The venom of jellyfish, *Chrysaora pacifica*, induces neurotoxicity via activating Ca^{2+} -mediated ROS signaling in HT-22 cells

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Abstract Stings of jellyfish, which frequently occur in a warm season, cause severe pain, inflammation and sometimes irreversible results such as the death. Harmful venoms from jellyfish, therefore, have been studied for finding the therapeutic agents to relieve pain or to neutralize toxic components. However, it is still unclear if and how jellyfish venom reveal neuronal toxicity even though pain induction seems to result from the activation of nociceptors such as nerve endings. In this study, using HT-22 cell line, we investigated neurotoxic effects of the venom of *Chrysaora pacifica* (CpV) which appears in South-East ocean of Korea. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, CpV significantly reduced the viability of HT-22 cells in a dose-dependent manner. Additionally, in 2',7'-Dichlorofluorescein diacetate fluorescence test under the culture condition lacking dominant inflammatory factors, CpV remarkably increased the production of intracellular reactive oxygen species (ROS). Reduced responsive

fluorescence to Rhodamine123 and increased expression of intracellular cytochrome *c* were also observed in HT-22 cells treated with CpV. These indicate that CpV-reduced viability of HT-22 cells may be due to the activation of apoptotic signalings mediated with oxidative stress and mitochondrial dysfunction. Furthermore, removing Ca^{2+} ion or adding N-acetyl-L-cysteine remarkably blocked the CpV effect to reduce the viability of HT-22 cells. The findings in this study clearly demonstrate that CpV may activate Ca^{2+} -mediated ROS signalings and mitochondrial dysfunction resulting in neuronal damage or death, and suggest that blocking Ca^{2+} pathway is a therapeutic approach to possibly block toxic effects of jellyfish venoms.

Keywords Apoptosis · Ca^{2+} signaling · *Chrysaora pacifica* · Jellyfish venom · Neurotoxicity · Reactive oxygen species

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Introduction

For decades, venoms of jellyfish have been studied to identify the mechanisms that cause inflammatory responses and peripheral pain, as well as to find the therapeutic agents that relieve pain or neutralize the toxic components. According to several *in-vitro* model studies previously reported, various jellyfish venoms exhibit potent and irreversible cytotoxicities in human or animal cell lines [1-3]. These reports commonly suggest that jellyfish venoms may influence apoptotic signaling to induce the damage or death of cells, indicating that their harmful effects may be caused by intracellular cytotoxic mechanisms. Additionally, these venoms have been reported to be associated with oxidative signaling in human cancer-cell lines by affecting the regulation of reactive oxygen species (ROS) production [4]. Because neurons that have dynamic membrane pathways permeable to Ca^{2+} ions, have a higher metabolic rate of mitochondria than of non-neuronal cells, and are critically sensitive to ROS-mediated toxicity, it is

also important to know if jellyfish venoms can activate ROS signaling that causes peripheral pain or neuronal cell death[5-7]. For this issue, we tried to test ROS-mediated neurotoxic effects of the venom extracted from *Chrysaora pacifica* (CpV) in HT-22 cells. *Chrysaora pacifica*, a jellyfish that frequently appears on the east coast of South Korea in summer, has brown tentacles that contain nematocysts with toxic venom that induces a burning sensation, muscle pain, pruritus, tentacle mark, blisters, superficial ulceration or necrosis, and local soft-tissue edema and angioedema in contacted human bodies [8].

In this study, we confirmed that treatment with CpV significantly reduced the viability of HT-22 cells in a dose-dependent manner and increased ROS production. ROS-mediated neuronal damage when treated with CpV depended on Ca^{2+} influx and seemed to be associated with mitochondrial damage and dysfunction. These results strongly suggest that CpV may lead Ca^{2+} influx to activate ROS signaling and to induce mitochondrial damage, resulting in neuronal damage or death. The findings of our study provide evidence for the neurotoxicity of CpV and its possible mechanism by which the jellyfish venom can affect the nervous system via oxidative signaling.

Materials and Methods

Venom preparation

Jellyfish *Chrysaora pacifica* was collected in East coast of South Korea, in July 2017. The tentacles were manually dissected and their nematocysts were isolated using the method described by Bloom *et al.* (1998) with a slight modification. In brief, dissected tentacles were gently swirled with the addition of distilled water three times, to remove debris and sea water. After decanting the supernatant, tentacle pellet settled down at the bottom were resuspended in about double volume of distilled water and shaken vigorously for 3 min. The detached nematocysts were separated by filtering tentacle preparation through 4 layers of medical gauze. This was repeated for two more times with adding distilled water. All procedures for nematocysts isolation were performed on ice. The filtrates were centrifuged at 700 g for 20 min at 4 °C. The pellets were then sonicated in a cold external solution containing the following (in mM): 145 NaCl, 5 KCl, 2 CaCl_2 , 1.3 MgCl_2 , 10 HEPES and 10 glucose, and pH was adjusted at 7.4 with NaOH. The resultant fluid was clarified by centrifugation (22000 g) for 30 min at 4 °C and used as the venom of *Chrysaora pacifica* (CpV). Concentrations of CpV used in this study were determined by the method of Bradford [9].

