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Cobra cardiotoxin-induced cell death in fetal rat cardiomyocytes and cortical neurons: different pathway but similar cell surface target

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Abstract

Cobra cardiotoxins (CTXs) are basic polypeptides with diverse pharmacological functions that are cytotoxic to many different cell types through both necrotic and apoptotic cell death pathways. In this comparative study of the action of CTX A3 from the Taiwan cobra (*Naja atra*) on fetal rat cardiomyocytes and cortical neurons, it was shown that CTX A3 induced different patterns of elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), CTX internalization, caspase-3 activity and viability. Application of an anti-sulfatide monoclonal antibody, O4 specific for 3-sulfo-galactose lipid, but not in the control experiments using anti-GM3 monoclonal antibody, reduces CTX-induced $[Ca^{2+}]_i$ elevation, CTX internalization and toxicity. Therefore, CTX may target similar sulfo-containing cell surface receptors in both fetal rat cardiomyocytes and cortical neurons, but induce cell death through different pathways specific to each cell type.

Keywords: Cardiotoxin; Neurons; Cardiomyocytes; Apoptosis; Necrosis

1. Introduction

Cardiotoxins (CTXs) from cobra snake venom are basic β-sheet amphiphilic polypeptides with a characteristic three-finger loop structure (Forouhar et al., 2003). Many different targets of the venom have been suggested based on in vitro studies of CTXs action on several cell lines and model membrane systems (Dufton and Hider, 1991; Forouhar et al., 2003; Sue et al., 2002). These targets include negatively charged and zwitterionic phospholipids, glycosaminoglycans, Na^+/K^+ ATPase, ion channels and protein kinase C (Chien et al., 1994; Chiou et al., 1995; Guo et al., 1993; Harvey, 1991; Su et al., 2003; Vyas et al ,1998). The wide range of potential targets of CTX action on cell membranes could explain its diverse pharmacological functions in CTX-induced toxicity in many different cell systems. It is also possible that the toxin targets a similar receptor in all cell types, but induces distinct cell-type specific responses.

Identification of a specific target for toxin action could help in the development of drugs to treat patients suffering from the toxins effects and also to be used as tools to study cell function. For instance, the binding specificity and affinity of cobra α-neurotoxin toward the acetylcholine receptor has allowed for the development of purification methods for the receptor and for the study of its cellular distribution during development (Nirthanan and Gwee,

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2004). In addition, the bacterial cholera AB_5 toxin, known to bind specifically to gangliosides GM1 in the plasma membrane, exerts its toxicity by hijacking the cellular endocytic pathway to move from the plasma membrane through *trans*-Golgi and endoplasmic reticulum (ER) to the cytosol of the host cells (Lencer et al., 1999; Sandvig and van Deurs, 2002). These toxins have become major tools to understand the processes of endocytosis. Also, viral pro-apoptotic and anti-apoptotic proteins can target mitochondria and control cell death by binding to host-specific apoptosis-modulatory proteins such as activated caspase-3 (Boya et al., 2004).

The effort to develop cytotoxic CTX as an anticancer drug has revealed its preferential cytotoxicity toward cancer cells, possibly acting through an inhibition of protein kinase C activity (Chiou et al., 1995; Guo et al., 1993). Induction of apoptosis in human leukemia K562 cells and enhancement of activation-induced apoptosis of $CD8^+$T lymphocyte have been reported for CTX A3 (Yang et al., 2005; Su et al., 2003). In contrast, CTX-induced myocyte cell death in the envenomed tissue is usually due to a necrotic pathway (Fletcher et al., 1996; Lee et al., 1968). Furthermore, the major CTX from Taiwan cobra, i.e. CTX A3, has recently been shown to target mitochondria and disrupt its network in H9C2 myoblast (Wang and Wu, 2005). It was suggested that CTX-induced fragmentation of mitochondria play a role in controlling CTX-induced necrosis of myocytes.

