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Apoptotic responses of *Carassius auratus* lymphocytes to nodularin exposure in vitro

Hangjun Zhang*, Dandan Shao, Yingzhu Wu, Chenchen Cai, Ciming Hu, Xiaolu Shou, Bingru Dai, Binhui Ye, Mengdi Wang, Xiuying Jia*

Department of Environmental Sciences, Hangzhou Normal University, Xuelin Road 16#, Xiasha Gaojiao Dongqu, Hangzhou, Zhejiang Province 310036, China

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A B S T R A C T

Nodularin, a metabolite of *Nodularin spumigena*, is widely detected in water blooms worldwide and causes serious negative effects on fish. The apoptosis-related cytotoxic effects and mechanisms of nodularin on *Carassius auratus* lymphocytes were investigated. Transmission electron microscopy results showed that nodularin-treated lymphocytes display a series of morphological changes, including condensed cytoplasm, nuclear chromatin agglutination and marginalization. DNA fragmentation was verified by the DNA-ladder and formation of sub-G1 DNA peaks. These cell characteristics confirmed the occurrence of apoptosis in lymphocytes. Flow cytometric results showed that the percentages of apoptotic cells incubated with 1, 5, 10, and 100 μg/L nodularin for 12 h reached 15.76%, 17.36%, 20.34% and 44.21%, respectively; controls showed low rates of apoptosis (2.4%). The mechanism of apoptosis induced by nodularin was determined, and results showed that nodularin exposure caused a significant increase in intracellular reactive oxygen species (ROS), loss of mitochondrial transmembrane potential in a dose-dependent manner, upregulation of intracellular Ca^{2+}, downregulation of Bcl-2 and upregulation of Bax expression at the mRNA and protein levels, and activation of caspase-3 and caspase-9 without caspase-8. In summary, all the results suggest that nodularin induces lymphocyte apoptosis via the mitochondrial apoptotic pathway and destroys the immune response of fish.

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1. Introduction

Cyanobacterial blooms, which frequently occur in freshwater, reservoirs, ponds, and coastal waters around the world due to exacerbated water eutrophication, pose ecological risks because they can release algal toxins [1]. The structure and toxicity of many algal toxins resemble those of nodularin and microcystins (MCs), both of which inhibit the eukaryotic protein serine/threonine PP1and PP2A in vitro and in vivo with similar potency [2–4]. Nodularin has the structure cyclo(-D-erythro-β-methylAsp(iso)-L-Arg-Adda-D-Glu(iso)-2-(methylamino)-2-(Z)-dehydrobutyric acid); Adda has the structure 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E), 6(E)-dienoic acid [5]. Nodularin is produced by *Nodularin spumigena*, which can be found in many freshwater bodies. The concentration of nodularin in phytoplankton typically ranges from 0.1 mg/g to 2.59 mg/g (dry weight), with a maximum concentration of 18.1 mg/g (dry weight) [6,7]. Its extracellular concentration can reach up to 95 μg/L when algal cells fracture [8]. Bioaccumulation of nodularin has been detected in blue mussel, Baltic clam, flounder, cod, and three-spined stickleback, as well as at low concentrations in herring and salmon [9,10]. For mice, nodularin has been proved to accumulate in the liver, kidney, spleen, blood, and other organs [11]. Recently, Qiu et al. found that fish kidney impairment from chronic exposure of toxic cyanobacterial blooms might be the first step of fish chronic toxicosis [12]. The effect of nodularin on aquatic organisms, especially fish, is of particular concern because fish may be exposed to toxins through oral uptake of toxin-containing cells or absorption via epithelial surfaces.

The bioaccumulated nodularin has been demonstrated to be toxic to aquatic organisms. Lehtonen and colleagues [13] reported that acetylcholinesterase activity changes in the clam *Macoma balthica* during short-term exposure to nodularin. Toxic effects have also been observed in flounder and sea trout (*Salmo trutta* m. trutta L.) orally exposed to toxic *N. spumigena* [14,15]. Abnormal development and increased mortality in embryo and larvae have been found in herring directly exposed to cyanobacteria [16]. Stunted and anomalous growth of juvenile three-spined

* Corresponding authors. Tel./fax: +86 571 28865327.
E-mail addresses: zhanghangjun@gmail.com (H. Zhang), hznujiaxiuying@126.com (X. Jia).

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sticklebacks exposed to nodularin from *N. spumigena* blooms has also been reported [17]. The liver tissue of flounder injected intraperitoneally with nodularin exhibits alterations in GST and CAT activities, suggesting the role of oxidative stress in nodularin-mediated toxicity [18]. Furthermore, induction of apoptosis has been demonstrated to be a key toxic mechanism for mammal cells treated with nodularin [19–22]. However, little is known about the difference of apoptotic cytotoxicities between fish cells and mammal cells induced by nodularin.

Nodularin has been identified as a direct environmental carcinogen [23], but recent studies have reported that nodularin may cause weakening of the immune system of dogs [24] and it can also encroach on immune actions by increasing the spontaneous adhesion of human peripheral polymorphonuclear leucocytes [25]. The immune system is suggested to be a susceptive target of the toxicity of nodularin. Previous studies have found the toxic effects of herbicides, pesticides, heavy metals, and algal toxins on the impairment of immune responses in fish [26–29]. For instances, microcystins, which include structures related to nodularin, can damage fish gills and even cause immune functions [30]. In eutrophic water, fish can directly suffer from chronic exposure to nodularin, especially in areas with warmer climates where *N. spumigena* blooms may persist for long periods of time. Determination of the immunotoxicity and mechanism of nodularin on fish is therefore important. However, immune effects of fish in response to nodularin are seldom known.

