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Ciguatoxins activate the Calcineurin signalling pathway in Yeasts: Potential for development of an alternative detection tool?

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ABSTRACT

Ciguatoxins (CTXs) are lipid-soluble polyether compounds produced by dinoflagellates from the genus *Gambierdiscus* spp. typically found in tropical and subtropical zones. This endemic area is however rapidly expanding due to environmental perturbations, and both toxic *Gambierdiscus* spp. and ciguatoxic fishes have been recently identified in the North Atlantic Ocean (Madeira and Canary islands) and Mediterranean Sea. Ciguatoxins bind to Voltage Gated Sodium Channels on the membranes of sensory neurons, causing Ciguatera Fish Poisoning (CFP) in humans, a disease characterized by a complex array of gastrointestinal, neurological, neuropsychological, and cardiovascular symptoms. Although CFP is the most frequently reported non bacterial food-borne poisoning worldwide, there is still no simple and quick way of detecting CTXs in contaminated samples. In the prospect to engineer rapid and easy-to-use CTXs live cells-based tests, we have studied the effects of CTXs on the yeast *Saccharomyces cerevisiae*, a unicellular model which displays a remarkable conservation of cellular signalling pathways with higher eukaryotes. Taking advantage of this high level of conservation, yeast strains have been genetically modified to encode specific transcriptional reporters responding to CTXs exposure. These yeast strains were further exposed to different concentrations of either purified CTX or micro-algal extracts containing CTXs. Our data establish that CTXs are not cytotoxic to yeast cells even at concentrations as high as 1 μM, and cause an increase in the level of free intracellular calcium in yeast cells. Concomitantly, a dose-dependent activation of the calcineurin signalling pathway is observed, as assessed by measuring the activity of specific transcriptional reporters in the engineered yeast strains. These findings offer promising prospects regarding the potential development of a yeast cells-based test that could supplement or, in some instances, replace current methods for the routine detection of CTXs in seafood products.

1. Introduction

Ciguatera is the most common non bacterial food-borne poisoning worldwide, with an estimated number of victims per year between 50 000 and 500 000 taking into account the high under reporting rate which characterizes this disease ((EFSA Panel on Contaminants in the Food Chain, 2010; Reich et al., 2008) and Center for Disease Control and Prevention). This poisoning provokes a wide range of gastrointestinal, neurological, neuropsychological, and cardiovascular symptoms whose severity may vary from moderate to lethal. It is caused by large cyclic polyethers, ciguatoxins (CTXs) (Legrand et al., 1995) that are produced by benthic dinoflagellates of the genus *Gambierdiscus* which proliferate episodically in highly degraded coral ecosystems (Lehane and Lewis, 2000). Algal ciguatoxins gradually bioaccumulate along the food chain, first in herbivorous fishes grazing on microalgal turfs covering dead coral, then in carnivorous fish that prey on these toxic herbivorous fishes. Ciguatera eventually affects humans when they consume contaminated fishes. This bioaccumulation process is accompanied by an oxidative biotransformation of the less oxidized forms of algal CTXs into more oxidized and more toxic CTX congeners (Ikehara et al., 2017).

CTXs are non-immunogenic, highly stable compounds, which stay active even after fishes have been frozen, cooked, salted or smoked. After ingestion of contaminated fish or shellfish, CTXs rapidly reach the bloodstream and freely circulate, before binding with a remarkably high affinity in the nanomolar range (Dechraoui et al., 1999) on

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mammalian voltage gated sodium channels (VGSCs) present on the membrane of sensory neurons. This binding provokes an abnormal increase of their open conformation occurrence and hence a subsequent membrane depolarization (Legrand et al., 1995; Lombet et al., 1987; Ramsdell, and Bottein Dechraoui, 2003). Consequently, the binding of CTXs on VGSCs significantly alters the ordinary functioning of neuronal cells resulting in a complex array of gastrointestinal (diarrhea, vomiting), cardiovascular (hypotension, bradycardia) as well as neurological (*e.g.* paresthesia, pruritis, cold allodynia, myalgia, arthralgia) and neuropsychological symptoms, which may last days, weeks, or months (Reich et al., 2008). Remarkably, different VGSC mammalian isoforms mediate different effects of the toxins. Among them, NaV1.6 and 1.7 are notably involved in spontaneous dermal pain behaviour while NaV1.8 plays a predominant role in gastrointestinal disturbances (Inserra et al., 2017). Depending on the severity of the poisoning event and on the patient's specific sensitivity, it may take up to several months for the organism to overcome ciguatera illness.

Originally limited to localized regions in the Pacific, Caribbean and Indian Oceans (Skinner et al., 2011), the incidence of ciguatera has spread to more temperate locations within the last decades due to the expansion of travel, tourism, increased importation of fish from the tropics but also to the effects of global warming and environmental pollution damages on marine ecosystems (Gingold et al., 2014). Ciguatera is currently responsible for numerous “imported cases” in the US, Canada, France or Germany (Mattei et al., 2014) while recent observations also suggest expansion of the bio-geographical range of toxic *Gambierdiscus* spp. (Aligizaki et al., 2008; Fraga et al., 2011; Fraga and Rodríguez, 2014; Pisapia et al., 2017) and ciguatoxic fishes (Boada et al., 2010; Bravo et al., 2015; Otero et al., 2010) in previously non-endemic areas such as Madeira, the Canary Islands and the Mediterranean Sea.