In our ongoing effort to understand the mechanism of action of CTX on cardiomyocytes, it was recently demonstrated that sulfated glycosaminoglycans, such as heparan sulfate in the extracellular matrix, can bind specifically to different CTX homologues and help in retaining specific CTXs on the cell surface in the presence of citrate (Lee et al., 2005). NMR and X-ray studies of CTX complexed with heparin-derived mimetics have further revealed that CTXs consist of an anionic binding pocket that appears to be suitable for the binding of sulfo-containing compounds (Sue et al., 2001; Ke et al., 2005). Therefore, sulfatide located on the outer membranes of many cells including neurons, might be a target for CTX action. With the availability of an anti-sulfatide monoclonal antibody, anti IgM O4, known to bind to sulfo-containing lipids with specificity toward 3-sulfo galactose, including galactosylceramide sulfate (SM4s), galactosylalkylacylglycerol sulfate (SM4g), lyso-SM4g and HSO_3-cholesterol (Ishizuka, 1997), comparative studies of the action of CTX A3 from the Taiwan cobra on fetal rat cardiomyocytes and cortical neurons were conducted to test this hypothesis.

2. Materials and methods

Crude snake venom (*Naja atra*) was purchased from a local snake farm (Tainan, Taiwan). Fluorescent dyes were purchased from Molecular Probes (Eugene, OR, USA). Cell culture supplies were purchased from GIBCO/Life Technologies, Inc. (Gaithersburg, MD, USA). Anti-sulfatide (O4) was obtained from Chemicon, Inc. (Temecula, CA, USA). Anti-GM3 monoclonal antibody (GMR6) was obtained from Seikagaku Corp. (Chuo-ku, Tokyo, Japan). Ac-DEVD-MCA was purchased from Bachem Bioscience (King of Prussia, PA, USA). Other chemicals were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) and Merck Frost (Montreal, Canada).

2.1. CTX preparations

Natural cardiotoxin (CTX) were purified from *Naja atra* venom by SP-Sephadex C-25 ion exchange column chromatography (Chien et al., 1994) and reverse phase high performance liquid chromatography (HPLC). Rhodamine B isothiocyante (0.5 mM) and CTX A3 (0.2 mM) in denaturation buffer (6 M Gn.HCl, 0.1 M NaH_2PO_4, pH 8) were mixed and incubated at 37 °C overnight. Labeled and unlabeled CTX A3 were separated by reverse phase HPLC and confirmed by mass spectroscopy. The concentration of the toxins was estimated from its absorbance at 277 nm ($\varepsilon^{1\ mM}=4.185$) for natural CTX, and 559 nm ($\varepsilon^{1\ mM}=105$) for Rh-CTX.

2.2. Cell cultures

Cardiomyocytes were isolated from day 18 fetal Wistar rats. Hearts were immersed in ice-cold Hanks balanced salt solution (HBSS) immediately after their removal. After a thorough washing, the minced ventricles were placed in sterile HBSS containing 0.25% trypsin at 37 °C for 30 min. To obtain purified cardiomyocytes, the dissociated cells were plated for 1 h and the unattached cardiomyocytes were collected, counted and seeded in culture medium (72% Dulbecco's Modified Eagle's medium, 18% Medium 199, 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin) on poly-L-lysine-coated plates or glass coverslips.

Rat cortical neurons were cultured as described (Small et al., 1998). The brains of day 18 fetal Wistar fetal rats were isolated and the cortices were dissected and triturated to dissociate cells. The cells were counted and seeded in culture medium (80% minimum essential medium (MEM), 10% horse serum, 10% fetal bovine serum, and 2 mM L-glutamine) on poly-L-lysine-coated plates or glass coverslips. To minimize glial growth, the cultures were treated with the mitotic inhibitors 15 μg/ml 5-fluoro-2′-deoxyuridine and 35 μg/ml uridine on day 4 of culture for 2 days at which time one-half of the medium was removed and replaced with growth media consisting of 90% MEM and 10% horse serum. Since cortical neurons grow on supporting layers of astrocytes, image analysis was performed by focusing on the top or bottom cell layers, to observe CTX-induced responses in neurons and astrocytes, respectively.