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The nematocysts venom of *Chrysaora helvola* Brandt leads to apoptosis-like cell death accompanied by uncoupling of oxidative phosphorylation

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ABSTRACT

The present work investigated the effects of the nematocysts venom (NV) from the *Chrysaora helvola* Brandt (*C. helvola*) jellyfish on the human nasopharyngeal carcinoma cell line, CNE-2. The medium lethal concentration (LC_{50}), quantified by MTT assays, was 1.7 ± 0.53 μg/mL (n = 5). An atypical apoptosis-like cell death was confirmed by LDH release assay and Annexin V-FITC/PI staining-based flow cytometry. Interestingly, activation of caspase-4 other than caspase-3, -8, -9 and -1 was observed. Moreover, the NV stimuli caused a time-dependent loss of mitochondrial membrane potential ($\Delta\Psi_m$) as was an intracellular ROS burst. These results indicated that there was uncoupling of oxidative phosphorylation (UOP). An examination of the intracellular pH value by a pH-sensitive fluorescent probe, BCECF, suggested that the UOP was due to the time-dependent increase in the intracellular pH. This is the first report that jellyfish venom can induce UOP.

1. Introduction

Chrysaora helvola Brandt (*C. helvola*) (Class: Scyphozoa, Order: Semaeostomeae, Family: Pelagiidae) is a virulent jellyfish inhabiting the Beibu Gulf. It propagates in the summer and autumn and compromises the tourism and fishery industries there. A sting by *C. helvola* leads to burning pain within a few seconds, followed by bleeding and inflammation-like syndrome. In cases of very severe, local dermal necrosis and systemic effects including vomiting, fever, headache and dyspnea have been observed. In the worse cases, the jellyfish may even cause death.

Venoms/toxins of jellyfish have been studied for a long time. The cytotoxicities of several different jellyfish venoms/toxins have been reported (Cao et al., 1998; Konstantakopoulos et al., 2009). Moreover, Sun et al. (2002) reported that apoptosis can be induced in human U251 and rat C6 malignant glioma cells and in transformed vascular endothelial ECV304 cell lines by CqTX, a toxin from the virulent box jellyfish *C. quadrigatus*. An increase in p53 expression was also observed in the U251 cells. It was suggested to be one mechanism by which CqTX induced apoptosis.

In the present work, we first studied the cytotoxicity of the NV from *C. helvola* on a human nasopharyngeal carcinoma cell line, CNE-2. The type of cell death of the CNE-2 cells induced by the NV was also investigated. Furthermore, we have performed a preliminary analysis of the activation of caspases, which are frequently involved in apoptosis. However, it seems that the UOP, as well as endoplasmic reticulum (ER) stress-related pathways, may also be involved, rather than the caspase-mediated intrinsic and extrinsic apoptotic pathways. The results of our study provide a preliminary evaluation of the cytotoxicity of the NV from *C. helvola*. This study also provides important information on the action mechanism of the NV on cells.

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2. Materials and methods

2.1. Nematocyst isolation and NV preparation

Mature specimens of *C. helvola* were collected in the Beibu Gulf, Guangxi Province, China, and were transported to the laboratory on dry ice. Nematocysts were isolated as described by Bloom et al. (1998) with minor modifications. Briefly, the excised tentacles were allowed to undergo continuous autolysis in fresh distilled water at 4 °C for 4 days. The residue was centrifuged at 3000 × g at 4 °C for 15 min, and the harvested pellets were assumed to be nematocysts. A total of 0.1 g of nematocysts was sonicated (Sonoplus, 70 Hz, 8 s at 12 s intervals, 90 times) on ice in 10 mL of PBS (pH 7.4). The resultant suspension was centrifuged at 13000 × g at 4 °C for 1 h. The supernatant was used as the NV.

The protein concentration of the NV was measured by the method described by Bradford (1976) using a Bio-Rad Protein Assay Kit (Bio-Rad) by comparison of samples with BSA protein concentration standards.

2.2. Cell lines and cultures

The human nasopharyngeal carcinoma cell line, CNE-2, was obtained from the ATCC. The cells were maintained in advanced RPMI1640 culture media (SH30809.01B, HYClone, USA) supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Measurement of cytotoxicity

The cytotoxicity of NV was examined by the MTT method as described by Sun et al. (2000) with slight modification. Briefly, CNE-2 cells were seeded at a density of 5×10^4 cells/well in 96-well plates. After 24 h, the cells were treated with various concentrations of NV (diluted with culture medium), and incubated for another 48 h. The cell viability was calculated as the absorbance ration of NV-treated vs. untreated cells × 100 to yield the percentage of cells. Five independent experiments were carried out for each NV concentration.

