Toxicon 106 (2015) 57–67

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Comparative study of the toxic effects of *Chrysaora quinquecirrha* (Cnidaria: Scyphozoa) and *Chironex fleckeri* (Cnidaria: Cubozoa) venoms using cell-based assays

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ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form
9 September 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
Jellyfish
Venom
Chrysaora quinquecirrha
Chironex fleckeri
Cell line
CfTX

ABSTRACT

The venoms of jellyfish cause toxic effects in diverse biological systems that can trigger local and systemic reactions. In this study, the cytotoxic and cytolytic effects of *Chrysaora quinquecirrha* and *Chironex fleckeri* venoms were assessed and compared using three *in vitro* assays. Venoms from both species were cytotoxic to fish gill cells and rat cardiomyocytes, and cytolytic in sheep erythrocytes. Both venoms decreased cell viability in a concentration-dependent manner; however, the greatest difference in venom potencies was observed in the fish gill cell line, wherein *C. fleckeri* was 12.2- ($P = 0.0005$) and 35.7-fold ($P < 0.0001$) more potently cytotoxic than *C. quinquecirrha* venom with 30 min and 120 min cell exposure periods, respectively. Gill cells and rat cardiomyocytes exposed to venoms showed morphological changes characterised by cell shrinkage, clumping and detachment. The cytotoxic effects of venoms may be caused by a group of toxic proteins that have been previously identified in *C. fleckeri* and other cubozoan jellyfish species. In this study, proteins homologous to CfTX-1 and CfTX-2 toxins from *C. fleckeri* and CqTX-A toxin from *Chironex yamaguchii* were identified in *C. quinquecirrha* venom using tandem mass spectrometry. The presence and relative abundance of these proteins may explain the differences in venom potency between cubozoan and scyphozoan jellyfish and may reflect their importance in the action of venoms.

1. Introduction

Jellyfish venoms are complex mixtures of bioactive compounds that cause toxic effects on various cell types. These toxic cocktails are injected into prey or predators upon stimulation of specialized stinging capsules (i.e. nematocysts), mostly found in the tentacles of jellyfish (Beckmann and Özbek, 2012). The venomous content of nematocysts is mainly composed of proteins and peptides that modify cellular processes by disruption of ion channels, formation of membrane pores or through enzymatic mechanisms (Lassen et al., 2010; Lee et al., 2011; Yanagihara and Shohet, 2012). These cytotoxic effects contribute to prey capture or predator deterrence (Kordiš and Gubenšek, 2000).

Chrysaora quinquecirrha (Cnidaria: Scyphozoa) and *Chironex fleckeri* (Cnidaria: Cubozoa) are jellyfish of medical significance as their venoms can also affect humans causing mild to severe envenomation. Specifically, stings from the Atlantic sea nettle *C. quinquecirrha* can cause skin rash, pain, headaches, cramps, cough, lethargy, nausea and loss of consciousness (Newman-Martin, 2007; Williamson et al., 1996). The box jellyfish *C. fleckeri*, considered the most dangerous cubozoan jellyfish, causes painful and potentially life-threating stings. Envenomation symptoms following *C. fleckeri* stings include excruciating pain, cutaneous inflammation, dermonecrosis, pulmonary oedema, cardiovascular

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http://dx.doi.org/10.1016/j.toxicon.2015.09.014
0041-0101/

dysfunction and cardiac failure which can lead to death (Brinkman and Burnell, 2009). Experimental studies of *C. quinquecirrha* and *C. fleckeri* venoms using *in vivo* and *in vitro* approaches have revealed their cardiotoxic, haemolytic, cytotoxic, myotoxic, lethal and dermonecrotic properties (Baxter and Marr, 1969; Burnett and Goldner, 1969, 1970; Burnett and Calton, 1976; Endean, 1987; Freeman and Turner, 1971; Hughes et al., 2012; Long and Burnett, 1989; Neeman et al., 1980a). Among these biological activities, the *in vitro* cytotoxicity of these venoms has been examined in various cell lines. For instance, the venom of *C. quinquecirrha* caused cessation of spontaneous beating of a primary culture of embryonic chick cardiocytes (Cobbs et al., 1983; Kelman et al., 1984) and induced mitogenic activity, nuclear alterations and dissolution of intercellular collagen in Chinese hamster ovary K-1 cells (CHO K-1) (Neeman et al., 1980a, 1980b). In human hepatocytes, venom caused an initial increase in metabolic activity, followed by a sharp decrease and cell death within minutes (Cao et al., 1998). Venoms from *C. quinquecirrha* and *C. fleckeri* also caused metabolic activity alterations in the human Chang liver cell line (Bloom et al., 2001). The venom of *C. fleckeri* caused a concentration-dependent inhibition of viability of rat aortic smooth muscle cells A7r5 (Brinkman et al., 2014; Konstantakopoulos et al., 2009; Winter et al., 2010) and whole venom and partially purified fractions caused cell detachment, reduction of cell metabolism and death of human cardiac myocytes and human skeletal myocytes (Chaousis et al., 2014; Pereira and Seymour, 2013; Saggiomo and Seymour, 2012). However, few of these studies have reported comparable toxic units (e.g. LC_{50} values) making the comparison of venom potencies difficult. Moreover, results also differ greatly among reports due to the intrinsic variability of venoms (i.e. high thermolability, protein aggregation) and the methods used for venom extraction and fractionation (Brinkman and Burnell, 2008).

