Cobrotoxin Inhibits Prostate Carcinoma PC-3 Cell Growth Through Induction of Apoptotic Cell Death Via Inactivation of NF-κB

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Abstract

We previously found that cobrotoxin inhibited NF-κB activity by reacting with signal molecules of NF-κB which is critical contributor in cancer cell growth by induction of apoptotic cell death. We here investigated whether cobrotoxin inhibits cell growth of human prostate cancer cells through induction of apoptotic cell death, which is related with the suppression of the NF-κB activity. Cobrotoxin (0.78 nM) inhibited prostate cancer cell growth through increased apoptosis in a dose dependent manner. Cobrotoxin inhibited DNA binding activity of NF-κB, an anti-apoptotic transcriptional factor. Consistent with the induction of apoptosis and inhibition of NF-κB, cobrotoxin increased the expression of pro-apoptotic proteins caspase 3.

Cobrotoxin, a venom of Vipera lebetina turanica, is a group of basic peptides composed of 233 amino acids with six disulfide bonds formed by twelve cysteins. NF-κB is activated by subsequent release of inhibitory IkB and translocation of p65. Since sulphydryl group is present in kinase domain of p65 subunit of NF-κB, cobrotoxin could modify NF-κB activity by protein–protein interaction. And Cobrotoxin down regulated Akt signals. Salicylic acid as a reducing agent of Sulphhydryl group and LY294002 as a Akt inhibitor abrogated cobrotoxin-induced cell growth and DNA binding activity of NF-κB. These findings suggest that nano to pico molar range of cobrotoxin could inhibit prostate cancer cell growth, and the effect may be related with the induction of apoptotic cell death through Akt dependent inhibition of NF-κB signal.

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I. Introduction

Prostate cancer is the most common cancer as well as the second leading cause of cancer-related deaths in men of Western countries. One out of nine men over 65 years of age is frequently diagnosed with prostate cancer in the United States. Dietary pattern has been identified as one of the major factors for the difference in prostate cancer incidence between Western and Asian countries. At present, there is no effective therapy available for the treatment of androgen-independent stage of prostate cancer, which usually arises after hormonal deprivation/ablation therapy. Cytotoxic chemotherapies or radiotherapy also do not show any significant improvement in patient condition due to the high recurrence of apoptosis resistance hormone refractory prostate cancer, which is responsible for 28,000 deaths per year. The clinical impact of advanced prostate cancer has led to the exploration of novel treatment modalities as well as anticancer agents.

Current therapy for prostate cancer is limited by the propensity of the disease to progress from androgen-dependent to an androgen-independent state. In the normal prostate, organ homeostasis is maintained by a dynamic balance between the rate of cell proliferation and the rate of apoptosis. Distruption of the molecular mechanisms that regulate apoptosis and cell proliferation among the stroma and epithelial cell populations may underlie the abnormal growth of the gland that characterizes neoplastic development of the prostate. Thus apoptosis induction provides a relevant endpoint for testing new drugs for therapeutic efficacy against prostate growth disorders, benign prostatic hyperplasia (BPH), and prostate cancer. Recently studies have reported that NF-κB is constitutively activated in human prostate cancer tissue, androgen-insensitive human prostate carcinoma cells, and prostate cancer xenografts. Syrovena et al reported that inhibition of NF-κB activity by acetylsalicylic acids promotes apoptosis in androgen-independent PC3 cells in vitro and in vivo. Contrast, it was reported that activation of NF-κB by lysophosphatidic acid promotes survival of PC3 cells. It has been also well established that activation of Akt pathway promotes prostate cancer cell survival and growth through phosphorylation and inactivation of downstream pro-apoptotic targets. Akt may be activated by various growth factors through phosphatidylinositol-3-kinase (PI3K), and it may be inactivated by the tumor suppressor genes, which have been found to be mutated in primary or metastatic prostate cancers. We also previously found that neuroblastoma cell death was accompanied with inactivation of NF-κB. Therefore, agents capable of suppressing NF-κB and/or Akt pathway may be potentially useful in the prevention and management of prostate cancer.

