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Apoptosis induction is involved in UVA-induced autolysis in sea cucumber *Stichopus japonicus*

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ABSTRACT

Autolysis easily happens to sea cucumber (*Stichopus japonicus*, *S. japonicus*) for external stimulus like UV exposure causing heavy economic losses. Therefore, it is meaningful to reveal the mechanism of *S. japonicas* autolysis. In the present study, to examine the involvement of apoptosis induction in UVA-induced autolysis of *S. japonicas*, we investigated the biochemical events including the DNA fragmentation, caspase-3 activation, mitogen-activated protein kinases (MAPKs) phosphorylation and free radical formation. Substantial morphological changes such as intestine vomiting and dermatolysis were observed in *S. japonicus* during the incubation after 1-h UVA irradiation (10 W/m^2). The degradation of the structural proteins and enhancement of cathepsin L activity were also detected, suggesting the profound impact of proteolysis caused by the UVA irradiation even for 1 h. Furthermore, the DNA fragmentation and specific activity of caspase-3 was increased up to 12 h after UVA irradiation. The levels of phosphorylated p38 mitogen activated protein kinase (MAPK) and phosphorylated c-Jun.-N-terminal kinase (JNK) were significantly increased by the UVA irradiation for 1 h. An electron spin resonance (ESR) analysis revealed that UVA enhanced the free radical formation in *S. japonicas*, even through we could not identify the attributed species. These results suggest that UVA-induced autolysis in *S. japonicas* at least partially involves the oxidative stress-sensitive apoptosis induction pathway. These data present a novel insight into the mechanisms of sea cucumber autolysis induced by external stress.

1. Introduction

Sea cucumber *Stichopus japonicus* (*S. japonicus*) is one of the most important holothurian species in coastal fisheries. In China, its total production of *S. japonicus* has reached more than 200,000 tons in 2014 with an overall economic value of more than 6 billion US dollars [40]. Autolysis is a common phenomenon that takes place in many aquatic animals [36,38,41], including sea cucumber, and causes substantial deterioration of the overall quality, especially during handling and storage, leading to heavy economic loss [25]. In response to external stimuli, the body of a certain species of sea cucumber becomes slackened because of part of the initially firm body wall flowed out in a sticky mass. Such a massive tissue autolysis of sea cucumber, also called "melting" or "local degeneration", has been reported in a number of publications [38].

UV radiation has long been recognized as one of the important stress factors in the marine systems and the direct absorption of specific wavelengths by macromolecules such as DNA, protein and chlorophyll is the primary cause of the harmful effects [25]. The DNA damage includes the formation of single strand breaks and double strand breaks (DDBs) [9]. The UV radiation also causes sunburn and immune suppression [11]. In sea cucumber cells, chromatin condensation and marginalization as well as cytoplasmic condensation have been observed with apoptosis or apoptosis-like cell death [38]. Oxidative stresses are resulted from the accumulation of reactive oxygen species (ROS) beyond the capacity of the cells to quench them [18]. UV irradiation has been shown to result in the activation of mitogen-activated protein kinases (MAPKs) superfamily, *e.g.* the extracellular signal-regulated kinase (ERK), c-Jun. N-terminal kinase (JNK) and p38 MAPK [6]. The ERK signaling pathway plays a critical role in the regulation of cell growth, differentiation and development. JNK, also known as stress activated protein kinase (SAPK), is preferentially activated by the cytokines, growth factors or cellular damage [13], while the p38, a stress-responsive MAPK, is triggered by the environmental stresses, including ionizing radiation, oxidative stresses and heat shock [4]. The activation of JNK and p38 has been reported to play an important role in the induction of cell death [14].

Abbreviations: UVA, ultraviolet A; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinases; JNK, cJun-N-terminal kinase; ERK, extracellular signal-regulated protein kinase; CL, cathepsin L; ESR, electron spin resonance; DMPO, dimethyl pyridine N-oxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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In order to gain a thorough understanding on the molecular mechanism of UVA-induced autolysis in sea cucumber *S. japonicus*, we examined the effect of UVA on the protein degradation, cathepsin L (CL) activity, as well as apoptosis-related biochemical events such as DNA fragmentation and caspase-3 activity. The present results demonstrate that UVA irradiation induce severe autolysis, possibly through the apoptosis induction accompanied with the enhanced free radical formation and phosphorylation of MAPKs.

2. Materials and Methods

2.1. Materials and Chemicals

Sea cucumber *S. japonicus* (one year old, 3–4 cm) were obtained from Dalian Bangchuidao Seafood Group Ltd. (E122°17′, N38°55′) from November 2014 to June 2015. Antibodies were used for western blotting including anti-phospho-p38 MAP Kinase (Thr180/Tyr182) rabbit polyclonal antibody (Cell Signaling 9211, 1:1000); anti-phospho-SAPK/JNK (Thr183/Tyr185) rabbit polyclonal antibody (Cell Signaling 9251, 1:1000); anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit polyclonal antibody (Cell Signaling 4370, 1:2000), anti-β-actin rabbit polyclonal antibody (Cell Signaling 4967, 1:1000) and a secondary anti-rabbit IgG (HRP-linked antibody, Cell Signaling 7074) were purchased from Cell Signaling (Beverly, MA, USA). Sodium dodecyl sulphate (SDS), *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250, DL-dithiothreitol (DTT) and Bovine serum albumin (BSA) were from Sangon Biotech Co., Ltd. (Shanghai, China). Caspase-3 activity kit was from Beyotime Institute of Biotechnology (Haimen, China). All the other chemicals used in this study were of analytical grade.

