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Authors

Han, John
Nguyen, Carolyn
Thrasher, Julianna
et al.

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The Nrf1 transcription factor is induced by patulin and protects against patulin cytotoxicity

John J.W. Han[1],

Carolyn D. Nguyen[1],

Julianna P. Thrasher,

Anna DeGuzman,

Jefferson Y. Chan[*]

Department of Laboratory Medicine and Pathology, University of California, Irvine, D440 Medical Sciences, Irvine, CA 92697, USA

Abstract

Patulin is a mycotoxin produced by a variety of molds that is found in various food products. The adverse health effects associated with exposure to patulin has led to many investigations into the biological basis driving the toxicity of patulin. Nevertheless, the mechanisms through which mammalian cells resists patulin-mediated toxicity is poorly understood. Here, we show that loss of the Nrf1 transcription factor renders cells sensitive to the acute cytotoxic effects of patulin. Nrf1 deficiency leads to accumulation of ubiquitinated proteins and protein aggregates in response to patulin exposure. Nrf1 expression is induced by patulin, and activation of proteasome genes by patulin is Nrf1-dependent. These findings suggest the Nrf1 transcription factor plays a crucial role in modulating cellular stress response against patulin cytotoxicity.

Keywords

Cellular stress; Nrf1; Transcription factor; Protein homeostasis; Mycotoxin

1. Introduction

Patulin (PAT) is a mycotoxin produced by a wide range of molds including Penicillium and Aspergillus (Saleh and Goktepe, 2019). It is a significant food contaminant frequently detected in fruits, fruit products, feeds, and the most common being apple juice. While many countries including the United States and European Union have set regulatory limits for patulin at 50 ug/L, levels beyond the maximum can still be found in some commercialized products (Harris et al., 2009; Saxena et al., 2008; Yurdun et al., 2001; Zaied et al., 2013;

[*]Correspondence to: Department of Laboratory Medicine and Pathology, University of California, Irvine, D440 Medical Science 1, Irvine, CA 92697, USA. jchan@uci.edu (J.Y. Chan).

[1]Both authors contributed equally to this work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Zhong et al., 2018). Patulin is known to have toxic effects on various organ systems in the body primarily targeting the kidneys, liver, gastrointestinal tract, and the immune system (Escoula et al., 1988; McKinley et al., 1982; Speijers et al., 1988). The molecular basis of its toxicity in cells remains unclear (Pal et al., 2017). Patulin is an electrophile, which exerts its damaging effects directly by forming covalent adducts with sulfhydryl groups in proteins, amino acids and other essential cellular molecules (Fliege and Metzler, 1999, 2000). Patulin has been shown to lower glutathione content in cells and induce oxidative stress by generating reactive oxygen species (ROS) (Pfeiffer et al., 2005; Zhang et al., 2015). Patulin has also been demonstrated to disrupt endoplasmic reticulum (ER) homeostasis and induce DNA strand breaks and apoptosis (Boussabbeh et al., 2015).

Despite extensive research on the importance of patulin in food safety and its cellular effects, little is known about the genes that protect against patulin in mammalian cells. Previously, it was found that the expression of the yeast transcription factor, Rpn4, is strongly induced by patulin (Guerra-Moreno and Hanna, 2017). Rpn4 serves as an important mediator in maintaining protein quality control and stress response through its role in coordinating expression of proteasome genes, as well as genes involved in protein ubiquitination and DNA repair in yeast (Karpov et al., 2013; Wang et al., 2010). Rpn4 is a short-lived protein that is rapidly degraded by the proteasome, and the negative feedback circuit between Rpn4 and the proteasome provides an efficient and sensitive means to control the abundance of proteasome in cells (Ju et al., 2004). It is increasingly evident that Nrf1 plays this role in mammalian cells (Motosugi and Murata, 2019).

Nrf1 (Nuclear factor erythroid-derived 2-related factor-1, also known as NFE2L1) belongs to the Cap-N-Collar (CNC) family of transcription factors (Kim et al., 2016). Nrf1 plays a crucial role in maintaining cellular homeostasis through its role in regulating expression of cytoprotective genes important for stress response (Kwong et al., 1999; Lee et al., 2013, 2011; Myhrstad et al., 2001; Ohtsuji et al., 2008; Radhakrishnan et al., 2010). The Nrf1 gene encodes several isoforms, and long protein isoforms include TCF11 and Nrf1a (also been designated previously as Nrf1). TCF11, consisting of 772 amino acids, is the longest form, while Nrf1a, which is 742 amino acids in size, is generated through alternate splicing of exon 4 of the Nrf1 gene, and it lacks a Neh4 subdomain (aa 242–271 of TCF11) that contains a transactivation domain (Kim et al., 2016; Luna et al., 1995). Both TCF11 and Nrf1a are located in the endoplasmic reticulum membrane, and they undergo retro-translocation into the cytoplasm and processing prior to gaining entry into the nucleus to activate a similar set of target genes (Radhakrishnan et al., 2014; Sha and Goldberg, 2014; Steffen et al., 2010). Under basal conditions, TCF11 and Nrf1a are ubiquitinated and degraded by the proteasome, resulting in low levels of these proteins in the cell. Under conditions of stress however, TCF11 and Nrf1a levels are upregulated largely through protein stabilization (Biswas et al., 2013; Han et al., 2021).