Elucidating important molecular interfaces of specific toxin-receptor/ion channel complexes have been largely studied in drug discovery initiatives. Cobrotoxin, a snake venom toxin of Vipera lebetina turanica, is a group of basic peptides, and important factor V activator composed of 235 amino acids with six disulfide bonds formed by twelve cysteins. It was reported that long chain snake toxin possessing five disulfide bonds has higher affinity to the nicotinic acetylcholine receptor, and reduced fifth disulfide bond lowers binding affinity to AchR. Kachalsky et al reported that cys192-193 residue of subunit of AchR was binding target of snake toxin, and the
disulfide bond of snake toxin may be core or additional specific binding amino acid residues\(^{22-24}\). Park et al recently also found that cobrotoxin inhibited NF-κB activation and target gene expression through the interaction with the signal molecule (p50 and IKKs) in the NF-κB pathway\(^{25}\).

In this study, We conducted an in vitro analysis to evaluate the prostate cell response to cobrotoxin in order to determine the ability of this venom toxin therapeutic agent to suppress prostate cell growth by affecting cell proliferation and apoptosis, and determine possible mechanisms related with inactivation of NF-κB and other cell survival signals.

II. Material and Method

1. Cell Culture

The PC-3 prostate cancer cell was obtained from ATCC (American Type Culture Collection, Rockville, MD). Prostate cells were cultured in RPMI-1640 medium (Life Technologies Inc., Gaithersberg, MD) supplemented with 10% fetal calf serum (FCS; Collaborative Biomedical Products, Bedford, MA) and antibiotics, penicillin/streptomycin (100 unit/ml, Bioproducts, Walkersville, MD). Cell cultures were then maintained at 37°C in a humidified atmosphere of 5% CO₂.

2. Cell viability assay

To determine the appropriate dose that is not cytotoxic to the cells, the cytotoxic effect was evaluated in the cells cultured for 24, 48 and 72 hr using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide MTT assay [28]. Briefly, the cells plated on 96 well plates at a concentration of 1.5 × 10⁴ cells/cm². The cells were incubated at 37°C in 5% CO₂, and were treated with TNF-α (150U/ml) and HCA (0, 10, 20, 40 and 80 M). After the incubation for 12, 24, 36 or 72 hr, the cells were washed twice with 1×PBS, followed by the addition of 1 ml of PBS, MTT was dissolved in without phenol red at a concentration of 10 g/ml, 10 l of this solution was then added to cell cultured for designed time. After 4 hr, cultures were removed from the incubator and the formazan crystals dissolved by adding 100 Isolubilization solution (0.04 N HCl in isopropanol). Metabolic activity was quantified by measuring light absorbance at 570 nm.

3. Apoptosis Evaluation

Apoptosis assays were performed using the 4,6-diamidino-2-phenylindole (DAPI) staining. PC-3 cells were cultured in the absence or presence of increasing concentrations of cobrotoxin, and apoptosis induction were evaluated after 24 hr. Apoptotic cells were determined by the morphological changes after DAPI staining under fluorescence microscopic observation (DAS microscope, 100 or 200×; Leica Microsystems, Inc., Deerfield, IL). For each determination, three separate 100-cell counts were scored. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells counted.

Apoptosis was also evaluated by TUNEL staining assay. In short, cells were cultured on 8-chamber slides. After treatment with cobrotoxin (1~8 nM) for 24 hr, the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at room temperature. TUNEL assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Total number of cells in a given area was determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted 100.
4. Immunofluorescence Staining

PC3 cells were plated in chambered tissue culture slides at a density of 5 × 10^4 cells/well in RPMI. The cells were then cultured with cobrotoxin (1–8 nM) or vehicle. Twenty–four hours later, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 min, membrane–permeabilized by exposure for 2 min to 0.1% Triton X-100 in phosphate-buffered saline, and placed in blocking serum (5% bovine serum albumin in phosphate-buffered saline) at room temperature for 2 h. The cells were then exposed to primary rabbit polyclonal antibody for active caspase 3 (1:50 dilution) overnight at 4°C. After washes with ice-cold PBS followed by treatment with an anti-rabbit secondary antibody Alexa Fluor 568 (Molecular Probes Inc., Eugene, OR, USA), 1:100 dilution, for 2 hr at room temperature, immunofluorescence images were acquired using a confocal laser scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany) equipped with a 63×oil immersion objective.

