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Yessotoxin triggers ribotoxic stress

Mónica Suárez Korsnes [a,*], Susan Skogtvedt Røed [b], Michael A. Tranulis [b], Arild Espenes [b], Berit Christophersen [b]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU), Campus Ås, P.O. Box 5003, NO-1432 ÅS, Norway
[b] Norwegian University of Life Sciences (NMBU), Campus Adamstuen, P.O. Box 8146, NO-0033 OSLO, Norway

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ABSTRACT

This work tests the hypothesis that the marine algal toxin yessotoxin (YTX) can trigger ribotoxic stress response in L6 and BC3H1 myoblast cells. YTX exposure at a concentration of 100 nM displays the characteristics of a ribotoxic stress response in such cells. The exposure leads to activation of the p38 mitogen-activated protein kinase, the stress-activated protein kinase *c-jun*, and the double-stranded RNA-activated protein kinase (PKR). YTX treatment also causes ribosomal RNA cleavage and inhibits protein synthesis. These observations support the idea that YTX can act as a ribotoxin.

1. Introduction

Yessotoxin (YTX) is a marine polyether compound produced by dino-flagellates and which can concentrate in filter feeding bivalves (Satake et al., 1997; Draisci et al., 1999; Ciminiello et al., 2003; Paz et al., 2004). It can induce apoptosis in different model systems (Korsnes and Espenes, 2011). The toxin can also induce non-apoptotic cell death in BC3H1 myoblast cells, primary cortical neurons and glioma cells (Korsnes et al., 2011; Alonso et al., 2013; Rubiolo et al., 2014).

The complexity of cellular responses to YTX exposure has recently called attention for possible medical applications (López et al., 2008, 2011b; Korsnes, 2012; Alonso et al., 2013; Kornienko et al., 2013). The understanding of mechanisms of action of YTX is developing. Its effects on cells seem to be cell-specific and concentration-dependent (De la Rosa et al., 2001; Malaguti et al., 2002; Leira et al., 2002; Alfonso et al., 2003; Malagoli et al., 2006; Korsnes et al., 2007; Callegari and Rossini, 2008; Dell'Ovo et al., 2008; Ronzitti and Rossini, 2008; Young et al., 2009; Orsi et al., 2010; López et al., 2011a; Martín-López et al., 2012; Pang et al., 2012).

The present contribution adds further complexity to the debate on cytotoxic responses to YTX. It indicates, for the first time, that YTX can induce ribotoxic stress response which is a cellular reaction to a site-specific damage in the 28s rRNA (Iordanov et al., 1997). These findings may enhance the attention to YTX within medical research.

The ribotoxic stress response is conserved between prokaryotes and eukaryotes. It involves a universal and evolutionary conserved function of the ribosome in both sensing stress in highly conserved regions of the 28S rRNA and inducing subsequent cellular response (Iordanov et al., 1998). Ribotoxic stress response has been defined as a damage within a conserved (the alpha-sarcin) loop of the 28S ribosomal RNA (28S rRNA), leading to inhibition or partial inhibition of protein synthesis, transcriptional activation of the immediate-early genes *c-jun* and *c-fos* and activation of stress kinases (Uptain et al., 1997; Iordanov et al., 1997, 1998; Shifrin and Anderson, 1999; Laskin et al., 2002).

The 28S rRNA consists of highly conserved domains as well as so-called divergent domains (D1 to D12). The divergent domains (D1 to D12) represent RNA which has diversified during eukaryotic evolution and they constitute nearly half of the 28S rRNA in higher eukaryotes (Gutell and Fox, 1988). D domains have no known function, however, they probably take part in the translational machinery as riboregulators, protein anchoring regions, or as domains for RNA-RNA interactions (Raué et al., 1988; Gutell and Fox, 1988; Houge and Døskeland, 1996; Degen et al., 2000). 18SrRNA and 28SrRNA are constituents of the 80S ribosomal complex participating in translation of mRNAs. Their cleavage may contribute to inhibition of protein synthesis (Degen et al., 2000).

* Corresponding author. Tel.: +47 67230000.
E-mail address: monica.suarez.korsnes@nmbu.no (M.S. Korsnes).

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Damage of the ribosomal 28S rRNA can lead to activation of the stress kinases JNK/SAPK1, p38 and transcriptional induction of immediate early genes such as *c-fos* and *c-jun* (Iordanov et al., 1997; Shifrin and Anderson, 1999; Laskin et al., 2002; Zhou et al., 2003, 2005; He et al., 2012). The molecular linkage between ribosome interaction and MAPK signalling remain incompletely understood (He et al., 2012). Shifrin and Anderson (1999) suggested, however, that the ribotoxic stress response not always requires active translation of the proteins. The 28S rRNA has therefore been reported as a specific sensor for stress induced by a subset of compounds inhibiting protein synthesis (Iordanov et al., 1997).

