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Effect of yessotoxin on cytosolic calcium levels in human hepatocellular carcinoma cells *in vitro*

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Abstract. Yessotoxin (YTX) and its analogs are a type of marine toxins found in marine environments in numerous coastal countries. These toxins tend to accumulate in filter-feeding molluscs and may threaten the shellfish industry and public health. Several previous studies indicated that YTX may induce apoptosis in different types of cell lines, although the exact underlying mechanisms have not yet been elucidated. The aim of this study was to mainly focus on the effect of YTX on cytosolic Ca^{2+} levels in human hepatocellular carcinoma cells. In order to investigate the exact mechanism of YTX-evoked Ca^{2+} increase, laser scanning confocal microscopy was used, with the addition of the chelator ethylene glycol tetraacetic acid (EGTA) and nifedipine, an L-type Ca^{2+} channel blocker, to the reaction system. The results demonstrated that YTX caused cytosolic Ca^{2+} level increase in Bel7402 cells and the YTX-evoked Ca^{2+} increase was successfully blocked by EGTA and nifedipine. Therefore, our results indicated that YTX may cause apoptosis via inducing Ca^{2+} entry in Bel7402 cells.

Introduction

Yessotoxin (YTX) and its analogs are a group of lipophilic marine toxins mainly produced by the dinoflagellates *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum*. These toxins tend to accumulate in filter-feeding molluscs, have been found in numerous countries worldwide (1-6) and were first detected in shellfish samples collected from Chinese coastal areas in 2009 (7).

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Abbrevations: AM, acetoxymethyl ester; DSP, diarrheic shellfish poisoning; FACS, fluorescent-activated cell sorting; HBSS, Hanks' balanced salt solution; LSCM, laser scanning confocal microscopy; NRC, National Research Council; YTX, yessotoxin

Key words: yessotoxin, toxicity, Bel7402 human hepatocellular carcinoma cells, Ca^{2+} level

YTX and its analogs were first isolated from *Patinopecten yessoensis* in Japan (8) and were long classified as one of the categories of diarrheic shellfish poisoning (DSP) toxins (9). However, as these toxins cannot induce diarrhea, nor inhibit protein phosphatase 2A, like other DSP toxins, they were classified as an independent group by the European Union Commission in 2002 (10).

Apoptosis is a programmed form of cell suicide. The process of apoptosis is controlled by genes and is crucial in disease outbreaks, including cancer. Once the signaling pathway of apoptosis is activated, the process cannot be easily undergone, even by tumor cells. Thus, there is an increasing number of studies on tumor cell apoptosis, with the aim to design an effective cancer treatment (11-13). Several studies indicated that YTX may induce apoptosis in different types of cell lines (14-21). However, the exact underlying mechanisms have not been elucidated.

As the chemical structure of YTX is similar to that of brevetoxins and ciguatoxins, which were shown to interfere with voltage-gated sodium channels, the effects of YTX on transmembrane ion channels were previously investigated (22,23). The present study mainly investigated the YTX-induced alterations in intracellular Ca^{2+} levels in Bel7402 human hepatocellular carcinoma cells and the possible underlying mechanisms.

Materials and methods

Reagents. Pure YTX was purchased from the National Research Council (NRC; Ottawa, ON, Canada). Fluo-3 acetoxymethyl ester (AM) solution (5 mM) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Nifedipine was purchased from Sigma (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany). All other chemicals were of analytical reagent grade or higher purity.

The Hanks' balanced salt solution (HBSS) used for cell washing consisted of 137 mM NaCl, 5.6 mM KCl, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.38 mM Na_2HPO_4, 0.44 mM KH_2PO_4 and 4.2 mM $NaHCO_3$. The pH of the HBSS was adjusted to 7.4 with 0.1 M HCl and NaOH.

Cell culture. The Bel7402 human hepatocellular carcinoma cell line was purchased from the Cell Bank of the Shanghai

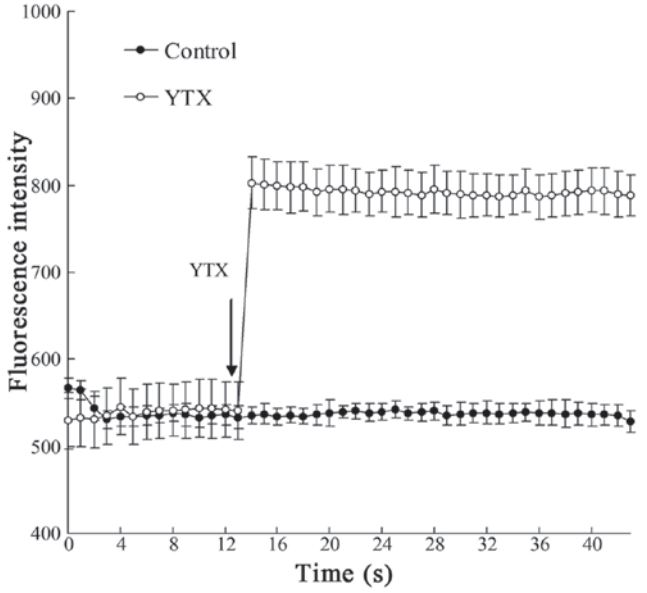

Figure 1. Effect of yessotoxin (YTX) on the Ca^{2+} levels in Bel7402 human hepatocellular carcinoma cells, compared to the control group. The attached Bel7402 cells in a 24-well microplate were loaded with Fluo-3 AM and bathed in a balanced salt solution containing 1 mM $CaCl_2$. At the time point indicated by the arrow, 100 ng YTX were added to the bathing medium.

