Amphiphilic β-sheet cobra cardiotxin targets mitochondria and disrupts its network

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Abstract Recent advance in understanding the role of toxin proteins in controlling cell death has revealed that pro-apoptotic viral proteins targeting mitochondria contain amphiphilic β-helices with pore-forming properties. Herein, we describe that the pore-forming amphiphilic β-sheet cardiotoxins (or cytotoxins, CTXs) from Taiwan cobra (Naja atra) also target mitochondrial membrane after internalization and act synergistically with CTX-induced cytosolic calcium increase to disrupt mitochondria network. It is suggested that CTX-induced fragmentation of mitochondria play a role in controlling CTX-induced necrosis of myocytes and cause severe tissue necrosis in the victims.

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1. Introduction

Cardiotoxins (CTXs) from cobra snake venom are basic β-sheet polypeptides with a characteristic three-finger loop structure (Fig. 1A). The available three-dimensional structures determined by X-ray and NMR in the presence and absence of membrane lipids indicate that CTXs bind to phospholipids and sphingolipid membranes and a continuous hydrophobic patch flanked by cationic lysine residues is involved in this interaction [1–3]. In addition, membrane-induced CTX oligomerization, followed by the re-arrangement of membrane lipid to form pores ranging from 20 to 30 Å, are suggested to be mainly responsible for the general cytotoxicity of CTX on many cell types, including cardiomyocytes [4,5]. Interestingly, CTXs have also been shown to bind to cardiolipin, a lipid component specifically located at mitochondria, in model membrane study and induce lipid re-organization [6–8]. CTXs also bind to intracellular ligands of nucleotide triphosphate (ATP and GTP) [9]. The CTX pore formation in plasma mem-

brane is likely to be involved in the CTX-induced perturbation of cytosolic calcium homeostasis and hypercontracture in rat ventricular myocytes [5,10]; however, evidence indicating that CTX may target intracellular organelle within the cell is lacking.

A large number of protein toxins from bacteria and virus have been extensively studied in order to understand their mechanism of action as well as of intracellular membrane trafficking [11–13]. For instance, cholera toxin and related AB5-subunit bacterial toxin have been shown to follow a specific glycolipid pathway to move from plasma membrane through trans-Golgi and endoplasmic reticulum (ER) to the cytosol of host cells [14,15]. Furthermore, anti-apoptotic and pro-apoptotic viral proteins from Vaccinia and Hepatitis B virus, respectively, act on the mitochondria and control apoptosis of infected cells [16,17]. Boya et al. have suggested that almost all known pro-apoptotic viral proteins proposed to translocate to mitochondrial membrane consist of amphiphilic basic β-helices with pore-forming properties lacking mitochondria targeting sequence. These proteins also induce mitochondrial membrane permeabilization (MMP), which is often accompanied by mitochondrial swelling and fragmentation [18].

Here, we study the internalization of the major CTX from Taiwan cobra, i.e., CTX A3, using both fluorescein isothiocyanate (FITC)-labeled CTX and CTX-antibody to identify its intracellular location. Although CTX A3 lacks mitochondria targeting sequence and the β-helical structure, it targets the mitochondrial membrane and induces mitochondrial swelling and fragmentation accompanied by CTX induced perturbation of calcium homeostasis. The significance of this observation is discussed to shed light on the structural property of mitochondrial targeting protein, the internalization of amphiphilic polypeptides and the action mechanism of CTXs.

2. Materials and methods

2.1. Materials

Crude snake venom (Naja atra) was purchased from local snake farm (Tainan, Taiwan). Antivenin of Bungarus multicinctus and Naja atra was purchased from Center for Disease Control (Taipei, Taiwan). Fluorescence dyes were purchased from Molecular Probes (Eugene, OR, USA). Ac-DEVD-MCA was purchased from Bachem Bioscience (King of Prussia, PA, USA). Fetal calf serum was purchased from Hyclone (Utah, USA). Culture medium and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).
2.4. Internalized CTX quantifications

H9C2 cells were cultured on cover-slips at a concentration of 10^4 cells/cm^2. For toxicity assay and internal CTX quantifications, H9C2 cells (ATCC number: CRL-1446) were maintained in Dulbecco’s modified eagle’s medium with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 g/ml streptomycin. For image recording, H9C2 cells were cultured on cover-slips at a concentration of 10^6 cells/plate. For toxicity assay and internal CTX quantifications, H9C2 cells were cultured on 24-well plates at a concentration of 10^5 cells/plate.

2.5. Toxicity and cell death assays

CTX A3 at different concentration was applied to cells for 24 h. Then, cells were trypsinized, collected and stained by 5 µM FITC-labeled annexin V (for apoptosis) and 2 µg/ml propidium iodide (for necrosis) at 4 °C for 30 min. The ratio of necrotic and apoptotic cells were quantitated by flow cytometry. 5 × 10^3 cells were used for each quantification. Caspase-3 activity and DNA laddering assays were also used for testing the apoptotic cell death. Cells were treated with 5 µM CTX A3 for 2–8 h. Then 10 µM Ac-DEVD-MCA for each 60 µg cell extracted proteins were mixed for 30 min at 37 °C. The activity of caspase-3 was expressed as the amount of Ac-DEVD-MCA cleaved. 5 µM CTX A3 was applied to cells for 12 or 24 h for DNA laddering assays. DNA extract was examined by electrophoresis on a 1% agarose gel, stained with ethidium bromide and photographed with UV trans-illumination.

