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Yessotoxin triggers ribotoxic stress

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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.
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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).

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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

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