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Down-regulation of the JAK2/PI3K-mediated signaling activation is involved in Taiwan cobra cardiotoxin III-induced apoptosis of human breast MDA-MB-231 cancer cells

Kuei-Li Lin [a], Jung-Chen Su [a], Ching-Ming Chien [a], Pei-Wen Chuang [a,b], Long-Sen Chang [c,d], Shinne-Ren Lin [a,d,*]

[a] Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
[b] Kaohsiung Municipal Min-Sheng Hospital, Kaohsiung, Taiwan, ROC
[c] Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan, ROC
[d] National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, Kaohsiung, Taiwan

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ABSTRACT

Cardiotoxin III (CTX III), a basic polypeptide with 60 amino acid residues isolated from *Naja naja atra* venom, has been reported to have anticancer activity. Exposure of MDA-MB-231 cells with 0.03, 0.09, and 0.15 μM of CTX III for 18 h, CTX III-induced cell apoptosis, as evidenced by accumulation of sub-G1 population, externalization of phosphatidylserine, loss of mitochondrial membrane potential ($\Delta\Psi m$) with subsequent release of cytochrome c, and activation of both capases-9 and caspase-3. This correlated with up-regulation in Bax and Bad, and down-regulation of various anti-apoptotic proteins, including Bcl-2, Bcl-X_L, and survivin in CTX III-treated cells. Mechanistic studies showed that CTX III suppressed the phosphorylation of JAK2, STAT3, Akt, and activation of PI3K. Moreover, the PI3K inhibitor wortmannin blocked activation of STAT3 and Akt without affecting the JAK2 activation, whereas JAK2 inhibitor AG490 suppressed the levels of phospho-STAT3, phospho-Akt, and PI3K, suggesting that PI3K activation occurs after JAK2 phosphorylation, and both PI3K and JAK2 kinases cooperate to mediate STAT3 and Akt phosphorylation. Both AG490 and wortmannin also led to up-regulation in Bax and Bad, and down-regulation of Bcl-2, Bcl-X_L, and survivin in MDA-MB-231 cells. Taken together, these results indicate that CTX III induces apoptosis in MDA-MB-231 cells via concomitant inactivation of the JAK2, STAT3, PI3K, and Akt signaling pathways.

1. Introduction

Breast cancer is a leading cause of morbidity and mortality in woman in developed and developing countries (Jemal et al., 2008). This pathology is currently controlled using surgery and radiotherapy, and is frequently supported by adjuvant chemo- or hormone-therapies. However, breast cancer is highly resistant to radiation and conventional chemotherapeutic agents. This resistance is associated with a poor prognosis for this metastatic disease, especially in hormone-independent cancer cases (Bange et al., 2001; Cuzick et al., 2004; Roy et al., 2005; Houssami et al., 2006). Novel therapies are therefore needed to deal with the increasing incidence of human breast cancer.

Cytoplasmic Janus-activated kinase (JAK) proteins are crucial components of diverse signaling transduction pathways that govern cellular survival, proliferation, differentiation, and apoptosis (Rane and Reddy, 2000; Thompson, 2005). The constitutive activation of JAKs is

* Corresponding author. Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC. Fax: +886 7 3123443.
E-mail address: shreli@cc.kmu.edu.tw (S.-R. Lin).

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frequently detected in breast carcinoma cells (Cance et al., 1993; Liovera et al., 2000). Once activated, JAK phosphorylates and activates cellular cytokine receptors and signaling molecules, such as those regulated by the phosphoinositide-3-kinase (PI3K), and signal transducer and activator of transcription (STAT; Rane and Reddy, 2000). The PI3K is a dimeric enzyme composed of an inhibitory/regulatory (p85) and a catalytic (p110) subunit. The p85 subunit is anchored to erbB receptor docking sites, and the p110 subunit is responsible for the phosphorylation and activation of the protein serine/threonine kinase Akt (Yarden and Sliwkowski, 2001). Akt plays a major role in the regulation of cell survival, apoptosis, and oncogenesis (Dillon et al., 2007). STAT proteins have been shown to have a major role in survival, proliferation, angiogenesis, and immune evasion of tumors (Battle and Frank, 2002; Yu and Jove, 2004). STAT activation is initiated upon phosphorylation of a critical tyrosine residue by growth factor receptors or Janus kinases, and involves dimerization between two phosphorylated STAT monomers followed by translocation of the dimers into the nucleus (Yang et al., 2008; Singh et al., 2009). One of these members, namely STAT3, is constitutively expressed in breast cancers. Thus, persistent activation of JAK2, STAT3, PI3K, and Akt results in deregulation of downstream gene expression that contributes to malignant progression in cancer cells. Additionally, members of the Bcl-2 family of proteins regulate the initiation of mitochondrial apoptotic pathway, and can be subdivided into anti-apoptotic (for example, Bcl-2, Bcl-XL, and Mcl-1) and pro-apoptotic members (for example, Bax and Bad). Decrease in the expression of pro-apoptotic Bcl-2 proteins or overexpression of anti-apoptotic Bcl-2 proteins is associated with enhanced oncogenic potential and poor response rate to chemotherapy (Antonsson and Martinou, 2000; Cory and Adams, 2002).