The double-stranded RNA-activated protein kinase R (PKR) is a widely expressed serine/theonine protein kinase containing two double stranded (ds) RNA-binding domains (Sadler and Williams, 2007). Ricin, Shiga Toxin 1 and interferon are examples of agents which can activate PKR via these dsRNA-binding domains (Williams, 2001; Gray et al., 2008). PKR associates with the ribosome in close proximity to the peptidyl transferase center, which is a site in the ribosome where peptide-bond formation occur. Some thrichothecene mycotoxins, for example, can trigger activation of PKR during a process cleaving the 28S rRNA (Bae et al., 2010). PKR association with the ribosome can therefore serve as a sensor for 28S rRNA damage (Zhou et al., 1997; Kumar et al., 1999).

PKR is a critical upstream mediator of ribotoxic stress induced by deoxynivalenol and other translational inhibitors (Zhou et al., 2003, 2005; He et al., 2012). PKR can also mediate activation of MAPK signalling pathways and induce rRNA cleavage (Williams, 2001; Zhou et al., 2003).

Many protein inhibitors can induce ribotoxic stress in different cellular systems through activation of JNK/SAPK1 and p38 MAPK pathways culminating in apoptosis. Induction of apoptosis is typically cell-specific. Examples of such inhibitors are the trichothecene mycotoxins, anisomycin, Shiga toxin 1, Deoxynivalenol (DON), T2-triol, ricin A, the tumor promoter palytoxin and ribosome inactivating proteins (RIPs) (Iordanov et al., 1997; Iordanov and Magun, 1999; Shifrin and Anderson, 1999; Kojima et al., 2000; Yang et al., 2000; Laskin et al., 2002; Narayanan et al., 2005; Pestka et al., 2004; Pestka, 2010).

Anisomycin, which is a well known ribotoxic stressor, can induce rapid apoptosis in lymphoid cells (Polverino and Patterson, 1997), but weakly in HeLa cells (Lee et al., 2005). Discrepancies in cell death response may be due to binding of anisomycin to the different site on ribosomes generating different signalling pathways that activate downstream kinases (Ouyang et al., 2005). Trichothecene mycotoxins are known ribotoxic stressors activating the MAP kinases, but not all of them are effective inhibitors of protein synthesis. Activation of the MAP kinases therefore seems not to be a requirement for initiating the ribotoxic stress response (Laskin et al., 2002).

Different types of mammalian cells can undergo ribotoxic stress (Houge et al., 1995; Iordanov et al., 1997, 1998; Shifrin and Anderson, 1999; Kojima et al., 2000; Pestka et al., 2004; Pestka, 2010). However, there is still dispute about the nature of signals that can trigger it. Bunyard et al. (2003) proposed that the signals may include a "pattern-recognition" of receptors in the cell surface after interaction with the chemical compound. The ribotoxic stress response might therefore be a complex response in which interactions of the toxic agent with the ribosomes are highly selective.

The present work evaluates the capacity of YTX to induce the ribotoxic stress response in L6 and BC3H1 myoblast cells exposed to 100 nM YTX. It attempts to identify some upstream and downstream signalling events typical for a ribotoxic stress response triggered by YTX exposure.

2. Materials and methods

2.1. Toxins

YTX was provided by Dr.Christopher O. Miles at the National Veterinary Institute of Norway. YTX was dissolved in methanol as a 50 μM stock solution. The stock solution was diluted in Dulbecco's modified Eagle's medium (DMEM, Sigma), achieving a final concentration of 100 nM YTX in 0.2% methanol. Treated cells were incubated with 100 nM YTX and control cells were incubated with 0.2% methanol as vehicle. Control cells and treated cells were exposed to different end points (24 h, 40 h, 48 h and 72 h). Okadaic acid was provided by Dr. John A.B. Aasen at the Norwegian School of Veterinary Science. Okadaic acid was dissolved in methanol as a 25 μM stock solution. The stock solution was diluted in Dulbecco's modified Eagle's medium (DMEM, Sigma), achieving a final concentration of 50 nM. Treated cells were incubated with 50 nM okadaic acid and control cells were incubated with 0.2% methanol as vehicle. Okadaic acid treated cells in the RNA fragmentation assay were exposed to two different end points (3 h and 24 h). Experiments for every specific assay were independently carried out more than three times with the exception of the protein synthesis assay which was performed independently two times six months apart.