Thus, the objective of this study is to examine the apoptotic responses of immune systems of fish upon exposure to nodularin using isolated lymphocytes from *Carassius auratus*. In particular, the mechanism to induce apoptosis responses by nodularin is investigated. Our results show that nodularin is able to induce apoptosis on *C. auratus* lymphocytes by the mitochondrial apoptotic pathway which is regulated by intracellular Ca^{2+}, reactive oxygen species (ROS), mitochondrial membrane potentials (MMP), caspase-3, and -9 activities, and Bcl-2/Bax expressions.

2. Materials and methods

2.1. Chemicals and toxin

Purified nodularin (CAS No. 118399-22-7, $C_{41}H_{60}N_8O_{10}$, MW = 824.96) was purchased from Sigma (St. Louis, MO, USA).

2.2. Experimental fish

C. auratus (6–12 months old) of both sexes, weighing approximately 400–500 g each, were obtained from the hatchery of the Freshwater Fisheries Institute of Zhejiang (China). All experimental fish were reared and maintained in re-circulating water controlled at 25 ± 1 °C. They were fed with pellet foods at a daily ration of 0.7% of their body weight. The healthy fish for studies were held in the laboratory for at least 10 days prior to experimental uses.

2.3. Isolation of lymphocytes and cell culture

Based on the method described by Kemenade et al. [31], *C. auratus* were killed by decapitation, and then the head, kidney, and spleen were removed. Single-cell suspensions were obtained by teasing the tissue (in serum-free RPMI-1640 culture medium) through a nylon sieve. The cells were washed twice in ice-cold culture medium without serum, collected and layered on 1.5 vol. lymphoprep (Huadong Pharmaceutical, Hangzhou, China, density adjusted to 1.077 g/mL). Following 30 min centrifugation at 650 × g, the lymphocyte layer was collected, and washed three times with phosphate buffered saline (PBS). The number of cells was determined by a hemocytometer, and the cells were cultured in antibiotic-free RPMI-1640 medium (Hangzhou Keyi Shengwu, Hangzhou, China) with 5% fetal calf serum (FCS) in a CO_2 atmosphere at 27 °C for 5 h to remove the adherent cells; then the nonadherent lymphocytes were carefully collected and recultured in RPMI-1640 medium.

2.4. Apoptosis detection and flow cytometry PI staining

Three concentrations were adopted for apoptosis detection in fish lymphocytes. Briefly, 1×10^6 cells were exposed to nodularin (0, 1, 5, 10, and 100 μg/L) for 12 h. After incubation, cells were collected, washed with ice-cold PBS, and then fixed in 70% ethanol for at least 24 h at 4 °C. Cells were washed twice with PBS and then treated with 100 μg/mL RNase (Sigma) and 50 μg/mL PI (propidium iodide, Sigma) staining buffer for 30 min at room temperature. Afterward, the cells were filtered using a BD Falcon circular tube (No. 352235, Becton Dickinson, New Jersey, USA) prior to analysis using a Guava easyCyte 8HT flow cytometer (Merck Millipore, Darmstadt, Germany). PI was excited at 488 nm and detected at 630 nm.

2.5. Electron microscope observation

After 12 h of nodularin exposure, 1×10^6 cells lymphocytes were washed with PBS and fixed overnight in 2.5% glutaraldehyde. The lymphocytes were then post-fixed in 1% osmium tetroxide, dehydrated through a graded alcohol series and acetone, and embedded in Epon 812. Ultra-thin sections were prepared and stained with uranyl acetate and lead citrate and then viewed under a Philips TECNAL-10 transmission electron microscope (Philips, Amsterdam, Dutch).

2.6. DNA ladder assay

The DNA ladder was detected through gel electrophoresis as previously described [32]. 1×10^6 lymphocytes were exposed to nodularin (0, 1, 5, 10, and 100 μg/L) for 12 h. After induction, the cells were harvested and washed twice with cold PBS. DNA extraction was conducted using an AxyPrep Genomic DNA small kit (Axygen Biotechnology, Hangzhou, China). Electrophoresis was then performed using 1% agarose gel. Afterward, DNA samples were stained with 30 ng/mL ethidium bromide and photographed on a Kodak Gel Logic 200 Imaging System (Kodak, New York Rochester, USA) using 5000 kb as a size marker.

2.7. Intracellular Ca^{2+} level detection

Intracellular Ca^{2+} levels in the current experiment were examined using previously described methods [33]. After incubation with nodularin and TG (Thapsigargin; Sigma), 1×10^6 lymphocytes were harvested and stained with Fluo3-AM (Beyotime Institute of Biotechnology, Haimen, China) at a final concentration of 5 μg/mL for 40 min at room temperature. Afterward, the cells were washed twice with cold PBS and collected for analysis on a Guava easyCyte 8HT flow cytometer in Incyte mode.

2.8. ROS assay

After exposure to nodularin and NAC (*N*-acetyl-L-cysteine; Sigma) for 12 h, 1×10^6 cells were collected and homogenized in cold PBS. DCFH-DA (2,7-Dichlorofluorescin; Sigma) was set at a final concentration of 10 μM for 20 min at room temperature. Cells were analyzed using a Shimadzu RF-3501 fluorescence

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