2.2. UVA Irradiation

S. japonicus were transferred to plastic petri dishes with ten individuals in each. Some were exposed to UVA irradiation for 10, 20, 30, 60, and 120 min using UVA lamps (wavelength range between 320 and 400 nm, with total intensity of 10 W/m^2) for the analysis of ROS levels and MAPKs signaling pathways. In addition, for other assays, the treatment of *S. japonicus* was done as previously reported with some modification [26,27]. After 1 h exposure, *S. japonicus* samples were kept at 25 ± 1 °C in dark incubator with little seawater for 6, 12, and 24 h, respectively. The 24 h samples were chosen to examine the morphological changes and images were captured at regular intervals. Control samples were the fresh *S. japonicus* which were captured from the feeding field without UVA irradiation and directly stored at −80 °C after taking photos. All the irradiated and control samples were stored at −80 °C for further experiments.

2.3. Protein Degradation

Protein degradation amount was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The samples were ground and protein in samples were extracted with protein extraction solution (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid disodium salt, 10 mM DTT, 1% Triton X-100, 0.1% SDS) at a sample to extraction solution ratio of 1:3 (*w*/*v*) on ice for 20 min. Then the extracts were centrifuged at 12,000 *g* for 15 min, at 4 °C, and supernatants were diluted with SDS-PAGE sample buffer (0.25 M Tris–HCl, pH 7.4, 8 M Urea, 5% SDS, 5% mercaptoethanol) to obtain a protein concentration of 2 mg/mL [3]. The diluted sample solution was boiled for 5 min and an aliquot of the boiled sample (10 μL) was analyzed with 10% polyacrylamide gel electrophoresis using a Mini Protein system (Bio-Rad Laboratories). After separation and staining, the gel was documented using GelCapture software (DNR Bio-Imaging Systems, Jerusalem, Israel).

2.4. Cathepsin L (CL) Activity Assay

S. japonicus from each incubation time (6 h, 12 h and 24 h after UVA radiation for 1 h) were homogenized in protein extraction solution (50 mM phosphate buffer at pH 7.0, 1 mM EDTA, 0.1% Triton X-100) at 4 °C by 1:3 (*w*/*v*). The homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C, supernatants were collected for analysis of CL activities using Z-Phe-Arg-Mec at the concentration of 20 μM as a specific substrate for CL. The activity of CL was analyzed in tubes containing 25 μL of assay buffer (350 mM KH2PO4, 480 mM Na2HPO4, 4 μM disodium EDTA, pH adjusted to 6.0 with 1 M HCl, 8 μM cysteine, 8 μM DTT) and 50 μL of extract sample. The assay was started by adding 25 μL of 20 μM substrate solution and incubated for 15 min at 37 °C. The reaction was stopped by adding 100 μL of 10% trichloroacetic acid. The CL activity determined by the fluorescence intensity detected in a supernatant was recorded at excitation 380 nm and emission 460 nm using a fluorospectrophotometer (Hitachi, Japan, F-2700). The activity of CL was expressed in U per mg of protein at saturating substrate concentrations.

2.5. DNA Fragmentation

The treated *S. japonicus* for various times were collected, and DNA was isolated using procedures described by Miyoshi et al. procedure [22]. Extract DNA samples were analyzed by electrophoresis in 2% agarose gels, followed by staining with gel red and imaged with an UV system (Bio-Rad, US).

2.6. Caspase-3 Activity Assay

Caspase-3 activity was measured by colorimetric assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's instructions. Briefly, *S. japonicus* kept for different time intervals were homogenized with lysis buffer and the lysates were centrifuged at 12,000 *g* for 15 min at 4 °C. Subsequently, the supernatants were incubated with acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) substrate at 37 °C for 30 min. Samples were read at 405 nm in enzyme-labeled instrument (Tecan Infinite, Switzerland, M200). The protein concentrations were measured by Brad-ford method [3]. Caspase-3 activity determined in UVA-treated samples was reported as a relative increase to the control group (non-UVA-treated samples).

2.7. Western Blot

S. japonicus exposed to UVA radiation were lysed on ice in a lysis buffer (20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 10 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 1% Trion X-100, 0.1% SDS, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF). Lysates were centrifuged at 12,000 rpm for 10 min, at 4 °C, and supernatants were collected. Total protein concentration in supernatants was measured by the Bradford method. 20 μg protein was subjected to 12% SDS-PAGE and the proteins separated on the gel were transferred to Polyvinylidene Fluoride (PVDF) membranes (Millipore, Billerica, America). The membranes were blocked and then incubated with primary antibody overnight at 4 °C followed by the secondary antibody. Proteins were detected by chemiluminescence (Nacalai Tesque, Kyoto, Japan). Films were scanned by the ChemiDoc system (DNR Bio-Imaging Systems, Jerusalem, Israel).

2.8. ESR Measurement

The *S. japonicuss* irradiated for different time were directly lyophilized, sealed in lucifugal bags and stored at −80 °C. 0.02 g of powder was transferred into a glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and placed in the ESR cavity. The ESR spectra were recorded at room temperature using A200 from Bruker (Karisruhe,