2.2. Cell culture

L6 cell lines were isolated from primary cultures derived from rat thigh muscle (ATCC Number CRL-1458). L6 cells fuse in culture to form multi-nucleated myotubes and striated fibres. BC3H1 cell lines were isolated from primary cultures derived from mouse (ATCC Number CRL-1443). Recent data suggest that BC3H1 cells closely resemble cells in an arrested state of skeletal muscle differentiation than smooth muscle cells. Both cell lines were purchased from the American Type Culture Collection (Manassas, USA) at a seeding density of 2×10^6 cells/cm^2. L6 Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, MedProbe). BC3H1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum. Cells were maintained undifferentiated at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Western blotting analysis

The analysis of phospho-p38 MAPK, phospho *c-jun*, *β*-actin and PKR was performed by using the anti-p38, anti-*c-jun*, anti-*β*-actin and anti-PKR antibodies (Cell signalling, Upstate, USA and Millipore) Briefly, control and YTX-treated cells were scraped on ice cold PBS and centrifuged at 600*g* for 10 min at 4 °C. 2×10^6 cells were resuspended in 100 μl of RIPA extraction buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, o.25% NeDoc, 1% NP40 and protease inhibitors.

The samples were incubated one hour at room temperature and lysates were often vortexed. The homogenates were transferred to an eppendorf tube and centrifuged at 700*g* for 10 min at 4 °C. The resultant supernatants were collected as cytosolic fractions. 120 μg of protein were separated on a 12% Bis-Tris polyacrylamide gels (BioRad) for one hour at 200 V, transferred onto a PVDF membrane and blocked with 5% nonfat dry milk in PBS. The membrane was probed with anti-p38, anti-*c-jun*, anti PKR diluted 1:500, and for *β*-actin 1:10.000 overnight at 4 °C. The membrane was washed with PBST (3×10 min) and incubated with a secondary antibody (goat anti rabbit) labelled with alkaline phosphatase, diluted 1:2:500. Immunoblotted bands were visualised with a variable

mode fluorescence imager (Typhoon 9200, GE Healthcare) after incubation with the ALP substrate (ECF, GE Healthcare).

2.4. Measurement of protein synthesis

Inhibition of protein synthesis was measured by the L-[U-^{14}C]leucine incorporation assay, with small modifications of the method of (Lindbäck and Granum, 2006). Briefly, 300×10^5 cells were added to each well of a 24-well tissue culture plate and cultured for (24 h, 48 h, and 72 h). Cells were exposed to 100 nM YTX concentration for (24 h, 48 h, and 72 h). Preheated 37 °C low-leucine medium was added to each well.

Cells were then washed with MEM medium (low-leucine medium) to remove the toxin. Cells were incubated for two hours in a low-leucine medium. 8 ml of preheated low-leucine medium with 16 µl isotope were mixed and the mixture added to each well. Cells were incubated for one hour at 37 °C. The radioactive medium was removed and 1 ml of 5% of trichloroacetic acid (TCA) was added to each well and incubated at room temperature for 10 min. TCA was removed and the wells were washed twice with 1 ml 5% TCA. After removing the TCA, 300 µl of 0.1 M KOH was added to each well and incubated at room tempearture for 10 min. The content of each well was transferred to liquid scintillation tubes with 2 ml of liquid scintillation cocktail. Tubes were vortexed and radioactivity measurements were performed in a scintillation counter for 1 min.

2.5. RNA isolation and analysis

RNA was isolated by using the RNeasy Mini kit (QIAGEN). Briefly, 2×10^6 Control and YTX-treated cells were washed in PBS, lysed with RTL lysis buffer supplied with the kit, mixed thoroughly and homogenize the lysate by vortexing for 1 min. The mixture was transferred into a QIAshredder spin column and centrifuged for 2 min at full speed. One volume of 70% ethanol was added to homogenized the lysate, mixed well by pipetting. Samples were transferred to an RNAse spin column placed in a 2 ml collection tube, centrifuged for 15 s at 10,000 rpm. The flow-through was discarded. The column was washed twice with washing buffer and centrifuged for 15 s at 10,000 rpm. A third wash was done for 2 min at 10,000 rpm to dry the spin column membrane and avoid residual ethanol. Residual ethanol may interfere with downstream reactions. RNA was eluted in 50 µl of RNAse-free dH2O and collected in an eppendorf tube. Total RNA concentration was determined by spectrophotometry using the NanoDrop 1000 (Thermo Scientific). Total RNA was stored at −85 °C. Electrophoresis was performed in 1.5% agarose gels with 1X Tris–acetate-EDTA (TAE) as running buffer. Samples containing 2 µg totRNA were diluted in 2X formamid/orangeG loading buffer giving a final concentration of 47.5% formamide. Immediately before electrophoresis the samples were denatured at 75 °C for 10 min and then chilled on ice for 5 min. Electrophoresis was performed at 7 V/cm. The gel was stained in a SybrSafe solution (Life Technologies), according to the manufacturer's manual.