Cardiotoxins (CTXs), a group of highly basic polypeptides of 60 amino acid residues, are present abundantly in *Naja naja atra* (Taiwan cobra) venom and show very diverse pharmacological functions including hemolysis, cytotoxicity, and depolarization of the muscles (Dufton and Hider, 1991). Moreover, these toxins showed cytotoxicity toward cancer cells, probably mediated by inhibiting protein kinase C activity or through a membrane fusion effect (Chien et al., 1991; Guo et al., 1993; Chiou et al., 1993). In our previous studies, CTX III has been shown to induce a dose- and time-dependent apoptosis in K562 cells via the intrinsic pathway involving the mitochondrial membrane permeability, the release of cytochrome c, and subsequent caspase activation (Yang et al., 2005a,b). Nevertheless, the cellular signaling pathway involved in the underlying process mechanism remains elusive. In the present investigation, CTX III was shown to be involved as an apoptotic-inducing factor against the solid tumor cell MDA-MB-231 mediated through JAK/PI3K and downstream signaling pathways.

2. Materials and methods

2.1. Chemicals

DMEM/F12 medium, fetal bovine serum (FBS), trypan blue, penicillin G and streptomycin were obtained from Gibco BRL (Gaithersburg, MD, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ribonuclease (RNase), rhodamine 123, AG490, and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). The following antibodies were used for Western blotting: anti-Akt, STAT3, survivin, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Bcl-2, Bcl-X_L, Bax, and Bid (BD Biosciences); anti-phospho-Akt (Upstate Temecula, CA) and anti- phospho-STAT3 antibodies (Cell Signaling Technology, Beverly, MA); anti-cytochrome c (PharMingen, San Diego, CA). The colorigenic synthetic peptide substrate, Ac-DEVD-pNA and Ac-LEHD-pNA, as well as the JAK2-specific inhibitor AG490 and PI3K-specific inhibitor wortmannin were purchased from Calbiochem (San Diego, CA). Annexin V and PI double staining kits were a product of PharMingen (San Diego, CA). Anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL). Hybond ECL transfer membrane and ECL Western blotting detection kit were obtained from Amersham Life Science (Buckinghamshire, UK).

2.2. Cell culture

The human breast cancer cell line, MDA-MB-231, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM/F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Isolation of CTX III

CTX III was purified from the venom of *Naja naja atra* (Taiwan cobra) by chromatography on Sephadex G-50 and SP-Sephadex C-25 as previously described (Lin et al., 2002). Solutions of CTX III were prepared in phosphate-buffered saline (PBS) and sterilized by filtration.

2.4. Cell viability assay

The viability of cells was determined by the MTT assay and the trypan blue dye exclusion assay was performed to confirm and verify cell viability. Cells were seeded at a density of 1×10^5 cells/3 mL cell culture medium into a 12-well plate. After 24 h of incubation, the cells were treated with vehicle (PBS) or 0, 0.03, 0.09, and 0.15 μM concentrations of CTX III in medium for 8, 12 and 18 h, respectively. MTT solution was added to each well (1.2 mg/mL) and incubated for 4 h. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 492 nm in an ELISA plate reader. For the trypan blue dye exclusion assay, cells were seeded at density of 1×10^5 cells/well onto a 12-well plate for 24 h, then CTX III was added to the medium at various indicated times and concentrations. After incubation, cells exposed to 0.2% trypan blue were counted in a hemocytometer.