2.6. Cell image recording

MitoTracker Red or MitoTracker Green in Hanks’ balanced salt solution (HBSS) was added to cells at 37 °C for 30 min for mitochondrial staining. Cell surface GM1 was labeled by Alexa555-CT B. Then, 5 µM FITC-labeled CTX A3 or unlabeled CTX A3 in HBSS was added and living cells were visualized by Zeiss LSM 510 confocal microscopy. For control experiment to exclude non-specific binding, CTX-antibody with FITC-conjugated secondary antibody or FITC-conjugated secondary antibody itself was also studied for locating unlabeled CTX A3 after cells fixation. All images were recorded with z section of 0.7 µm. Disruption of Mitochondrial network was quantified based on the estimated mitochondria size as determined by using software of ImageJ (National Institutes of Health, USA) and Origin 7 (OriginLab). The mono color images obtained from Zeiss LSM image browser were digitized by using ImageJ software. The size and number of mitochondrial were quantified by using “analysis particles” function available on the same software package (pixels2; mean ± S.D.). The standard error on the mean (S.E.M.) was calculated by using Origin 7.

3. Results

3.1. Internalization of CTX and CTX-induced cell death in H9C2 myoblasts

Although it has long been established that amphiphilic polypeptides of CTX A3 induces muscle contraction and systolic heart arrest, due to irreversible cell membrane depolarization, the long term effect of CTX action on muscle to cause tissue necrosis and perturbed wound healing process is less understood [21–23]. In fact, as shown in Fig. 1B, flow cytometric study on H9C2 cells after treated with FITC-labeled CTX A3 indicated that most of the CTX A3 was internalized within one hour, resulting in a recovery in membrane potential and cell morphology. This allows us to study the long-term effect of the CTX A3 action on H9C2 in a dose dependent manner. Fig. 1C shows that CTX A3 treatment on H9C2 for 24 h results in mostly necrotic myoblast cell death. This conclusion is also confirmed by the lack of CTX-induced caspase-3 activity and DNA laddering (data not shown), in contrast to the CTX-enhanced caspase-3 activity on cortical neuron 1 (Wang et al., submitted). Since CTX A3 also induces necrotic cell death in fetal rat cardiomyocytes 1, our results suggest that the CTX action on both the plasma membrane and intracellular organelles may contribute to the switch between apoptotic and necrotic cell death of different cell type and cause the cell type

dependent toxicity of CTX action. Interestingly, most of the CTX A3 remains on the plasma membrane of neuron without significant internalization. In the latter case, CTX A3-induced neuron death is mainly through apoptotic process, as also reported recently that CTX A3 induces apoptosis in human leukemia K562 [24].

3.2. CTX targets mitochondrial membrane

In order to study the CTX A3 internalization process and its intracellular location, confocal microscopy was used to image the living H9C2 cell treated with FITC-labeled CTX A3 in the presence of MitoTracker Red for staining mitochondria. As shown in Fig. 2, most internalized CTX A3 appears to colocalize with MitoTracker Red at 15 min, indicating that mitochondria is a potential intracellular site for CTX A3 binding. The observed location is not due to the possible artifact introduced by the FITC probe because similar colocalization of CTX A3 with MitoTracker Red can also be observed by using CTX A3 antibody staining method (data not shown). In addition, CTX A3 location is not dependent on mitochondrial membrane potential since pre-treated carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP) to completely depolarize mitochondria membrane does not affect its location. In fact, it slightly enhances the rate of CTX A3 internalization to mitochondria (Fig. 3). It is also noted that the mitochondria of CTX A3-treated cell at 15 min become fragmented, in contrast

Fig. 2. Confocal microscopic study of the CTX A3 internalization, mitochondrial fragmentation and swelling. Scale bar, 10 μm.
mitochondrial swelling after 1 h. The localization of CTX A3 is seen to colocalize with mitochondria (Fig. 2). The partial colocalization of CTX A3 with membrane raft domain as monitored by GM1 marker. Initially, CTX A3 lies mostly in the plasma membrane. This is evidenced by the partial colocalization of CTX A3 with plasma membrane GM1 as probed by using Cholera toxin B (CT B) subunit (Fig. 4). At 5 min, increasing amount of internalized CTX A3 is seen to colocalize with mitochondria (Fig. 2 and Fig. 3A). Despite the presence of abundant mitochondria in H9C2 myoblasts, the internalized CTX A3 also localizes at other intracellular organelles initially. It is likely that certain endocytic pathway is involved in the trafficking and our preliminary result indicates that a specific sulfoglycolipid-dependent pathway maybe responsible for the process.  