2.6. DNA isolation

DNA was isolated by using the DNeasy Tissue kit (QIAGEN) and used according to the manufacturer's instructions. Briefly, 2×10^6 Control and YTX-treated cells were pelleted and lysed by adding proteinase K to a final concentration of 20 mg/ml, resuspended in lysis buffer supplied with the kit and vortexed. The samples were incubated at 70 °C for 10 min. After adding 100% ethanol to the samples, the mixture was transferred to a DNeasy spin column and centrifuged at 6000 rpm for one minute to collect the DNA. The column was washed twice with washing buffer and centrifuged for one min at full speed to dry the membrane and avoid residual ethanol which may interfere with subsequent reactions. DNA was eluted in 200 µl of elution buffer supplied with the kit and collected in an eppendorf tube.

2.7. Agarose gel electrophoresis

Samples were subjected to 1.5 % agarose gel electrophoresis at 50 V for 2.5 h containing 0.1 µg/ml ethidium bromide. Tris Acetate EDTA (TAE) was used as the running buffer. DNA was visualized under UV light.

3. Results

The present experiments demonstrate that YTX can induce ribotoxic stress response in L6 and BC3H1 cells. The actual concentration were in this case 100 nM, and the exposure times were 24 h, 48 h, and 72 h. Such exposure leads to 28S rRNA cleavage, activation of PKR, *c-jun*, p38 MAP kinase as well as protein synthesis inhibition. These features are characteristic for ribotoxic stress response (Iordanov et al., 1997, 1998).

Agarose gel electrophoresis demonstrated the cleavage in the 28S rRNA. These analyses indicated a time-dependent decrease of the 28S rRNA band intensity for both cell lines. The 18S rRNA band appeared relatively intact in control and treated cells but giving rise to multiple cleavages in the conserved 28S rRNA regions flanking the D6 and D8 domains (Fig. 1). YTX appears to induce cleavage of the 28S rRNA without DNA fragmentation (Fig. 2).

He et al. (2012) showed that rRNA cleavage requires activation of PKR, p38, caspase 3, 8 and 9. Korsnes et al. (2006b) has already shown caspase 3 and 9 activation under YTX exposure. The results here (Fig. 3) suggest that YTX induces PKR activation in L6 and BC3H1 cells as evidenced by its own phosphorylation.

Iordanov et al. (1997, 1998) proposed that ribotoxic stress activates the mitogen-activated protein kinase p38 and the stress-activated protein kinase *c-jun*. The present experiments therefore analysed if YTX exposure can activate p38 and *c-jun*. It appeared to induce phosporylation of p38 MAPK in both L6 and

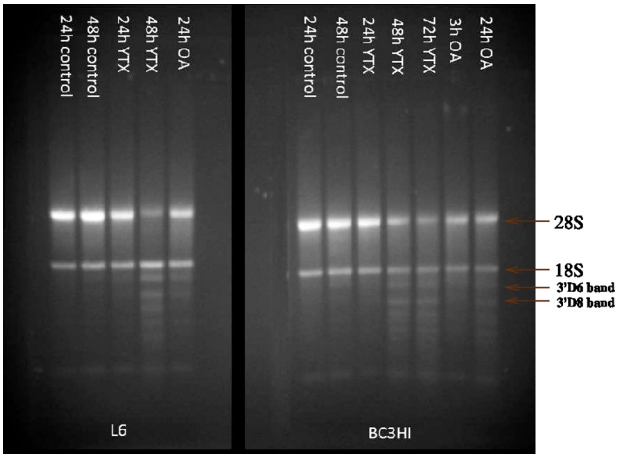

Fig. 1. Result from agarose gel RNA electrophoresis showing 28S rRNA cleavage in L6 and BC3H1 myoblast cell lines exposed to yessotoxin (YTX) and Okadaic acid (OA). Cells were treated with YTX (100 nM) and OA (50 nM) for indicated end points. The intact 28S rRNA band was reduced in a time-dependent manner. The 18S rRNA band appears relatively intact after exposure but giving rise to more fragmentation bands. The fragmentation next below the 18S band match for YTX and OA. These fragments might correspond to the conserved 28S rRNA regions flanking the D6 and D8 domains, showing 3'D6 and 3'D8 bands similarly reported in leukemia cells treated with okadaic acid and bovine endothelial cells treated with recombinant TNF combined with cycloheximide (Houge et al., 1995; Samali et al., 1997). Pictures are representative of more than three separate experimental setups.