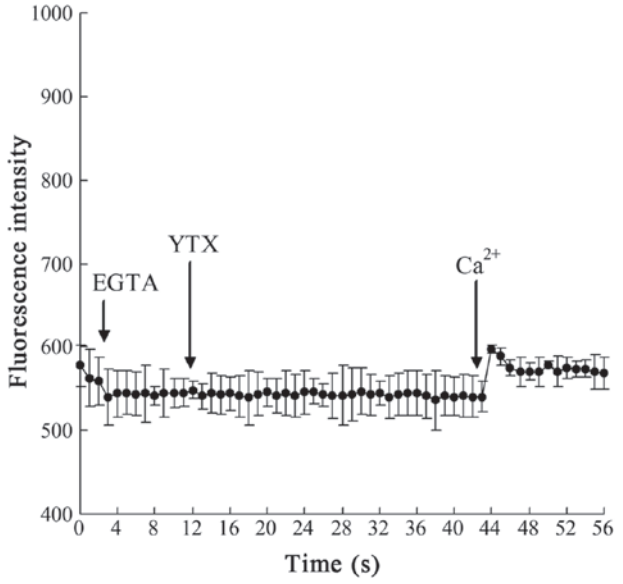

Figure 2. Effect of yessotoxin (YTX) on Bel7402 cells in a solution without Ca^{2+}. The attached cells in the microplate were loaded with Fluo-3 AM. Susequently, the Ca^{2+} in the extracellular medium was removed using EGTA (indicated by the first arrow). YTX (100 ng) was then added to the medium. There was no observed increase of Ca^{2+} levels in the cells. Approximately 30 sec later, $CaCl_2$ at a final concentration of 2 mM was added to the medium, with no resulting rise in Ca^{2+} levels. EGTA, ethylene glycol tetraacetic acid.

Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 300 mg/l L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, plus 10% fetal calf serum (TBD, Beijing, China) and incubated at 37˚C in a humidified 5% CO_2 atmosphere.

Analysis of intracellular Ca^{2+} levels. The Bel7402 cells were transferred to a 24-well microplate and incubated at 37˚C. When 90% confluence was reached, the cells were washed twice with HBSS and loaded with Fluo-3 AM at a final concentration of 5 μM for 1 h at 37˚C in the dark. The solution containing the dye was then removed and the cells were washed twice with HBSS.

The average fluorescence intensity of intracellular Ca^{2+} concentration in labeled cells was detected under a laser scanning confocal microscope (FluoView FV1000; Olympus Co., Tokyo, Japan). The wavelength of excitation was set at 488 nm and the emission wavelength at 525 nm for Fluo-3 fluorescence reading. Newly-developed FV300/FV500 Application software was used for the measurement and analysis of Ca^{2+} concentration.

Ethylene glycol tetraacetic acid (EGTA) and nifedipine, at a final concentration of 2 mM and 1 μM, respectively, were added to the reaction system to investigate the exact mechanism of YTX-evoked Ca^{2+} increase.

Results

Effect of YTX on intracellular Ca^{2+} levels. As shown in Fig. 1, the intracellular Ca^{2+} levels in Bel7402 cells were obviously increased following the addition of YTX to the medium, with the increase of Ca^{2+} levels lasting for >40 sec.

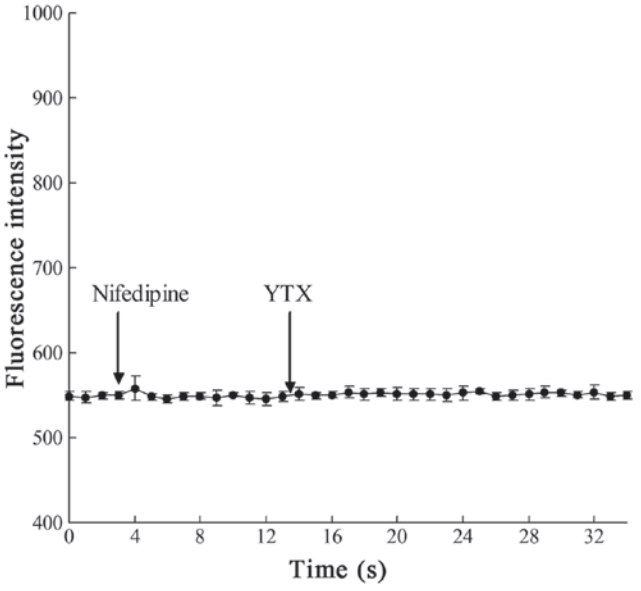

Figure 3. Effect of nifedipine on yessotoxin (YTX)-induced Ca^{2+} concentration elevation. Nifedipine is a well-known effective blocker of L-type Ca^{2+} channels. Following addition of nifedipine, YTX was added to the extracellular medium, with no resulting increase in the Ca^{2+} concentration.

