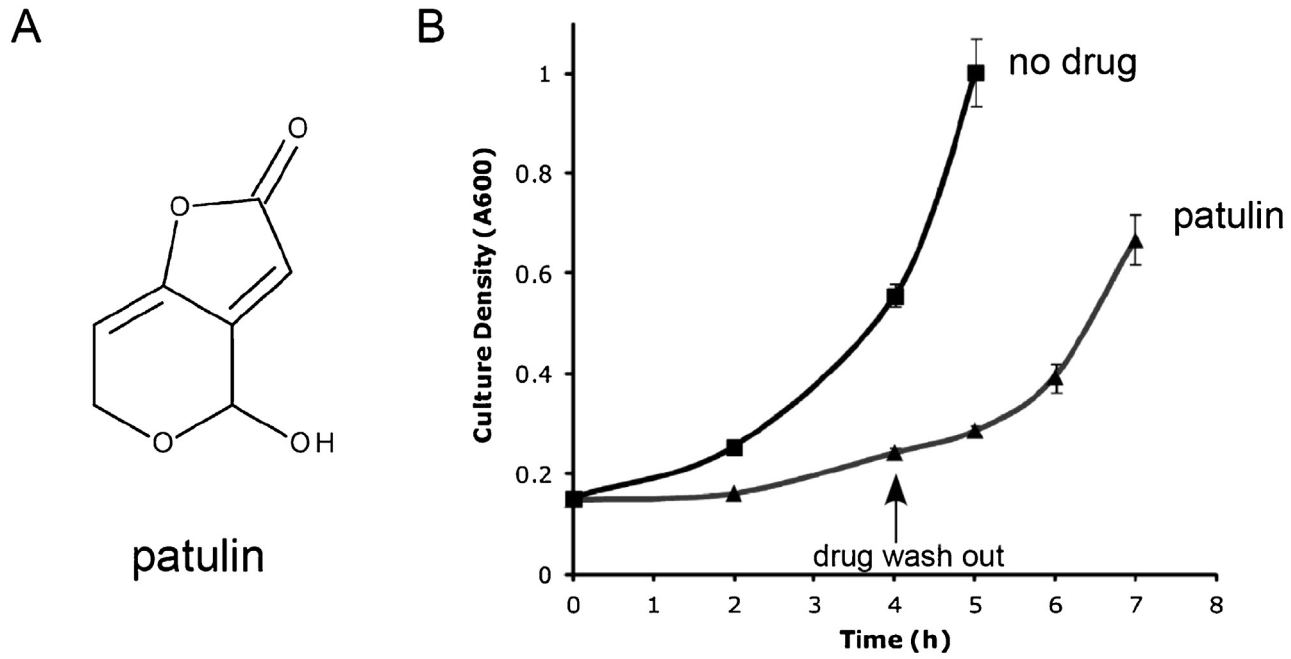

Fig. 1. Patulin structure and reversible toxicity.
A) Chemical structure of patulin. B) Growth of wild-type cells in liquid culture during treatment with patulin (50 μg/mL). After 4 hours of treatment, the drug was washed out. Error bars represent standard deviations from two independent cultures.

compromise or overwhelm the cell's capacity for protein quality control. Misfolded proteins are a major cause of proteotoxicity, and accordingly, cells have developed complex stress responses to identify and eliminate misfolded proteins. One such proteotoxic stress response is mediated by the transcription factor Rpn4 (Mannhaupt et al., 1999; Xie and Varshavsky, 2001). Rpn4 is capable of controlling proteasome abundance in cells by carrying out the coordinated transcription of all (~35) proteasome genes and a number of proteasome-interacting proteins. It does this by recognizing a promoter element known as the PACE motif (proteasome associated control element; Mannhaupt et al., 1999). Rpn4 is itself a substrate of the proteasome. Under normal conditions, Rpn4 is rapidly destroyed, resulting in low baseline levels (Xie and Varshavsky, 1999; Guerra-Moreno et al., 2015 Guerra-Moreno et al., 2015). However, any stress which inhibits or overwhelms proteasome function may result in stabilization of Rpn4 protein. This leads to increased Rpn4 transcriptional activity that promotes new proteasome synthesis until proteasome function is restored to levels adequate to carry out rapid Rpn4 degradation, creating a homeostatic feedback loop (Ju et al., 2004; Wang et al., 2008, 2010). This pathway is functionally conserved in higher organisms where the stress-inducible transcription factor Nrf1 plays a similar role (Radhakrishnan et al., 2010; Steffen et al., 2010).

Here we show that patulin is a potent inducer of the Rpn4 stress response. Cells lacking Rpn4 show increased sensitivity to patulin, indicating the physiologic relevance of this response. Patulin's regulation of Rpn4 appears to be largely post-transcriptional in nature. These results indicate that proteotoxicity represents a major aspect of patulin toxicity.

2. Materials and methods

2.1. Yeast strains and chemicals

Yeast were cultured in YPD medium at 30 °C unless indicated otherwise. YPD consisted of 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. The wild-type (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* and *rpn4Δ* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpn4:KAN*) strains have been previously described (Hanna et al., 2014). Patulin, sodium arsenite, α-amanitin, actinomycin D, cycloheximide, and tunicamycin were obtained from Sigma Aldrich. Patulin, actinomycin D, and tunicamycin were prepared in dimethyl sulfoxide (DMSO); sodium arsenite, cycloheximide, and α-amanitin were prepared in water. Chemical structure images were prepared with the Biovia Draw program.

2.2. Generation of the anti-Rpn4 antibody

The full length open reading frame of *RPN4* was cloned into pET-45b which provides for an N-terminal 6x-histidine tag. The protein was expressed in *E. coli* (BL21 DE3) and purified by Nickel-affinity chromatography. This protein was used to generate a rabbit polyclonal antibody (Covance). The antibody was validated using whole cell extracts from wild-type and *rpn4Δ* strains (Fig. 2A), which confirmed complete absence of the assigned immunoreactive band in the *rpn4Δ* mutant.

2.3. Immunoblot analysis

Whole cell extracts were prepared from exponential phase cultures using a lithium acetate/sodium hydroxide method which has been previously described in detail (Weisshaar et al., 2017). In the experiment of Fig. 5C, whole cell extracts were prepared by resuspending cell pellets in 1X Laemmli loading buffer and boiling for 5 min. Where indicated, actinomycin D (20 μg/mL) or α-amanitin (20 μg/mL) was used to inhibit mRNA transcription. Where indicated, cycloheximide (100 μg/mL) was used to inhibit protein synthesis. Analysis was by standard SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblot. The following antibodies were used: anti-Pgk1 (Invitrogen; 459250), anti-ubiquitin (Santa Cruz; SC-8017), anti-Rpn4 (this study), and anti-Rpn8 (Hanna et al., 2007).

2.4. Phenotypic analysis

Overnight cultures were normalized by optical density and spotted in three-fold serial dilutions onto YPD plates lacking or containing patulin (5 μg/mL) and incubated at 37 °C for the indicated times. The