One aspect of patulin toxicity in mammalian cells is thought to occur through induction of proteotoxicity (Guerra-Moreno and Hanna, 2017). As Nrf1 has been shown to be essential for transcriptional activation of proteasome genes in response to proteasome inhibition, we sought to determine its contribution in mediating protective responses to patulin toxicity. Here, we show that Nrf1 protein levels are strongly upregulated by patulin. We also show

that expression of proteasome genes is upregulated by patulin, and this response is Nrf1-dependent. Nrf1 knockout cells are sensitive to patulin-induced cytotoxicity. These results demonstrate that Nrf1 can act as a sensor for patulin-mediated activation of proteasome genes to increase proteasomal capacity in cells. Taken together, these results suggest that activation of Nrf1 is an adaptive intracellular response to patulin-induced stress, and Nrf1 plays a protective role against patulin-induced toxicity.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin and fetal bovine serum (FBS) were purchased from ThermoFisher Scientific (Waltham, MA). Bradford protein assay reagent was from BioRad (Hercules, CA). General chemicals for buffers and culture media were purchased from Sigma-Aldrich (St. Louis, MO). LDH Cytotoxicity Assay kit was purchased from Enzo Life Sciences (Farmingdale, NY). Primary antibodies against Tubulin-A (3873, mouse mAb), horseradish peroxidase linked anti-rabbit IgG (7074) and anti-mouse IgG (7076) antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit monoclonal antibody against TCF11/Nrf1a is also from Cell Signaling (D5B10) and it has been validated for specificity using Nrf1 knockout cells (Han et al., 2017).

2.2. Cells lines

Nrf1 knockout mouse embryonic fibroblast (MEF) cells and knockout cells reconstituted with Nrf1 cDNA were previously described, and they were grown in Dulbecco's modified Eagle's medium supplemented with 1% glutamine, 20% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Nrf1 knockout HEK293 cells were obtained from Canopy Biosciences (St. Louis, MO). HEK293 and HCT116 cells were obtained from ATCC (Manassas, Virginia) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2.

2.3. Western blotting

Cells were lysed in Loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromphenol blue, 42 mM dithiothreitol), and the mixture was sonicated for 1 min and boiled for 5 min. Lysates were then cleared by centrifugation for 15 min at 4 °C. Proteins were electrophoresed on SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl pH 8.0%, and 0.05% Tween 20) at room temperature for one hour, and then incubated with the indicated primary antibodies at 1:1000 dilution (unless otherwise indicated) overnight at 4 °C followed by incubation with 1:2000 dilution of horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody. Immunoreactive bands on the blots were visualized using chemiluminescent detection system and quantified by densitometric analysis on iBright (ThermoFisher Scientific) using the on-instrument software.

2.4. Aggresome staining

ProteoStat Aggresome dye (Enzo Life Sciences, PA) was used to detect misfolded and aggregated proteins in cells (Shen et al., 2011). Cells were grown on multi-chamber glass bottom slides and treated with vehicle or patulin. After 24 h, cells were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in 1 × assay buffer for 30 min on ice. Cells were washed with 1 × assay buffer for two times and stained for 30 min at room temperature with ProteoStat Aggresome dye. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Biotium) for 5 min. Stained cells were examined with a Nikon epifluorescent microscope using a Texas Red filter set for the ProteoStat dye, and UV for blue fluorescence for DAPI, respectively. Images were acquired using a 63 × objective lens with a Spot RT3 digital camera and Adobe Photoshop CS was used to layer the captured images. Fluorescence intensity per cell was quantified using NIH ImageJ analysis software to obtain mean corrected total cell fluorescence (CTCF) of 8–10 readings/area per high power field, and CTCF was calculated as: Integrated density– (Area of selected cell x mean background fluorescence).

2.5. Cell viability assay

Cell viability was assessed by Trypan blue exclusion or LDH-WST assay. For Trypan blue assay, MEF cells were seeded at a density of 3×10^5 cells/well in 6-well plate. After overnight growth, cells were incubated with patulin at 5uM concentrations for 24 h at 37 °C. At the end of incubation, the number of viable cells and non-viable (Trypan blue stained) were counted. Briefly, trypan blue solution was added to the cell suspensions in a 1:1 ratio, and percentage of living cells and dead cells were analyzed on a DeNovix (Wilmington, DE) automated cell counter. Triplicate values were averaged, and SEM calculated and graphed with Prism software (GraphPad). Cell viability was determined as follows: Viability (%) = (live/total cells) X 100%. For LDH release assays, HEK293 cells were seeded in 96-well plates in 6–8 replicates per condition at a density of 10^4 cells/well. After attaching, cells were treated with the indicated concentration of patulin for 24 hr, LDH released in the extracellular environment was measured by water-soluble tetrazolium salts (WST-1) conversion assay done according to manufacturer's recommendation. Absorbance reading at 490 nm was then measured using a Synergy H1 multi-well plate reader (Agilent; Santa Clara, CA). Wells containing media alone were used as blanks to subtract absorption by media components. Results were normalized to untreated cells as negative control (expressed as 100%). Positive control consisted of treating cells with lysis buffer to achieve 100% lysis. Cytotoxicity was determined as: Cytotoxicity (%) = (absorbance of sample - absorbance of negative control /absorbance of positive control - absorbance of negative control) X 100%.