2.4. LDH release assay

The NV-induced LDH release from the impaired cells was measured as described previously (Wu et al., 2015) with a minor modification. Briefly, the cell-free culture media supernatants were collected from each well after 48 h of NV treatment and then incubated with the appropriate reagent mixture according to the supplier's instructions (Jiancheng, China). The amount of formazan salt, formed by the reduction of iodonitrotetrazolium catalyzed by diapharose in the presence of NADH, was measured by the absorbance at 490 nm. The results were proportional to both the LDH activity and the number of damaged cells. The data were normalized to the absorbance of untreated cells (100%) and are expressed as the percentage of the control averaged from five independent experiments.

2.5. Annexin V-FITC/PI double staining analysis by flow cytometry

Annexin V-FITC/PI staining was performed using an apoptosis detection kit (Beyotime, China). Briefly, after treatment of the cells with NV for 48 h, they were washed twice with cold PBS and harvested through trypsinization. The cells were centrifuged at 1000 r/min for 5 min, and the pellet was resuspended in binding buffer and incubated with FITC-conjugated Annexin V and PI for 15 min at room temperature in the dark. Finally, the number of cells stained was analyzed using a Beckman Coulter flow cytometer with 518 nm and 620 nm emission filters. Approximately 10^4 cells were counted for each sample. The distribution of the cells in the different quadrants, which indicated that the cells were impermeable to both PI and AV (PI^-/AV^-), permeable to AV but not PI (PI^-/AV^+), permeable to PI but not AV (PI^+/AV^-), and permeable to both PI and AV (PI^+/AV^+) was determined using the Cell Quest Research software.

2.6. Measurement of caspase activities

The activity levels of caspases-1, -3, -8, -9, and -4 of the CNE-2 cells were measured by the cleavage of chromogenic caspase-specific substrates Ac-YVAD-*p*NA, Ac-DEVD-*p*NA, Ac-IETD-*p*NA, Ac-LEHD-*p*NA and Ac-LEVD-*p*NA, respectively (Beyotime, China). Assays were carried out according to the methods described by Zhang et al. (2010). Briefly, the harvested cells were lysed and centrifuged. The supernatants were transferred to 96-well plates, and then the substrates were added to each sample at a concentration of 2 mM. The reaction was initiated at 37 °C for 24 h, and the concentrations of the products, *p*NA (*p*-nitroaniline) were monitored by absorbance at 405 nm. Three independent experiments were carried out for each condition.

2.7. Mitochondrial membrane depolarization assay

The changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) were measured using a fluorescent probe, JC-1 (Beyotime, China) according to the method described by Xin et al. (2009) with a slight modification. Briefly, CNE-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After incubation for 24 h, the cells were loaded with JC-1 according to the manufacturer's instructions, followed by three washes with serum-free medium. The cells were then incubated with various concentrations of NV, and the fluorescence was immediately measured in a microplate reader (Infinite 200Pro, Tecan) every minute for 3 h, for excitation at 488 nm and emission at 590 nm. The positive control was incubated with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) instead of NV, while the negative control was incubated with culture medium alone.

2.8. Measurement of the intracellular ROS

The intracellular concentrations of ROS were measured using a fluorescent probe DCFH-DA (2′, 7′-dichlorodihydrofluorescein diacetate) (Beyotime, China) according to the method described by Zhang et al. (2012). DCFH-DA does not fluoresce itself, but can form a fluorescent product with intracellular ROS, which can be monitored by fluorimetry. Briefly, after incubation for 24 h, cells seeded in 96-well plates (5×10^4/well) were loaded with 10 μg/mL DCFH-DA for 30 min at 37 °C, followed by three washes with serum-free medium. They were then incubated with NV and the fluorescence was immediately measured in a microplate reader (Infinite 200Pro, Tecan) every minute for 3 h with excitation at 488 nm and emission at 525 nm.

2.9. Measurement of the intracellular pH

The intracellular pH was measured using a pH-sensitive fluorescent probe, BCECF-AM (2′, 7′-bis (2-carboxyethyl)-5, 6-carboxyfluorescein acetoxymethyl ester) (Beyotime, China), as described by Chen et al. (2012) with slight modification. Briefly, after incubation for 24 h, cells seeded in 96-well plates (5×10^4/well) were loaded with 10 μg/mL BCECF-AM for 30 min at 37 °C