Addition of EGTA. EGTA at a final concentration of 2 mM was used to investigate whether the YTX-evoked Ca^{2+} increase was caused by Ca^{2+} in the extracellular medium or released from cytoplasmic organoids. As shown in Fig. 2, in a Ca^{2+}-free solution, YTX was not able to induce an elevation of the Ca^{2+} concentration. Although 2 mM $CaCl_2$ was then added to the medium, the fluctuation of Fluo-3 fluorescence was marginal.

Addition of nifedipine. The possible involvement of Ca^{2+} channels in the elevation of Ca^{2+} concentration evoked by YTX was also investigated. Several drugs are known to block different types of Ca^{2+} channels. Nifedipine, at a final concentration of 1 μM, was used in this study to block the L-type Ca^{2+} channels; YTX was then added to observe the fluorescence change in the Bel7402 cells. As shown in Fig. 3, the YTX-evoked Ca^{2+} increase was completely blocked by nifedipine.

Discussion

YTX is a low-molecular weight compound that is toxic to different mammalian and insect cell types, including cancer cells (14,15,23-26). Our previous studies demonstrated the apoptosis induced by YTX in HeLa human cervical cancer cells, Bel7402 human hepatocellular carcinoma cells and HL7702 human liver cells (19-21). The identification of cell death signalling pathways and cellular factors involved in apoptosis induced by YTX may provide useful information for evaluating the potential use of YTX for therapeutic purposes, including cancer research. Botana Lopez *et al* (27) indicated that YTX may be used as an antitumor agent, with this possibility already considered in the European patent application EP1875906.

Apoptosis, the process of programmed cell death, is often characterized by an orderly progression of morphological changes, including cell shrinkage, condensation of chromatin and externalization of membrane phosphatidylserine, and is controlled by cell signals that may originate extracellularly or intracellularly (28). These signals may positively or negatively affect apoptosis. Extracellular signals, including toxins, hormones, growth factors, nitric oxide and cytokines (29), may either cross the plasma membrane or transduce into intracellular signals in order to affect other responses. Ca^{2+} is an important intracellular second messenger which is crucial in numerous processes, including cell proliferation, differentiation, DNA damage and apoptosis (30,31). Thus, the changes in the cytoplasmic Ca^{2+} concentration may induce the process of apoptosis (32,33). Previous studies indicated that YTX may induce the increase of Ca^{2+} levels in several types of cells (22,23,26). Our previous studies also demonstrated that YTX may induce Ca^{2+} entry in different types of human cells (21,34). Ca^{2+} ions are crucial in the process of apoptosis and cytoplasmic Ca^{2+} concentration is involved in the modulation of several cell functions. It was previously suggested that the capacitative Ca^{2+} influx through Ca^{2+} channels and the Ca^{2+} released in cytoplasm from intracellular organelles are apoptogenic (35-37). As described by Putney (38,39), the release of Ca^{2+} from intercellular stores may induce Ca^{2+} influx from the extracellular medium. However, de la Rosa *et al* (22) demonstrated that the Ca^{2+} influx induced by YTX was not affected by internal stores. Our previous study also indicated that YTX did not affect internal Ca^{2+} levels in a Ca^{2+}-free medium (21).

Although the changes in the Ca^{2+} levels may be associated with the apoptosis induced by YTX, the exact mechanism underlying this increase in Ca^{2+} has not been elucidated. The chemical structure of YTX is similar to that of brevetoxins, which are known to interfere with sodium channel function. Therefore, it is possible that YTX is also able to interact with cellular ion channels. We previously demonstrated that nifedipine, a specific L-type Ca^{2+} channel blocker, was able to inhibit the Ca^{2+} increase induced by YTX in several human cell lines (21,34). YTX may exert an effect on L-type Ca^{2+} channel in cells. Although YTX may induce the Ca^{2+} increase in HL7702 normal human liver cells and Bel7402 human hepatocellular carcinoma cells, the increasing trend of Ca^{2+} levels in Bel7402 cells was different from that observed in HL7702 cells (34). This difference may provide new insight into cancer therapy. Further studies are required to elucidate the exact mechanisms underlying apoptosis induced by YTX in tumor cells.

In conclusion, YTX was shown to exert a cytotoxic effect on normal human liver cells and human hepatocellular carcinoma cells. Although YTX may induce Ca^{2+} influx in these two cell types, the detailed increasing trend of Ca^{2+} levels was different. The difference between normal human liver cells and human hepatocellular carcinoma cells may provide new insight into cancer therapy.

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