2.6. RNA isolation and RT-PCR

Total RNA was extracted using Zymo DirectZol RNA Miniprep (Zymo Research, Irvine, CA), and iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA) was used for cDNA synthesis. Quantitative RT-PCR was performed using Roche FastStart DNA Green Master Mix (Roche LifeScience) with previously described primers (Han et al., 2021), in a QuantStudio-3 Real-Time PCR system running QuantStudio Design and Analysis Software

(v1.5.1, Applied Biosystems). PCR cycling conditions consist of 95 °C for 10 min and 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Results were calculated using the ΔΔCT method with ALAS1 as reference gene.

2.7. Statistical analysis

Data are expressed as means ± SD. Statistical analyses using Student's t-test or one way ANOVA done with Prism software (GraphPad), * indicates P values < 0.05 and considered significant.

3. Results

3.1. Nrf1 knockout cells are more susceptible to cytotoxicity induced by patulin

To determine whether Nrf1 protects against patulin-induced cytotoxicity, MEF cells derived from Nrf1 knockout mice were used. Cytotoxic effects of patulin on knockout cells were compared to isogenic knockout cells complemented with wild type Nrf1a cDNA. Cells were treated with 10 or 20 μM of patulin for 24 h, and cell death was determined by trypan-blue exclusion assay. The concentrations chosen were within the range used in previous studies done on similar cell lines, and they are also within the range that is encountered in cases where exposure is beyond the maximum recommended limits in humans (Pillay et al., 2020; Zhang et al., 2015; Zhong et al., 2018; Zhou et al., 2010). Compared to cells expressing Nrf1a, knockout cells showed decreased survival after treatment with patulin (Fig. 1A). To confirm these findings, the cytotoxic effects of patulin were also examined in HEK293 human embryonic kidney cell line. Wild type control and Nrf1 knockout HEK293 cells were treated with 5, 10 or 20 μM patulin, and cell death was measured by LDH leakage assay. Consistent with MEF-cell data, treatment of Nrf1 knockout HEK293 cells with increasing concentrations of patulin resulted in decrease in cell viability over a 24-hour period (Fig. 1B). Together, these results demonstrate that loss of Nrf1 sensitizes cells to patulin-induced cytotoxicity.

3.2. Patulin induces accumulation of ubiquitinated proteins and aggresomes in Nrf1 deficient cells

Next, we examined the impact of Nrf1 deficiency on proteotoxic stress induced by patulin. Cells were treated with patulin, and lysates were analyzed for ubiquitinated proteins by immunoblotting. In patulin treated cells, levels of global protein ubiquitination were significantly higher in Nrf1 knockout cells, compared with knockout cell rescued with Nrf1a. This is seen as enhanced smears in a broad region of membrane proteins (Fig. 2A and B). Similarly, Nrf1 knockout HEK293 cells showed increased levels of ubiquitinated proteins compared to wild type cells (Fig. 2C). To determine whether knockout cells are susceptible protein aggregation in response to patulin, formation of aggresome bodies were monitored using the ProteoStat Aggresome Detection dye. This dye has been validated to specifically detect protein aggregates and aggresomes in cells. Structures stained by ProteoStat dye were found in the perinuclear region, and in untreated cells, staining was slightly higher in knockout cells compared to knockout cells rescued with Nrf1a cDNA suggesting that basal level of proteostatic function is diminished in knockout cells (Fig. 2D top panels and 2E). After treatment with patulin, ProteoStat staining was much higher

in knockout cells than in cells expressing Nrf1a (Fig. 2D bottom panels and 2E). As aggresomes are formed when the protein degradation system is overwhelmed, this suggests that proteostatic response to patulin treatment is compromised by loss of Nrf1 function. Together, these findings suggest that expression of Nrf1 protects against patulin-induced accumulation of ubiquitinated proteins and aggresome formation.