The cytotoxicity of jellyfish venoms has been associated with a group of proteins that possess lethal, haemolytic, inflammatory, dermonecrotic, cardiovascular and pore-forming activities (Brinkman and Burnell, 2008; Brinkman et al., 2014; Nagai et al., 2000a, 2000b, 2002; Yanagihara and Shohet, 2012). These proteins have mostly been identified in the venom of *C. fleckeri* (CfTX toxins) and other cubozoan jellyfish; however, homologous proteins to CfTXs have also been isolated and/or predicted in cnidarians from Classes Scyphozoa and Hydrozoa (Brinkman et al., 2014; Lassen et al., 2011; Sher et al., 2005).

In this study, we used three cell-based assays to test and compare the toxic effects of *C. quinquecirrha* and *C. fleckeri* venoms and examined some potential venom components associated with these biological activities. The fish gill line RTgill-W1 was selected as it was previously shown to be sensitive to the action of jellyfish venoms (Helmholz et al., 2010) and immortalised rat cardiomyocytes and sheep erythrocytes were used to compare the toxic effects on mammalian cells. Due to the detection of protein bands in the molecular mass range of CfTX toxins, we investigated if similar proteins were present in the venom of *C. quinquecirrha* using tandem mass spectrometry. Three CfTX-like proteins were identified in *C. quinquecirrha* venom that may have similar roles as the CfTXs in *C. fleckeri* venom and explain in part the cytotoxic and cytolytic effects of this scyphozoan jellyfish venom.

2. Experimental section

2.1. Jellyfish collection

C. quinquecirrha specimens were obtained from the jellyfish-breeding facilities of the Tennessee Aquarium (Chattanooga, Tennessee, USA). A total of 25 specimens with an average umbrella diameter of 20 cm, and fully extended fishing tentacles of 80 cm in length were selected. Jellyfish were maintained in pseudokreisel aquariums and fed twice daily with live brine shrimps, bloodworms and other invertebrates. Prior to sample collection, jellyfish were fasted for 24 h to avoid contamination from food. Mature specimens of *C. fleckeri* were collected from coastal waters near Weipa (Queensland, Australia) by Christopher Mooney, Mark O′ Callaghan and Avril Underwood (James Cook University) and transported to laboratory facilities. Fishing tentacles from both species were excised from live specimens and stored immediately in artificial seawater at 4 °C for further processing.

For *C. quinquecirrha* handling, all procedures were approved by the Animal Health and Welfare Committee from the Tennessee Aquarium and the Tennessee Aquarium Conservation Institute (TNACI) under the proposal number 14-02. *C. fleckeri* is not an endangered or protected species; therefore, no specific permits were required for field studies and no specific permissions were required as the animals were collected from marine environments that are not protected or privately owned.

2.2. Venom extraction

For both species, nematocysts were isolated and cleaned similar to the methods described by Bloom et al. (1998) and Marchini et al. (2004). Briefly, excised tentacles were stored in seawater at 4 °C and nematocysts were obtained by spontaneous release from autolysed tentacle tissue. Nematocysts were allowed to settle and the supernatant was decanted and replaced with fresh seawater. Water exchanges were performed every 24 h for 10 days until the majority of visible tissue debris was discarded. Nematocysts were then cleaned using a discontinuous gradient of Percoll (Sigma) diluted with 35 g L^{-1} NaCl and centrifugation (300 × *g*, 4 °C, 1 h). The discontinuous gradient consisted of 100, 90 and 30% Percoll. Cleaned nematocysts were recovered and washed three times (300 × g, 4 °C, 3 min) with venom extraction buffer (VEB) composed of 20 mM PO_4^{3-} and 0.15 M NaCl (pH 6.7).

Venom was extracted from nematocysts using bead mill homogenization according to Brinkman and Burnell (2007). Cleaned nematocysts were resuspended in cold VEB and 0.5 mm glass beads were added. Samples were shaken using a tissue homogenizer (Precellys 24, Precellys) for 10 s bursts at 5000 rpm with chilled-on ice intervals of 1 min (7–9 homogenization cycles). Discharge of nematocyst capsules (>90%) was monitored microscopically. Venom extracts were centrifuged (20 k × *g*, 4 °C, 10 min), filtered (0.22 μm Millex-GV filters, Millipore) and kept on ice. Protein concentration was determined fluorometrically using a Qubit Protein Assay kit with the Qubit 2.0 system (Invitrogen). Venom samples were frozen at −20 °C (used within two weeks) and one aliquot of *C. quinquecirrha* venom was freeze-dried and stored at 4 °C until further use. Frozen venoms samples were thawed on ice prior to analysis and are referred to as “thawed” venom throughout this study.

2.3. Cell viability assays

The *in vitro* cytotoxic effects of *C. quinquecirrha* and *C. fleckeri* venoms were assessed in two cell lines, fish gill cells and rat cardiomyocytes. The epithelial gill cell line RTgill-W1 from the rainbow trout *Oncorhynchus mykiss* (CRL-2523) was obtained from the American Type Culture Collection (ATCC, USA) (Bols et al., 1994). The cell line was routinely cultured in 25-cm^2 culture treated flasks (Nunc) with Leibovitz's medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) (v/v) (Sigma–Aldrich) and an antibiotic-antimycotic solution (Sigma–Aldrich). Gill cells were continuously incubated at 21 °C in darkness. Confluent cells were detached with 0.25% trypsin-0.02% EDTA solution (Sigma–Aldrich)