A wide range of tests intended to detect CTXs in contaminated matrices are already available (see (Caillaud et al., 2010) for review): (i) the mouse bioassay, now questioned for ethical reasons (Nicolas et al., 2014); (ii) the neuroblastoma cell based-assay (CBA-N2a) which requires the use of additional toxin reagents (veratridine and ouabain) (Manger et al., 1993); (iii) the radioactive or fluorescent receptor binding assays (RBA) performed on either rat or porcine synaptosome membrane preparations (Darius et al., 2007; Dechraoui et al., 1999; Hardison et al., 2016; Lombet et al., 1987) and (iv) liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) techniques that allow discrimination between individual CTX congeners, provided that reference material (CTX standards) are available (Yogi et al., 2014). However, one major constraint of these tests is that they all require expensive reagents and/or highly sophisticated laboratory equipment, and are therefore not suitable for a quick use by fishery services, fishermen or consumers directly on the field. In this context, simpler tests have been foreseen and some have even reached the market (" CiguaCheck® ", " CiguaTect™ "). However, severe shortcomings in the reliability of these test kits have eventually driven their retraction from the market (Bienfang et al., 2011).

Thus, there is an urgent need to support initiatives to implement new detection techniques which would be easier, cheaper, and transportable. Yeast is a well-known unicellular organism which combines multiple advantages: it is easy to modify genetically, grows fast on inexpensive culture media, and last but not the least, belongs to the Eukaryotic Kingdom and therefore shows a very good conservation of most cellular signalling pathways with higher eukaryotic cells (Goffeau et al., 1996; Thewes, 2003). Hence, new bioassays based on yeast cells are emerging such as the one recently described to detect smaller marine micro-algal toxins able to enter the cells (Richter and Fidler, 2015). The aim of the present study was to establish whether yeast cells might be used as cellular model to detect CTXs either as purified toxin samples or in micro-algal extracts. We first investigated a possible cytotoxic or growth inhibitory effect of CTXs on yeast cells. Then, the variations of intracellular free Ca^{2+} content in yeast cells upon exposure to CTXs were assessed. Finally, the extent of activation of the conserved calcineurin signalling pathway was measured in different genetically modified yeast strains, in response to exposure to either pure P-CTX-3C or increasing concentrations of toxic *versus* non-toxic *Gambierdiscus* spp. micro-algal extracts.

Table 1
Yeast strains used in this study.

Strain	Genotype	Reference
BY4741a	*MAT***a** *his3–1 leu2–0 met15–0 ura3–0*	(Brachmann et al., 1998)
HM117	BY4742α *MATα his3–1 leu2–0 lys2–0 ura3–0 knr4::LEU2*	(Martin-Yken et al., 2003)
HMP0	BY4741**a** *knr4::Kan*^{r} YKO library (Open Biosystems) + plasmid pAL5 (Lagorce et al., 2003).	This study.
HMP1	BY4742α *knr4::leu2::PFKS2(6xCDRE)-yEYFP-kan*^{r}	This study.
HMP6	BY4742α *knr4::leu2::PFKS2(6xCDRE)-yEGFP-kan*^{r}	This study.

2. Material and methods

2.1. Yeast cell lines

Five different yeast cell lines of *Saccharomyces cerevisiae* were used or constructed for the present study. The full genotypes and references of the wild type (wt) strain BY4741a *vs.* genetically modified strains are described in Table 1.

2.2. Genetic design of Yeast reporter strains

Since yeast cells are normally surrounded by a thick cell wall which protects them from harmful environmental stress, a mutant strain with a more permeable cell wall was tested in addition to the wild type strain, to assess whether it would display an increased sensitivity to CTXs. The *knr4*Δ mutant strain HM117 deleted for *KNR4* gene, was chosen as a background host because its cell wall is known to be more permeable than the wild type yeast strain (Hong et al., 1994; Martin et al., 1999).

To follow the activation of the calcineurin signalling pathway by measuring the enzymatic activity of β-galactosidase, a reporter strain HMP0 has been constructed. The *knr4::Kan*^{r} mutant from the Yeast Knockout (YKO) Collection (Open Biosystems) was transformed by plasmid pAL5. On this plasmid, 6 tandem repetitions of the Crz1 transcription factor binding site have been integrated in the promotor of the yeast gene *PFKS2*, upstream of the lacZ gene encoding β-galactosidase (Lagorce et al., 2003). To construct the strains HMP1 and HMP6, a modified version of vector pJRL2 (Addgene) was used, in which the selection cassette was modified as described previously (Liu et al., 2015) and the YFP gene was replaced by the yeast adapted versions of either GFP or YFP (Cormack et al., 1997). *PFKS2 (6xCDRE)* promotor was amplified from plasmid pAL5 and inserted in the modified plasmid pJRL2 upstream of either yEYFP or yEGFP. Finally, cassettes containing promotor, fluorescent reporter gene and resistance gene *kan*^{r} were integrated by homologous recombination in HM117 at the original *KNR4* genomic locus. Correct integrations of the cassettes were verified by sequencing.

2.3. CTXs containing biological material

Pure Pacific ciguatoxin type 3 C (P-CTX-3C) used in our assays, was obtained from the bank of standards of the Laboratoire des Micro-Algues Toxiques at Institut Louis Malardé (Papeete, French Polynesia). Briefly, P-CTX-3C was purified from cell extracts of TB92, a highly toxic strain of *G. polynesiensis*, as previously described in (Chinain et al.,

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