3.3. Nrf1 expression is induced by patulin

Nrf1 is known to be responsive to cellular stresses. Accordingly, we sought to determine whether the expression of Nrf1 is induced by patulin exposure. Nrf1 proteins, as demonstrated by.

bands above and below the 130 kDa marker, were elevated by patulin in HEK293 cells in a dose-dependent manner (Fig. 3A). Western blotting also revealed a time-dependent induction of Nrf1 in HEK293 cells (Fig. 3B). An increase was observed 0.5 h after patulin treatment and levels increased further over time with a maximum amount seen by 2 and 4 h. Like HEK293 cells, Nrf1 expression was induced in mouse embryonic fibroblasts and HCT116 cells by patulin in a dose-dependent manner indicating that patulin-mediated upregulation is not cell specific (Fig. 3C and D). Together, these results demonstrate that Nrf1 expression is upregulated by patulin.

3.4. Patulin upregulates the proteasome pathway through Nrf1

Patulin is thought to mediate toxicity through a mechanism involving proteotoxic stress and has been shown to increase proteasome levels in yeast. RT-qPCR was done to examine the effect of patulin on Nrf1-mediated regulation of proteasome gene expression. For cells not treated with patulin, mRNA expression of several proteasome genes was lower in Nrf1 knockout cells compared with knockout cells reconstituted with Nrf1a (Fig. 4). Patulin treatment in Nrf1 knockout cells expressing Nrf1a elicited up-regulation of various proteasome genes. In contrast, induction of proteasome genes by patulin was not observed in Nrf1 knockout cells (Fig. 4). Expression of USP7 and YWHAZ, which are not known targets of Nrf1, remained unchanged between knockout and knockout cells expressing Nrf1a, and were not induced by patulin. The upregulation of proteasome genes observed might be a protective and adaptive response by cells to patulin toxicity.

4. Discussion

In this study, we identify patulin, a mycotoxin found commonly in agricultural products, as an activator of Nrf1 transcription factor. We demonstrate that patulin induces expression of proteasome genes through Nrf1 and that cells deficient in Nrf1 are more sensitive to patulin-induced cytotoxicity. The proteasome is responsible for elimination of damaged or misfolded proteins and protein aggregates that originate in the cell under both normal and pathological conditions, and their accumulation leads to proteotoxic stress and cell death (Ciechanover and Kwon, 2015; Dantuma and Lindsten, 2010; Gilda and Gomes, 2017; Jung and Grune, 2008; Obeng et al., 2006; Pohl and Dikic, 2019; Smith et al., 2011; Wu and Rapoport, 2018). A growing body of evidence indicates that Nrf1 transcription factor is crucial in maintaining protein homeostasis by regulating expression of proteasome subunits

(Lee et al., 2013, 2011; Radhakrishnan et al., 2010; Steffen et al., 2010). Rpn4, a speculated Nrf1 ortholog in yeast, was also found to be activated by patulin to induce proteasome pathway, and toxicity to patulin was exacerbated by a loss of Rpn4. These similarities between Rpn4 and Nrf1 suggest that the protective mechanism mediated by the proteasome degradation pathway in response to patulin toxicity is conserved in evolution.

Our results suggest that a mechanism of cytotoxicity caused by patulin in Nrf1 knockout cells is through induction of proteotoxic stress. Consistent with this, ubiquitinated protein levels and aggresomes were elevated by patulin treatment in Nrf1 knockout cells. Patulin has also been shown to have a high affinity for covalent binding to sulfhydryl groups as well as lysine, histidine, and α-amino groups that can lead to the generation and the accumulation of misfolded proteins (Fliege and Metzler, 1999). In addition, patulin is known to induce production of reactive oxygen species (ROS), which can directly inactivate peptidase function and thus reducing the flux of proteins through the proteasome (Farout et al., 2006). Moreover, excessive ROS could incur damages to proteins by cleavage, cross-linking reactions and oxidization of amino acid side chains and backbone thereby causing a toxic buildup of such proteins and protein aggregates overwhelming proteasome function and triggering proteotoxic stress (Fliege and Metzler, 2000; Zhang et al., 2015).

Nrf1 expression is induced by patulin. Whether this occurs through transcriptional induction or post transcriptional mechanism via protein stabilization remains to be determined. In this regard, a recent study suggests that patulin-mediated induction of Nrf2, a paralog of Nrf1 involved in cellular stress response, is achieved through its interaction with KEAP1 (Pillay et al., 2021). Under normal conditions, Nrf2 is sequestered by KEAP1 in the cytosol, prompting Nrf2 for degradation via the ubiquitin-proteasome pathway (Baird and Yamamoto, 2020). Intracellular accumulation of electrophilic substances, such as patulin, can modify the cysteine residues on KEAP1 causing a conformational change that triggers the release of KEAP1-bound Nrf2 which then translocates to the nucleus to activate gene expression (Pillay et al., 2021). As Nrf1 shares structural similarities with Nrf2 and is also known to interact with KEAP1, it is possible that patulin-mediated induction of Nrf1 is also achieved by its release from KEAP1. Furthermore, patulin was shown to activate various intracellular kinases including mitogen-activated protein kinases (MAPKs), p38 kinase, and c-Jun N-terminal kinase (JNK) (Liu et al., 2006). As Nrf1 also undergoes various post-translational modification including phosphorylation to modulate its stability and function, it is possible that the patulin-mediated induction of Nrf1 is achieved through modulating the activity of post-translational modification enzymes (Biswas et al., 2013; Han et al., 2017; Tsuchiya et al., 2013).