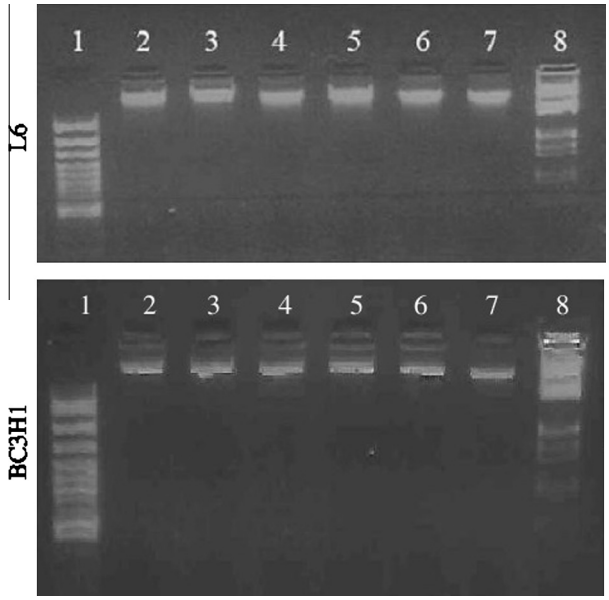

Fig. 2. Results from agarose gel DNA electrophoresis of L6 (upper) and BC3H1 cells (lower) exposed to 100 nM YTX showing no DNA fragmentation. Sample identities and incubation times for L6 cells as follows: (1) Lambda Hind III. (2) Control cells, 24 h. (3) Control cells, 40 h. (4) Control cells, 48 h. (5) 100 nM YTX, 24 h. (6) 100 nM YTX, 40 h. (7) 100 nM YTX 48 h. (8) 100 bp ladder. Sample identities and incubation times for BC3H1 cells as follows: (1) Lambda Hind III. (2) Control cells, 24 h. (3) Control cells, 48 h. (4) Control cells, 72 h. (5) 100 nM YTX, 24 h. (6) 100 nM YTX, 48 h. (7) 100 nM YTX 72 h. (8) 100 bp ladder. Pictures are representative of more than three separate experimental set-ups.

BC3H1 cells in a time-dependent manner. P38 MAP kinase was activated after 24 h and continued to be activated up to 48 h (Fig. 4).

The *c-jun* protein is a central nuclear target of the JNK signal transduction pathway. The activity of *c-jun* is regulated at both transcriptional and post-translational levels (Mielke et al., 1999). *C-jun* phosporylation appeared in the present experiments to be activated in both L6 and BC3H1 cells exposed for 24 h. The activity of *c-jun* in L6 cells was different as compared to BC3H1 cells. The level of *c-jun* phosphorylation was found increased at 24 h for L6 cells and persisted at slightly enhanced levels up to 48 h. Phosphorylated *c-jun* in BC3H1 cells also increased at 24 and 48 h and peaked at 72 h. *C-jun* phosphorylation in BC3H1 cells at 24 h was similar to that observed in the positive control anisomycin at 24 h exposure (Fig. 5).

Compounds acting through the ribotoxic stress response inhibit or partially inhibit protein synthesis in the same dose-range as their activation of stress kinases (Iordanov et al., 1998). They exert their action on ribosomes, thus leading to inhibition of protein synthesis. The present experiments determined protein synthesis inhibition by measuring the incorporation of leucine into cellular proteins (Figs. 6 and 7). These measurements were performed two times six months apart where the second set of measurements were done with newly ordered cell lines. The capacity of YTX to inhibit protein synthesis in L6 and BC3H1 cells at 100 nM YTX concentration was evident for both cell lines. Both sets of measurements show a clear reduction in protein synthesis over time. Inhibition of protein synthesis occurred faster in L6 cells after 24 h as compared to BC3H1 cells.

4. Discussion

A large variety of toxins and compounds can cause ribotoxic stress response. Examples of such agents are anisomycin, ricin A, α-sarcin, *E-coli*-derived Shiga toxin, UV, palytoxin and thrichothecene mycotoxins (Iordanov et al., 1997; Shifrin and Anderson, 1999; Smith et al., 2003; Zhou et al., 2005; Lee et al., 2006; Iordanov et al., 1998; Pestka, 2010; He et al., 2012; Pan et al., 2013). These toxins are termed ribotoxic stressors or ribotoxins.

