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Comparative effects of nodularin and microcystin-LR in zebrafish: 2. Uptake and molecular effects in eleuthero-embryos and adult liver with focus on endoplasmic reticulum stress

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ABSTRACT

Microcystin (MC) and nodularin are structurally similar cyanobacterial toxins that inhibit protein phosphatases. Additional modes of action are poorly known, in particular for nodularin. In our associated work, we showed that active cellular uptake is mediated by the organic anion transporting polypeptide drOatp1d1 in zebrafish (Faltermann et al., 2016). Here, we assessed the transcriptional expression of three genes encoding three uptake transporters during embryonic development from 24 h post fertilization (hpf) to 168 hpf. Transcripts of *drOatp1d1* and *drOatp2b1* are present at 24 hpf. The abundance increased after hatching and remained about constant up to 168 hpf. Transcripts of *drOatp2b1* were most abundant, while *drOapt1f* transcripts showed very low relative abundance compared to *drOatp1d1* and *drOatp2b1*. We further demonstrated the uptake of fluorescent labeled MC-LR in eleuthero-embryos and its accumulation in the glomerulus of the pronephros.

An important molecular effect of MC-LR in human liver cells is the induction of endoplasmic reticulum (ER)-stress. Here, we investigated, whether MC-LR and nodularin similarly lead to induction of ER-stress in zebrafish by analyzing changes of mRNA levels of genes indicative of ER-stress. In zebrafish liver organ cultures short- and long-term exposures to 0.15 and 0.3 μmol L^{-1} MC-LR, and 0.5 and 1 μM L^{-1} nodularin led to significant transcriptional induction of several ER-stress marker genes, including the chaperone *glucose regulated protein 78* (*bip*), the spliced form of *x-box binding protein* (*xbp-1s*), the *CCAAT-enhancer-binding protein homologous protein* (*chop*) and *activating transcription factor 4* (*atf4*). Furthermore, strong transcriptional changes occurred for *tumor necrosis factor alpha* (*tnfa*) and *dual specificity phosphatase 5* (*dusp5*), associated with mitogen activated protein kinase (MAPK) pathway. However, no alterations in transcript levels of pro-apoptotic genes *Bcl-2 like protein 4 (bax)* and *p53* occurred. In contrast to adult liver, MC-LR and nodularin did not result in detectable changes of mRNA levels of selected target genes involved in ER-stress in zebrafish eleuthero-embryos, nor was the abundance of transcripts belonging to the MAPK and pro-apoptosis pathways altered.

In conclusion, our data indicate that MC-LR and nodularin have similar transcriptional effects. They lead to changes in mRNA levels of genes that suggest induction of ER-stress, and furthermore, lead to increased level of *tnfα* mRNA in the adult liver, which suggests a novel (transcriptional) mode of action in fish. However, although taken up by eleuthero-embryos, no transcriptional changes induced by these cyanobacterial toxins were detected. This is probably due to action to specific organs such as liver and kidneys that could not be identified by whole-embryo sampling.

1. Introduction

Exposure of humans and animals to cyanobacterial toxins from cyanobacterial blooms is an increasing global problem due to inten-

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sifying eutrophication and global climate change (Paerl and Paul, 2012). In freshwater, microcystin (MC) producing cyanobacteria are of importance, whereas in brackish water, nodularin producing cyanobacteria are relevant. Cyanobacterial blooms may lead to toxicity in fish populations (Wiegand and Pflugmacher, 2005). Whereas the effects of MCs are fairly well investigated, effects of nodularin are less known. However, these cyclic heptapeptides share structural similarities, and toxicokinetic and toxicodynamic properties (Fujiki and Suganuma, 2011). Due to their large size, cellular uptake functions by active transport via organic anion transporting polypeptides (Oatp). In zebrafish liver, *Oatp2b1* and *Oatp1d1* are predominant (Popovic et al., 2010). In our associated paper, we showed that Oatp1d1 mediates cellular uptake of MC-LR and nodularin (Faltermann et al., 2016). In contrast to cellular expression, at present nothing is known about the expression of these uptake transporters in zebrafish embryos and eleuthero-embryos. Early embryos are protected from MC-LR (Oberemm et al., 1997), as long as they are not hatched, because the chorion probably acts as barrier for the cyanotoxin (Wang et al., 2005). Similar to MC-LR, nodularin accumulates in the liver. In the liver of European flounders, nodularin concentrations of up to 557 $\mu g\,kg^{-1}$ have been detected (Persson et al., 2009).

The main target organ of MC-LR, and potentially nodularin, is the liver (Svircev et al., 2010), which expresses high levels of many uptake transporters, including Oatps (Roth et al., 2012). One of the important molecular toxicological mechanisms of these toxins is the inhibition of serine/threonine specific protein phosphatases PP1 and PP2A (MacKintosh et al., 1990). This in turn leads to hyperphosphorylation of proteins, ultimately resulting in deterioration of cellular integrity.