In summary, we identify patulin as an activator of Nrf1 expression and function. Furthermore, Nrf1 deficient cells showed greater sensitivity towards patulin toxicity. Together, these findings provide a new context in which Nrf1 may have a protective role in response to patulin, a prevalent toxin associated with various health problems.

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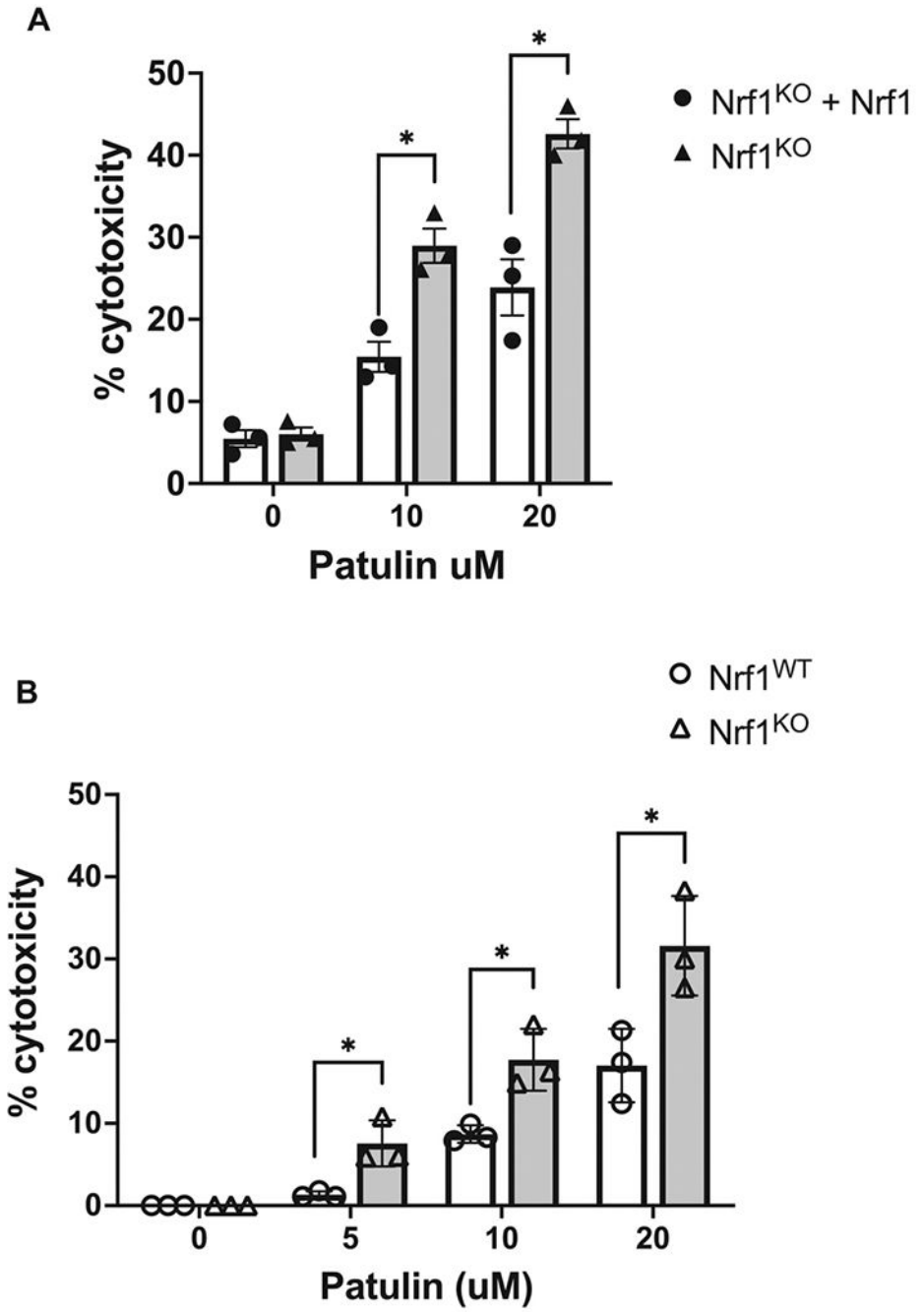

Fig. 1.

Nrf1 knockout cells show enhanced sensitivity to patulin-induced cytotoxicity. (A) Nrf1−/− and Nrf1−/− MEF cells complemented with Nrf1a cDNA were cultured with the indicated concentration of patulin. After 24 h, trypan blue dye exclusion assays were done. Percent dead cells was calculated as (dead cells/dead cells + live cells) x 100%. Data represents means ± SD for 3 independent experiments. Statistical analysis was done using Student's t-test (*) represents $p < 0.05$. (B) Nrf1 wild type and Nrf1 knockout HEK293 cells were treated with the indicated concentration of patulin. After 24 h, LDH-WST assays were

done. Percent cytotoxicity was calculated as (sample – negative control/positive control - negative control) x 100%. Data represents means ± SD for 3 independent experiments each containing 8 replicates. Statistical analysis was done using Student's t-test (*) represents $p < 0.05$.