Ribotoxic stress response includes a cascade of events such as 28S rRNA cleavage, protein synthesis inhibition, MAPK and PKR activation, transcriptional activation of the immediate early genes *c-fos* and *c-jun* and often induction of apoptosis (Iordanov et al., 1997; Shifrin and Anderson, 1999). The present study evidences these traits during YTX exposure. It shows, for example, that YTX exposure induces cleavage of the 28S rRNA, considered to be a highly specific event in the ribotoxic stress response (Iordanov et al., 1997; Shifrin and Anderson, 1999).

28S rRNA cleavage can correlate to apoptosis induction (Houge et al., 1993, 1995; Houge and Døskeland, 1996). It coincides with DNA fragmentation during apoptosis (Houge et al., 1995). However, 28S rRNA cleavage can take place without concomitant DNA fragmentation. This can for example happen for Molt-4 cells treated with okadaic acid which is known to be a general apoptotic inducer (Bøe et al., 1991; Houge et al., 1995; Samali et al., 1997).

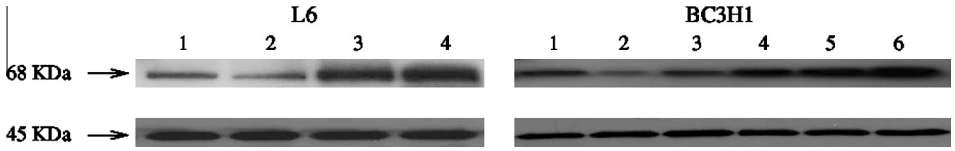

Fig. 3. Western blotting analysis of pPKR in L6 and BC3H1 cells exposed to 100 nM. For L6 cells (upper left): (1) Control cells 24 h. (2) Control cells 48 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. For BC3H1 cells (upper right): (1) Control cells 24 h. (2) Control cells 48 h. (3) Control cells 72 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. (5) Cells treated with YTX 72 h. Loading control β-actin (lower). Pictures are representative of more than three separate experimental set-ups.

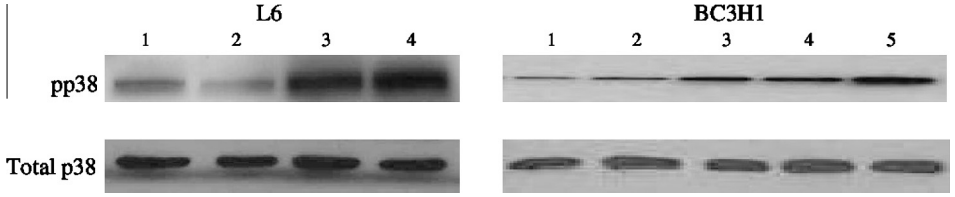

Fig. 4. Western blotting analysis of pp38 MAPK in L6 and BC3H1 cells exposed to 100 nM. For L6 cells (upper left): (1) Control cells 24 h. (2) Control cells 48 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. Total protein p38 (lower). For BC3H1 cells (upper right): (1) Control cells 24 h. (2) Control cells 48 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. (5) Cells treated with YTX 72 h; Total protein p38 (lower). Pictures are representative of more than three separate experimental set-ups.

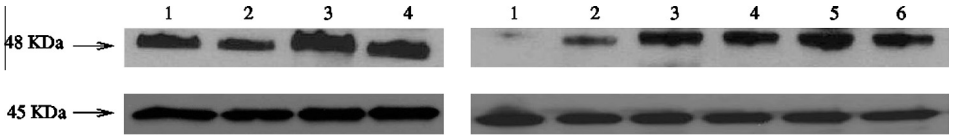

Fig. 5. Western blotting analysis of p*c-jun* in L6 and BC3H1 cells exposed to 100 nM. For L6 cells (upper left): (1) Control cells 24 h. (2) Control cells 48 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. For BC3H1 cells (upper right): (1) Control cells 24 h. (2) Control cells 48 h. (3) Cells treated with YTX 24 h. (4) Cells treated with YTX 48 h. (5) Cells treated with YTX 72 h. (6) Cells treated with Anisomycin 24 h. Loading control β-actin (lower). Pictures are representative of more than three separate experimental set-ups.