Finally, CTX A3 action on H9C2 cell induces significant mitochondrial swelling after 1 h. The localization of CTX A3 with swollen circular mitochondria membrane vesicle as emphasized by the enlarged image shown in the bottom of Fig. 2 suggests that CTX A3 is largely localized at mitochondrial membrane. We conclude that CTX A3 targets mitochondrial membrane as a result of membrane dependent internalization process after its initial action on plasma membrane to depolarize the cell.

3.3. CTX disrupts mitochondria network

The CTX A3-induced mitochondrial fragmentation in H9C2 cells deserves more attention in the light of recent interest in understanding the mechanism of mitochondrial dynamics during fusion/fission processes [8]. In order to see whether the phenomena is due to the indirect CTX A3-induced calcium effect or a direct action of CTX A3 on mitochondrial membranes, a calcium chelator, (acetoxymethyl)-1,2-bis(o-aminophenoxy)ethane N,N,N',N'-tetraacetic acid (BAPTA-AM), was pre-incubated with H9C2 cell (Fig. 5A) to remove intracellular calcium (Fig. 5B) before CTX A3 treatment [5,25]. Quantitative comparison between H9C2 cells treated in the presence and absence of CTX A3 and/or BAPTA-AM (Fig. 5C) reveals that CTX A3 can indeed promote mitochondria fragmentation in the absence of CTX A3-induced increase of intracellular calcium (blue traces in Fig. 5A and B). However, calcium appears to act synergistically to further promote the mitochondria fragmentation.

4. Discussion

Despite the well-documented long-term effect of cobra venom in perturbing wound healing process and inducing severe tissue necrosis of the victim survived from cobra snakebite, most previous studies have focused on the short-term death-causing toxicity of cobra venom. Therefore, identification of targets in the plasma membrane has become a focal point for the study of three-major protein toxins from cobra venoms, i.e., α-neurotoxin, CTXs and phospholipase A2 [19,26–28]. The establishment of cobra α-neurotoxin as a specific ligand to target acetylcholine receptor provides clear evidence to explain the neurotoxicity of cobra venom action. Recent demonstration of CTX as a pore forming polypeptide also justifies its role in causing irreversible membrane depolarization, cytosolic calcium homeostasis, muscle contraction and general cytotoxicity [3,5,10], although it is likely that many extracellular proteins and carbohydrate components may be also involved to impart the specificity observed in CTX actions on cardiomyocytes [21,29].

In this study, we demonstrate that β-sheet CTXs are internalized to mitochondria within minutes and the toxin is capable of disrupting the mitochondria network in the presence and absence of CTX-evoked cytosolic calcium. Unlike the effect of polycations and polyamines capable of causing the release of soluble mitochondrial intermembrane proteins from isolated mitochondria [30,31], CTX A3-treated H9C2 myoblasts display mainly necrotic characteristics as evidenced by the loss of plasma membrane integrity and the lack of caspase-3 activity and DNA laddering. Interestingly, neisserial porins PorB with β-barrel structure have also been reported to co-localize with mitochondria of target cells,
where they also protect cell from apoptosis via protein–protein interaction between a mitochondria porin, the voltage-dependent anionic channel (VDAC) and PorB [32,33]. This observation should open an avenue for future effort in identifying potential intracellular targets for CTX action and shed new light to understand the mechanism of CTX-induced myoblast necrosis.

Many bacterial, viral and plant toxins enter the cell by hijacking intrinsic membrane trafficking machinery and control cell death by perturbing related cell signaling processes [13,17]. In fact, studies of toxin trafficking have revealed the existence of new pathway and protein toxins become a useful tool to understand the transport and sorting devices in endocytic membrane traffic. In addition to targeting ER and Golgi, viral and bacterial proteins also regulate apoptosis at the mitochondrial level by multiple strategies [16]. Just as viral pro-apoptotic proteins translocating to mitochondria [18], cobra CTXs also target mitochondrial membrane and induce mitochondria swelling and fragmentation. However, instead of inducing apoptosis by involving pore forming amphiphilic α-helices as do mitochondria targeting viral proteins, the amphiphilic β-sheet CTX induce necrotic myoblast necrosis.

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Disruption of mitochondria network by CTXs is of current interest considering that many proteins involved in mitochondrial fusion and fission have recently been identified. Dynamic structural change of the mitochondrial network involves continuous remodeling by fusion and fission events and regulation of mitochondria calcium [25]. Interestingly, not only do the fusion events rely on a transmembrane GTPase of Fzo [8,38,39], but also the mitochondria fissions depend on dynamin-related protein-1 (Drp-1) [40]. Although most of the cellular activity with overexpressed Drp-1 and its recruiting protein, hFis1, remains normal [25], the disruption of mitochondria network as a result of mitochondria fragmentation does protect against calcium-mediated apoptosis by preventing calcium propagation within interconnected mitochondria [40]. It is likely that the toxicity of CTX also involves the intertwined CTX action on both plasma and mitochondrial membranes through not only the CTX-evoked cytosolic calcium, but also the disrupted mitochondria network as we demonstrated in this study.
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