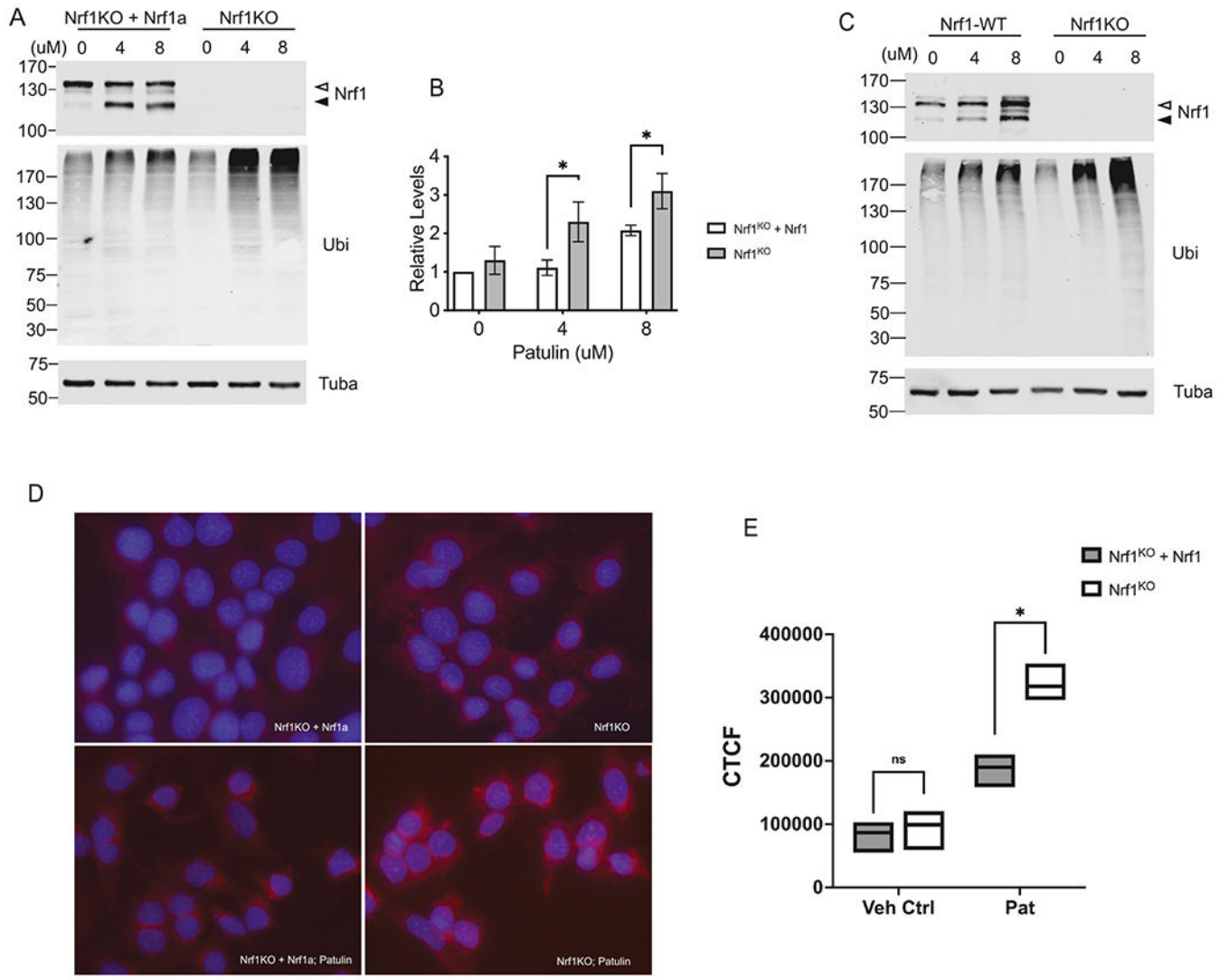

Fig. 2.