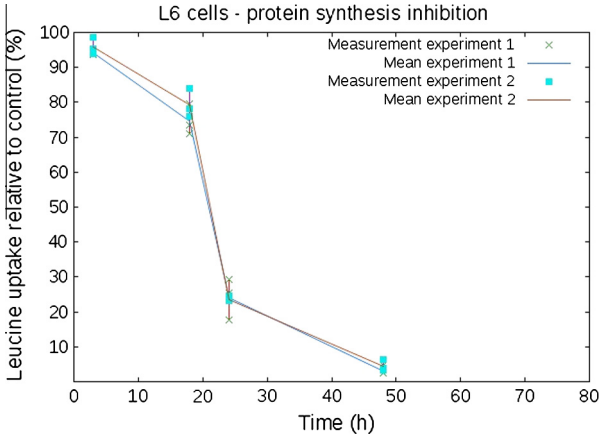

Fig. 6. Results from measurements of L-[U-^{14}C]leucine uptake in L6 cells exposed to 100 nM YTX for 3, 18, 24 and 48 h. The figures are from two independent experiments and three measurements per time point for each experiment. The percentage of inhibition of protein synthesis was calculated in relation to an untreated control sample.

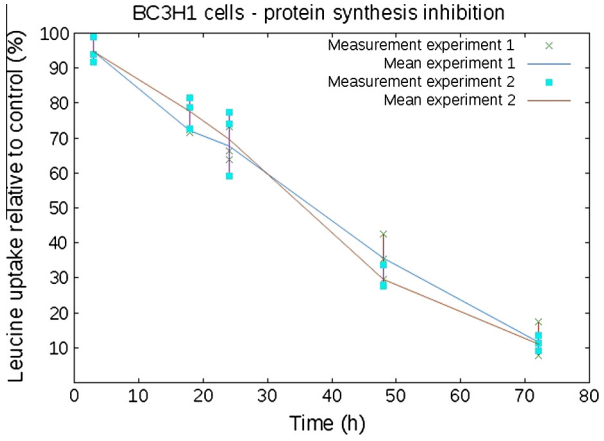

Fig. 7. Results from measurements of L-[U-^{14}C]leucine uptake in BC3H1 cells exposed to 100 nM YTX for 3, 18, 24 h and 48 h and 72 h. The figures are from two independent experiments and three measurements per time point for each experiment. The percentage of inhibition of protein synthesis was calculated in relation to an untreated control sample.

The 28S rRNA cleavage in such treated cells reveals multiple fragments under the 18S band. These fragments have been denoted as the 3'D6 and 3'D8 bands or B band localised in the D8 divergent domain of the 28S rRNA (Houge et al., 1995; Samali et al., 1997). The fragmentation pattern of the 28S rRNA in L6 and BC3H1 cells treated by YTX and okadaic acid (Fig. 1) conforms to the corresponding pattern for Molt-4 cells reported by Houge et al. (1995) and Samali et al. (1997). YTX, similar to okadaic acid induces also multiple cleavages under the 18S band.

Ribotoxic stress response is often associated with apoptosis (Iordanov et al., 1997; Shifrin and Anderson, 1999). However, it might be associated with non-apoptotic cell death since BC3H1 cells exposed to YTX also can undergo paraptotic-like cell death (Korsnes et al., 2011) which appears to be kinetically slower than apoptosis (Sperandio and deBelle, 2008). The detailed mechanisms of YTX toxicity are still unknown, and elaborations of small perturbations of the molecule have not so far revealed significant change in its toxicity (Korsnes et al., 2013). Further work might clarify induction of ribotoxic stress by YTX and its role in programmed cell death.

Ribotoxic stress can exhibit significant variation. Shifrin and Anderson (1999) reported differences in the cascade of events during ribotoxic stress response triggered by trichothecene mycotoxins. Some trichothecene mycotoxins inhibit protein synthesis, activate MAPK kinases and die by apoptosis (Iordanov et al., 1997; Shifrin and Anderson, 1999; Laskin et al., 2002; Zhou et al., 2003, 2005; Pestka et al., 2004; He et al., 2012). Other trichothecenes inhibit protein synthesis without activating MAP kinases and inhibit induction of apoptosis (Shifrin and Anderson, 1999). Intrinsic and extrinsic apoptotic signalling pathways, as well as cross-talk, may occur under ribotoxic stress (Zhou et al., 2005; Xia et al., 2007; He et al., 2012).

The ability of some trichothecenes to activate MAPK kinases resides probably in the absence of side groups interacting with ribosome target sites such as the peptidyl transferase site or displace the binding of ribosome associated molecules. Trichothecenes appear therefore to interact with the ribosomal peptidyl transferase site in a multifactorial way (Shifrin and Anderson, 1999).

YTX can induce p38 MAP kinase activation and *c-jun* phosphorylation in L6 and BC3H1 cells. Korsnes et al. (2011) previously reported SAPK/JNK activation from the same type of exposure. Ribotoxic stress response appears to be mediated by MAPK signalling pathways which are typical key modulators of stress responses.