Cell culture

HT-22 neurons as an immortalized hippocampal neuronal cell line were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 .

Measurement of cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate the effects of CpV on cell viability. CpV was treated to HT-22 cells for 30 min and completely washed out. One hour after adding 0.4 mg/mL MTT, medium was gently removed and DMSO was added into each well to dissolve formazan crystals. The cell viability was obtained by reading absorbance at 550 nm using a microplate reader (Model 550, Bio-rad, Philadelphia, PA, USA).

Measurement of intracellular reactive oxygen species (ROS)

CpV was treated for 30 min and then completely washed out. After the addition of 50 μM of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), fluorometric analysis was conducted at an excitation/emission wavelength of 485 nm/535 nm using a microplate reader (Spectra Fluor, Tecan, Austria).

Western blotting analysis

Cells were washed three times with phosphate-buffered saline (PBS; pH 7.4) and lysed with modified RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF, 1 $\mu\text{g/mL}$ aprotinin, and 1 $\mu\text{g/mL}$ leupeptin, pH 7.4). Proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electro-transferred onto a polyvinylidene fluoride membrane (BIO-RAD) using Towbin transfer buffer (192 mM glycine, 25 mM Tris, and 20% methanol, pH 8.3). The blots were incubated with 5% skim milk in TTBS (25 mM Tris, 150 mM NaCl, pH 7.4, containing 0.1% Tween 20) for 1 hour at a room temperature to block nonspecific binding. Subsequently, the membranes were incubated overnight at 4 °C with anti-cytochrome *c* and anti- β -actin antibody. The blots were washed three times with TTBS and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at a room temperature. After several washes, the blots were developed using enhanced chemiluminescence detection reagents (Intron Biotechnology, Sungnam, Korea) according to the manufacturer's instruction. Optical densities of bands were quantified with an Image J analyzer (<http://rsb.info.nih.gov/ij/>) and normalized with those of β -actin.

Statistical analysis

Data analysis and statistical significance were performed by using Excel (Microsoft) software and then data were represented as mean value $\pm$ SE Student's *t*-test and one-way ANOVA were performed to determine significance, set at $p < 0.05$ or 0.01.

Results

The effect of CpV in neurons is not fully known yet, even though it may affect peripheral nerve endings and fibers when the venom induces inflammation and pain. In this study, we first investigated

Fig. 1 CpV-induced neurotoxicity to reduce the viability of HT-22 cells. (A). A jellyfish, *Chrysaora pacifica*. Scale bar: 10 cm. (B). Microscopic views showing neuronal viability in MTT assay with or without CpV treatment (CpV; 0, 100, 250 or 500 ng/mL, 30 min). (C). Normalized data of neuronal viability. Venom (CpV) and bell proteins (Bell protein) were purified from brown tentacles containing nematocysts and bell of jellyfish, respectively. In all groups treated with CpV, the viabilities of HT-22 cells were significantly and dose-dependently reduced. Data indicate the mean $\pm$ SE and ** p < 0.01, compared with 0 ng/mL group

whether CpV might exhibit neurotoxicity to induce cell damage or death in HT-22 cells. Neurons were treated with 100, 250, 500 or 1000 ng/mL CpV for 30 min and then were completely washed out for measuring cell viability (Fig. 1). In an MTT assay, CpV significantly reduced the viability of HT-22 cells at all concentrations, showing the dose-dependent toxicity (Fig. 1C, CpV, 100 ng/mL = 52.73 ± 2.96 , 1000 ng/mL = $30.51 \pm 0.63\%$, p < 0.01, nine trials, compared with 0 ng/mL). This reduction of HT-22 viability was also compared with cells treated with proteins extracted from the jellyfish bell (Bell protein, Fig. 1C, p < 0.05) or not treated with any drugs (0 ng/mL CpV, Fig. 1C). These data indicate that CpV may have a specific cellular effect to induce neuronal damage or death of HT-22 cells.