Patulin induces accumulation of ubiquitinated proteins and aggresomes in Nrf1 deficient cells. (A) Representative western blot showing total ubiquitin-bound proteins in Nrf1 knockout MEF cells and knockout cells rescued with Nrf1a, at basal conditions and following treatment with 4 μM or 8uM patulin for 24 h. Whole-cell lysates were subjected to western blotting using anti-TCF11 antibody (top panel), anti-ubiquitin (middle panel) and anti-alpha-tubulin (bottom panel). The anti-TCF11 antibody recognizes the long isoforms of Nrf1 (TCF11 and Nrf1a). The band migrating above 130 kDa corresponds to membrane localized proteins (open arrowhead), while the band migrating below 130 kDa corresponds to membrane-free Nrf1 (filled arrowhead). Western blot against alpha-tubulin was used for protein loading control. (B) Bar graph shows densitometric analysis of ubiquitin immunoblots normalized to alpha-tubulin. Values are expressed in arbitrary units, and presented as mean ± SD. Data were analyzed using Student's t-test, (*$P < 0.05$). (C) Western blot showing ubiquitin-bound proteins in wild type and Nrf1 knockout HEK293 cells, at basal conditions and following treatment with 4 μM or 8uM patulin for 24 h. Whole-

cell lysates were subjected to western blotting using anti-TCF11 antibody (top panel), anti-ubiquitin (middle panel) and anti-alpha-tubulin (bottom panel). (D) Cells were incubated for 24 h with 4 μM patulin or vehicle control, fixed and then stained with ProteoStat dye. The nuclei were stained with DAPI (blue). Representative images are shown. (E) Box plots of ProteoStat staining in cells. Data are mean ± SD of 2 independent experiments. Corrected total cell fluorescence (CTCF) calculations were done using images from 3 to 4 different high-power fields each containing at least 10 cells/condition. Data were analyzed using Student's t-test, (*$P < 0.05$).

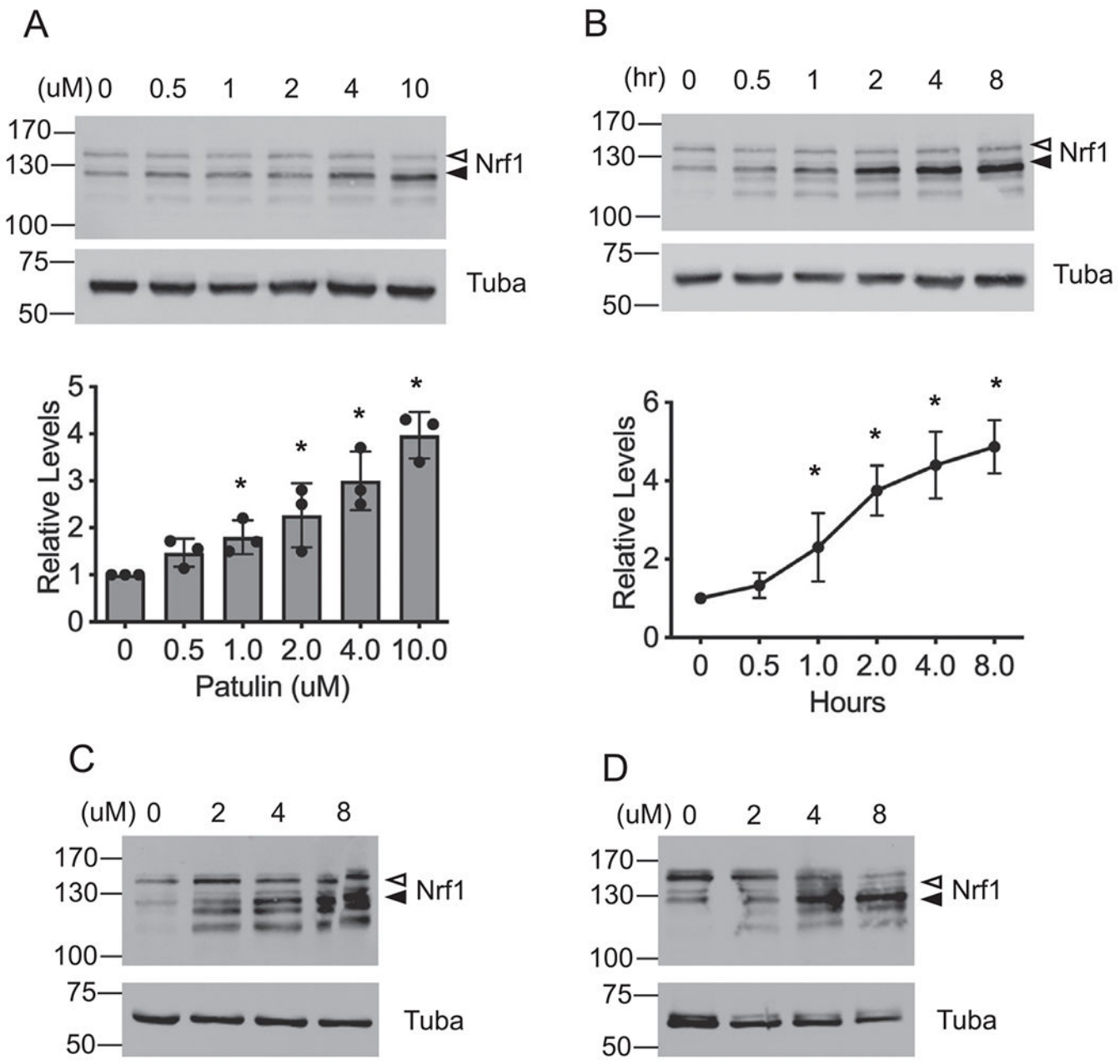

Fig. 3.