YTX exposure differently affects L6 and BC3H1 cells. It causes faster inhibition of protein synthesis in L6 cells than for BC3H1 cells. Efficient MAP kinases activation during exposure is achieved for both cell types even though protein synthesis is inhibited. YTX therefore seems to activate MAP kinases independent of its capacity to inhibit protein synthesis. Functional ribosomes are required for activation of MAP kinases signalling and protein synthesis inhibition (Iordanov et al., 1997). Cells containing translationally inactivated ribosomes, fail to activate MAP Kinases in response to anisomycin, ricin A and α-sarcin (Iordanov et al., 1997). YTX may therefore target translationally active ribosomes.

Ribotoxic stressors have in general a large range of medical applications. Their ability to inhibit protein synthesis can be useful for killing cells which are resistant to apoptosis such as melanoma cells (Risberg et al., 2009). The well known ribotoxic stressor anisomycin can for example sensitize glioblastoma cells to die by modulating the death receptor pathway (Xia et al., 2007). Protein synthesis inhibition specially affects cells with high requirement for protein synthesis such as transformed cells. Translation regulation of some of the proteins involved in cancer progression, can therefore be modulated by protein synthesis inhibitors (Chan et al., 2004).

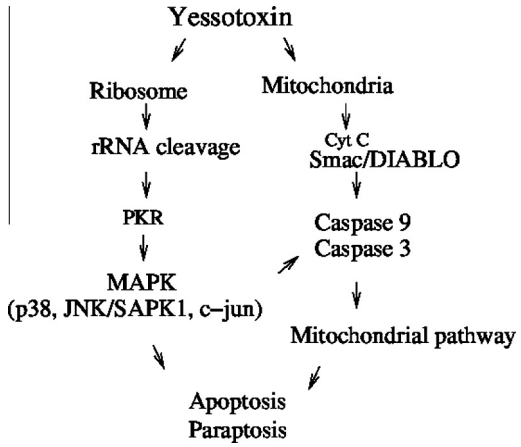

Fig. 8. Hypothetical model for ribotoxic stress signalling pathway.

Ribosomal rRNA cleavage has been shown to occur in parallel with both extrinsic and intrinsic pathways of programmed cell death (He et al., 2012). YTX induces rRNA cleavage and it also induces apoptosis through activation of the mitochondrial pathway (Korsnes et al., 2006a). It remains to clarify potential linkage between rRNA cleavage and induction of the intrinsic pathway.

This work corroborates that ribotoxic stress response appears to be complex and involve multiple mechanisms and cross-talk among signalling pathways. It may for example involve ER stress response (Pestka, 2010; Lee et al., 2010; Schmeits et al., 2014). Rubiolo et al. (2014) recently showed induction of autophagic cell death through endoplasmic reticulum (ER) stress in glioma cells exposed to YTX. Cross-talk between ribotoxic stress and ER stress response may take place. Further work might evidence such cross-talk.

Takizawa et al. (2002) and Zhou et al. (2003) reported a clear link between MAPK and PKR activation as a transducer of ribotoxic stress culminating in apoptosis. YTX exposure also induces PKR phosphorylation linked to 28S rRNA cleavage but culminating in a non-apoptotic cell death (Korsnes et al., 2011).

Iordanov et al. (1997) and Shifrin and Anderson (1999) suggested that ribotoxins like DON and anisomycin, which share common binding sites on rRNA, might initiate and activate identical signal transduction pathways even if they have different structure and activity (Zhou et al., 2003, 2005; Pestka, 2010; He et al., 2012).

The present work shows that YTX can induce p38 MAP kinase phosphorylation, *c-jun* and PKR activation, inhibit protein synthesis and cleavage the 28S rRNA. This cascade of signalling events induced by YTX are quite similar to those reported by ribotoxins like anisomycin and DON (Zhou et al., 2003, 2005; Pestka, 2010; He et al., 2012). YTX may act as a ribotoxin activating similar and conserved signalling pathways upstream to rRNA cleavage as the ribotoxic stressors DON and anisomycin. The probability that YTX might share common binding sites on rRNA with DON and anisomycin remains to be clarified.

Fig. 8 illustrates the present view of YTX mechanisms of action. The complexity here makes YTX a relevant tool for medical research as a ribotoxin or ribotoxic stressor, protein synthesis inhibitor, potent activator of MAP kinase signalling and programmed cell death inducer. Further work remains to clarify more precisely upstream and downstream signalling events under ribotoxic stress response and the linkage to programmed cell death mechanisms.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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