Neuronal cell death is dominantly caused by oxidative stress, which is directly induced by the activation of Ca^{2+} -mediated excitotoxic signaling cascades. Therefore, it is possible that the CpV-reduced viability of HT-22 cells, shown in Fig. 1, is caused by the oxidation-mediated excitotoxicity. To find out if CpV may affect the cellular ROS production in HT-22 cells, we measured the fluorescence intensities of DCF-DA under the CpV-treated conditions and compared them with those of non-treated cells (0 ng/mL, Fig. 2). In this experiment, increased intensities of DCF-DA fluorescence were observed in CpV-treated HT-22 cells,

demonstrating that CpV increases the production of intracellular ROS in a dose-dependent manner (CpV, 0 ng/mL = 18.14 ± 0.43 , 250 ng/mL = 32.74 ± 0.81 , 1000 ng/mL = $44.19 \pm 1.69\%$, p < 0.05, five trials). The pattern of changes in ROS production by CpV treatment was similar to that in cell viability, showing the dose-dependent cytotoxicity in Figure 1, indicating that CpV-induced cell death might be correlated with the activation of oxidative signaling.

Although CpV might have a cellular mechanism to activate oxidative signaling and to induce excitotoxicity resulting in neuronal death, it is also necessary to test if mitochondrial dysregulation is involved in the cytotoxic effects associated with CpV-increased ROS production. For this, we loaded rhodamine123 to each group of HT-22 cells, and measured the amount of active mitochondria by observing its fluorescent intensity in microscopic views (Fig. 3A). Both 100 and 250 ng/mL CpV significantly reduced the fluorescent intensity of rhodamine123, indicating that CpV induced mitochondrial depolarization. CpV-induced mitochondrial dysfunction was also confirmed by measuring cytochrome *c*, which is a typical reference of mitochondrial-mediated apoptosis. In western blot analysis, the expression level of cytochrome *c* in HT-22 cells treated with CpV was detected more than in control cells (Fig. 3B, CpV, 50 ng/mL = 188.39 ± 31.53 , p < 0.05; 250 ng/

Fig. 2 CpV-induced increase of intracellular ROS in HT-22 cells. (A). Phase contrast microscopic images of HT-22 cells. These images were obtained from HT-22 cells with or without 250 ng/mL CpV. (B). To measure the intracellular ROS level, HT-22 cells were loaded with 2',71-dichlorofluorescein diacetate (DCF-DA), and then fluorescence intensity was measured using a fluorescent microplate reader. The increased rate was calibrated with each cell viability measured by MTT assay. Data indicate the mean $\pm$ SE and * p < 0.05 or ** p < 0.01

mL = 239.98 ± 20.68 , p < 0.01; five trials). Thus, according to these results, CpV-induced neurotoxicity may be caused by oxidative signaling that is mediated with mitochondrial damage.

Because the Ca^{2+} ion is a major factor that triggers ROS signaling in neurons, ROS-mediated neurotoxicity shown in CpV-treated HT-22 cells indicates that Ca^{2+} -activated signaling may be involved. To test this issue briefly, we focused on two cellular processings in CpV-induced neurotoxicity. One was the involvement of Ca^{2+} influx and the other was the effect of N-acetyl-L-cysteine (NAC). The neuronal viability was again observed in the MTT assay under treatment with EGTA (5 mM) or NAC (1 mM). In Figure 4, EGTA treatment of HT-22 cells completely blocked the neurotoxic effect of CpV (500 ng/mL), showing a similar viability in the control group (Fig. 4, CpV = 43.39 ± 3.74 ; CpV + EGTA = $98.18 \pm 1.97\%$, p < 0.05, compared to CpV). NAC added to HT-22 cells also showed the significantly increased viability, compared to cells treated with CpV alone (Fig. 4, CpV + NAC = $67.24 \pm 6.70\%$, p < 0.05, compared with CpV). These results indicate that CpV-induced neurotoxicity involves Ca^{2+} -dependent ROS signaling in HT-22 cells.

Discussion

Toxic venoms of Jellyfish include risky components that cause skin ulceration and necrosis and induce pain, indicating that they are not only superficially but also invasively cytotoxic [1-3]. According to peripheral pain mechanisms, nerve endings are

likely to be targeted by jellyfish venoms, and sequential activation of inflammatory responses may be associated with short- or long-lasting pain induction. Therefore, the noxious effects of jellyfish venoms in inducing peripheral pain may be correlated with neurotoxic mechanisms in neurons. In this study, we tested the neurotoxic effects of venoms extracted from *Chrysaora pacifica* in cultured neurons. This jellyfish is commonly known as a brown species native to the northern Pacific Ocean; its sting is mild but can cause serious irritation and severe burning pain on the skin [10]. First of all, we confirmed that CpV exhibited critical neurotoxic effects to reduce the survival rates of HT-22 cells and that the compound of proteins extracted from the bell of jellyfish rather than from the tentacles showed no toxic effects. These findings strongly suggest the existence of venom-specific neurotoxicity in HT-22 cells (Fig. 1). Furthermore, CpV-induced neurotoxicity in HT-22 cells was dependent on Ca^{2+} , ROS, and mitochondria, indicating the involvement of intracellular and mitochondrial oxidative signaling. The antioxidant effect of NAC, which restored cell viability, also strongly suggests that the activation of ROS signaling may be a major factor that induces the neurotoxicity in HT-22 cells when treated with CpV. Although a predominant pathway of Ca^{2+} to activate ROS signaling has not been addressed in this study, our results provide evidence that removing extracellular Ca^{2+} ions and blocking ROS signaling might effectively reduce the cytotoxicity of jellyfish venoms.