Patulin activates Nrf1 expression. (A) HEK293 cells were treated for 2 h with vehicle or with the indicated concentration of patulin. Cell lysates were then prepared and immunoblotted for Nrf1 using anti-TCF11 antibody which recognizes the TCF11 and Nrf1a isoforms. Membrane form (open arrowhead) of TCF11/Nrf1a are seen as multiple bands migrating above 130 kDa and membrane-free forms (filled arrowhead) are seen as bands migrating below 130 kDa. Western blotting against alpha-tubulin was used for protein loading control. (B) HEK293 cells were treated with 4 μM patulin, and cell lysates were prepared at the indicated time points for western blotting with anti-TCF11 and alpha-tubulin antibodies. Histograms showing the quantification of TCF11/Nrf1a relative to total α-tubulin

in HEK203 cells. Density values are expressed in arbitrary units (AUs), and data are mean ± SD (n = 3 replicates), one-way ANOVA, *P < 0.05. Dose response of patulin-induced TCF11/Nrf1a protein expression in (C) HCT116 and (D) MEF cells after 2 h of culture.

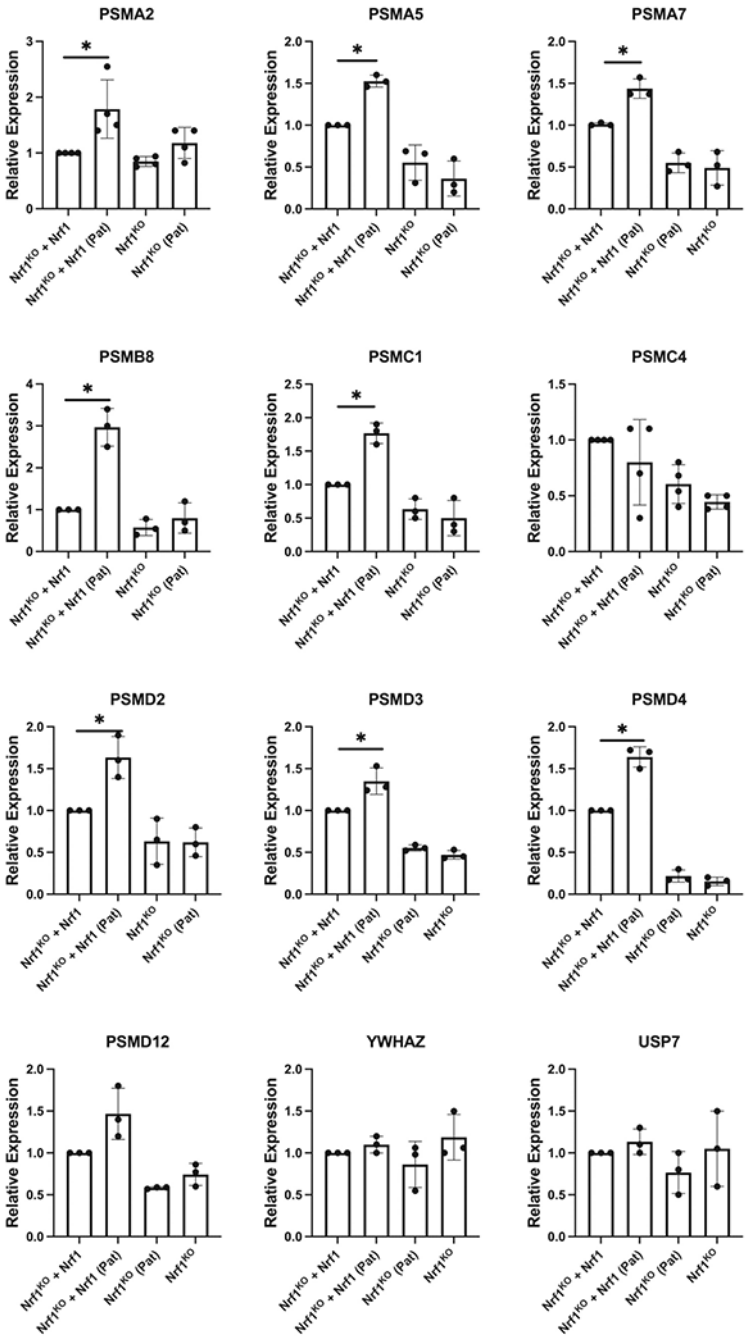

Fig. 4.

Patulin upregulates the proteasome pathway through Nrf1. Nrf1−/− MEF cells, or Nrf1−/− expressing Nrf1a were cultured with vehicle control or 2uM patulin. After 16 h, RNA was extracted and analyzed for expression of known Nrf1 target genes. Dot-plots depict relative expression of indicated genes. P values were calculated by Student's t-test (n = 3), (*) indicates $p < 0.05$.