Thus far, little is known about additional molecular effects of nodularin. For MC-LR our group demonstrated an induction of endoplasmic reticulum (ER)-stress and associated unfolded protein response (UPR) in a human hepatoma cell line (Christen et al., 2013). The ER has various important functions, including protein folding. Disturbance of protein structure and functions, either induced physiologically or toxicologically, results in accumulation of unfolded or misfolded proteins and leads to ER-stress (Malhotra and Kaufman, 2007). This molecular mechanism is involved in the pathogenesis of many diseases, including neurological (Paschen and Mengesdorf, 2005) or metabolic disorders (Ozcan and Tabas, 2012). During prolonged ER-stress, UPR is activated to restore the normal function of the ER and stabilize protein folding. The UPR is highly conserved in animals and acts via three different pathways resulting in decreased protein translation, increased protein degradation and up-regulation of molecular chaperones, like the glucose regulated protein 78, also called bip (Malhotra and Kaufman, 2007). Transcriptional induction of *bip*, as well as splicing of the *x-box binding protein 1* (*xbp-1*) are key processes in the UPR and can be used for ER-stress determination (Arensdorf et al., 2013). However, changes in mRNA levels need not consequently result in changes on the protein level. Nevertheless, also up-regulation of *chop* is an indicator of ER-stress and the encoded protein is mainly responsible for apoptosis, which is induced, when normal functions are not regained (Samali et al., 2010).

Oxidative stress is closely linked to ER-stress (Malhotra and Kaufman, 2007), which can result in apoptosis. Oxidative stress is reduced by several enzymes, including catalase, superoxide dismutase and glutathione peroxidase (Malhotra and Kaufman, 2007). Also, the phase II enzyme glutathione-S-transferase (GST) protects cells from oxidative stress and reactive oxygen species (Hayes and Pulford, 1995). Activation of oxidative stress by MC-LR and nodularin was shown in aquatic animals (Amado and Monserrat, 2010). Another pathway that is affected by MC-LR is the mitogen-activated protein kinases (MAPK) signaling pathway. Interaction of MC-LR and the MAPK signaling cascade occurs via induction of tumor necrosis factor alpha (TNFα), an endogenous tumor promotor (Fujiki and Suganuma, 2011; Christen et al., 2013). After binding to its receptor, TNFα can induce all three MAPK signaling pathways (p38, ERK1/2 and JNK) resulting in the regulation of numerous cell functions including proliferation, apoptosis and differentiation (Marques-Fernandez et al., 2013; Wajant et al., 2003).

The aim of our present work was to investigate the expression of *Oatps* during early life stage development, and to determine the uptake of MC-LR into zebrafish by use of a newly synthesized fluorescent derivative of MC-LR (Grundler et al., 2015). Furthermore, we aimed to investigate the ER-stress induction, as well as the oxidative stress response in liver organ cultures of adult zebrafish and in eleuthero-embryos exposed to MC-LR and nodularin. Our data are based on changes in mRNA levels (and not protein levels), but nevertheless suggest that MC-LR and nodularin act similarly by induction of ER-stress, representing a novel transcriptional mode of action in fish.

2. Material and methods

2.1. Chemicals and reagents

Microcystin-LR (MC-LR) and nodularin were purchased from Enzo life-science (Lausen, Switzerland). Reconstituted fish water was prepared as followed: Deionized water with ions added ($CaCl_2 \times 2H_2O$ 147.0 $mg\,L^{-1}$, KCl 2.9 $mg\,L^{-1}$, $MgSO_4 \times 7H_2O$ 61.6 $mg\,L^{-1}$, $NaHCO_3$ 32.4 $mg\,L^{-1}$) and a conductivity of 470–480 μS/cm. Texas red was purchased in form of succinimidyl ester from LuBioScience (Luzern, Switzerland).

2.2. Animals

Fertilized eggs and adult zebrafish were obtained from Harlan Laboratories Ltd. (Itingen, Switzerland). Adult zebrafish were maintained in the laboratory as previously described (Blüthgen et al., 2013).

2.3. Exposure of zebrafish early life stages to fluorescent MC-LR, cryo-sectioning and imaging

Exposure of zebrafish eleuthero-embryos started after hatching at 72 hpf in 48-well plates (Huberlab, Reinach, Switzerland), containing 500 μL reconstituted fish water per well, with 0.01% DMSO (solvent control), 1 $\mu mol\,L^{-1}$ MC-LR-Texas red (Grundler et al., 2015) or 1 $\mu mol\,L^{-1}$ Texas red (negative control). In addition, 1 $\mu mol\,L^{-1}$ MC-LR labeled with Alexa 488 (Grundler et al., 2015) has been employed for analysis of uptake into early embryos. The first fluorophor that we coupled to MC-LR was Alexa 488, therefore we did our first experiments with MC-Alexa 488. Afterwards, we also tested other fluorophores, including Texas red, that was used for labeling gentamycin for uptake experiments with zebrafish eleuthero-embryos (Wang and Steyger, 2009). MC-LR-Texas red was found to be best concerning uptake by drOatp1d1, signal intensity and conditions for imaging of zebrafish eleuthero-embryos in respect to autofluorescence.

After exposure for 96 h, 168 h post fertilization (hpf) eleuthero-embryos were fixed in 4% paraformaldehyde (Fluka, Buchs, Switzerland) for 4 h at room temperature, embedded in Tissue TEK (Hartenstein, Würzburg, Germany) and stored at −80 °C until sectioning. Fifty μm sections were generated with a cryostat (HM560 MV, Thermo Scientific) and analysed. Images were taken by an Olympus Laser scanning microscope FV100 (excitation: 561 nm).