The cellular mechanism of HT-22 cells to preserve themselves under neurotoxic conditions seems to be similar to that of neurons in an *in vivo* system, even though cultured HT-22 cells are not

Fig. 3 The involvement of mitochondrial signalings in CpV-induced neurotoxicity. (A). The fluorescent intensity of Rhodamine123 was tested in HT-22 cells with or without CpV (0, 100, 250 ng/mL) to observe the changed activities of mitochondria. Active mitochondria were significantly reduced in CpV-treated groups. (B). The expression of cytochrome *c* was analyzed by a western blot analysis. Cytochrome *c* was more expressed in CpV-treated HT-22 cells, indicating the increment of mitochondrial fractions. Data indicate the mean $\pm$ SE and * p < 0.05 or ** p < 0.01

fully conditioned as in an intact nervous system [11,12]. The survival rates of HT-22 cells, in this study, were critically dependent on the concentration of CpV added to the culture media, suggesting that the neuroprotective functions of cells might be activated at low concentrations of CpV. Additionally, Ca^{2+} chelation significantly restored the survival rates of cells at the high concentration of CpV, providing evidence that Ca^{2+} might act as a critical mediator for CpV-induced neurotoxicity. In normal or slightly toxic conditions, Ca^{2+} plays a role in regulating cellular signaling associated with protein synthesis, cellular metabolism, ATP productions in mitochondria, and neuroprotective functions [13]. However, the excessive influx of extracellular Ca^{2+} under neurotoxic conditions activates pathophysiological signaling, leading to a significant increase of ROS, which is due to the

facilitated oxidative process of mitochondria [13-16]. Although we did not quantitatively measure ROS production of mitochondria in this study, changes of Rhodamine123 fluorescence and cytochrome *c* expression demonstrate that CpV crucially induced mitochondrial dysfunction in HT-22 cells (Fig. 3). This provides evidence that mitochondria-mediated apoptotic signaling cascades may be activated in the neurotoxic mechanism of CpV. Membrane depolarization and dysfunction of mitochondria are dominantly dependent on excessive Ca^{2+} influx, and contribute to the activation of apoptotic processes in various types of mammalian cells [7,17-19]. In this experiment, we observed the reduction of cytochrome *c* level at 500 ng/mL treatment of CpV (Fig. 3B). The release of cytochrome *c* from mitochondria is a key factor for not only indicating but also initiating apoptosis [20,21]. The concentration

Fig. 4 Antioxidant effects against CpV-induced neurotoxicity. (A). Microscopic views showing the antioxidant effects of EGTA (CpV + EGTA) and NAC (CpV + NAC) in CpV-treated HT-22 cells. (B). Normalized viabilities of CpV-treated HT-22 cells under the antioxidant conditions. Both EGTA and NAC treatments significantly enhanced neuronal viability of CpV-treated HT-22 cells, indicating that CpV-induced neurotoxicity may result from oxidative stress. Data indicate the mean $\pm$ SE and $*p < 0.05$

of 500 ng/mL CpV seems to be critical to exhibit the maximal reduction of cell viability (as shown in Fig. 1), and there is a possibility that a potent apoptotic signaling may be activated and induce mitochondrial damage at this concentration of CpV. Therefore, the reduction of cytochrome *c* at 500 ng/mL CpV may be due to the apoptosis-reduced protein synthesis or severe damage of mitochondria showing the abnormal permeability of outer membrane [22]. Furthermore, neurons are more susceptible to apoptotic processings because of their dynamic Ca^{2+} signaling [23–27]. Although we did not figure out which pathways of Ca^{2+} influx contributed to CpV-induced neurotoxicity, the antioxidant effects of Ca^{2+} chelation and NAC on the survival rates of HT-22 cells indicate that CpV may lead to the neurotoxicity that is associated with Ca^{2+} -mediated ROS signaling, as is supported by previous reports [28–30].

In this study, we did not confirm if the neurotoxic effects of CpV shown in HT-22 cells were mediated by peripheral pain or inflammatory responses. However, our results suggest that CpV-activated Ca^{2+} signaling may participate in the peripheral pain mechanism induced by jellyfish venoms, as is consistent with previous studies reporting that Ca^{2+} influx and mitochondrial regulation contribute to pain modulation in the nerve endings of

the mammalian periphery [31,32]. Although further studies should be addressed, the regulation of Ca^{2+} signaling and mitochondrial functions may be a potent clinical approach to pain, inflammation, and cytotoxicities that are induced by jellyfish venoms.

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