5. Confocal immunocytochemistry

To determine whether cobrotoxin could be uptaken into the cells, cells (1 × 10^5 cells/cm^2) were cultured on the chamber slide (Lab-Tak II chamber slider system, Nalge Nunc Int., Naperville, IL, USA) and then treated by cobrotoxin labeled with superior Alexa Fluor 488 dye (Molecular Probe, Eugene, Oregon, USA). Cells were incubated for 24 hr at 37°C, and the cells were then fixed in 4% paraformaldehyde, membrane permeabilized by exposure for 5 min to 0.2% Triton X-100 in phosphate-buffered saline, and were placed in blocking serum (5% horse or goat serum in phosphate-buffered saline). Immunofluorescence images were acquired using a confocal laser scanning microscope (dual wavelength scan, MRC1024, Bio-Rod, Hercules, CA, USA) with a 60 X oil immersion objective.

6. Western blot analysis

Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igepal 630 (Sigma–Aldrich, St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000 g for 1 hr. Equal amount of proteins (80 μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked for 2 hr at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.03% tween-20. The membrane was incubated for 5 hr at room temperature with specific antibodies caspase-3, goat polyclonal pIκB antibody (1:1000) (Santa Cruz, CA, USA Santa Cruz Biotechnology Inc.). The blot was then incubated with the corresponding conjugated anti–rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

7. Preparation of nuclear extracts and electromobility shift assays

It was performed according to the manufacturer’s recommendations (Promega, Madison, WI). Briefly, 1 × 10^6 cells/ml was washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 g for 1 min, and the resulting supernatant was removed. Solution A (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 μg/ml phenylmethyl– sulfonyl fluoride, 1 μg/ml
peptatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotonin, and 0.5% Nonidet P-40 was added to the pellet in a 2:1 ratio (v/v) and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2:1 ratio (v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000 g for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-32P] ATP for 10 min at 37°C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 μl (50,000–200,000 cpm) of 32P-labeled oligonucleotide and another 20 min of incubation at room temperature. For supershift assays, nuclear extracts from cells treated with cobrotoxin (1 μg/ml) were incubated with specific antibodies against the p65, p50 and Rel-A NF-κB isoforms for 1 hr before EMSA. For competition assays, nuclear extracts from cells treated with cobrotoxin (4 nM) were incubated with unlabelled NF-κB oligonucleotide (50X, 100X and 200X) or labeled SP-1(100X) and AP-1(100X) for 30 min before EMSA. Subsequently 1 μl of gel loading buffer was added to each reaction and loaded onto a 6% nondenaturing gel and electrophoresed until the dye was three-fourths of the way down the gel. The gel was dried at 80°C for 1 hr and exposed to film overnight at 70°C. The relative density of the DNA-protein binding bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

8. Surface plasmon resonance analysis

Activated CM-dextran matrix carried out by mixing ethyl-N-(dimethylaminoethyl) carbodiimide and N-hydroxysuccinimide was surfaced on the sensor chip. Melittin was then layered onto the CM-dextran sensor chip, and then the chip was blocked by 1M ethanolamine, pH 8.5. Serial dilutions of BV, melittin or immunoprecipitated p50 extracted from nuclear fraction of cells treated by SNP were prepared using HEPES buffered saline buffer, and then flowed sequentially with increasing concentration. The regeneration of protein interaction was performed by changing of the pH of solution and then finally determined by pH12. The BIACORE 2000™ system continuously monitors the change in mass at the sensor surface, and the protein interaction kinetics were analyzed by BIAevaluation 3.0 software (BIACORE AB, 5-75450, Upplands, Sweden).

9. Statistical analysis

Data were analyzed using one-way analysis of variance followed by Tuckey test as a post hoc test. Differences were considered significant at p<0.05.

III. Results

1. Induction of apoptosis

To delineate whether the inhibition of cell growth by the cobrotoxin was due to increase of the induction of apoptosis, we evaluated change of the chromatin morphology of human prostate cancer cells using DAPI staining. Consistent with the loss of viability, apoptosis determined after 24 hr treatment was increased in a dose dependent manner. The percentages of the normal significantly increased by cobrotoxin 1, 2, 4, 8nM was 25.9±0.7, 54.8±2.6, 80.0±0.6 and 96.5% respectively(Fig. 1). We also evaluated PC3 cell apoptosis by TUNEL assay. As shown in Fig. 1 TUNEL- positive cells (stained green) were dose-dependently increased in cobrotoxin treated PC3 cells, and the nuclei (stained blue) were found to be condensed. But this result was reversed when the salicylic acid was employed.
Fig. 1. Apoptotic Cell Death determined with DAPI Staining (A), and TUNEL staining (B) and the Cell Death (C) of Prostate PC3 Cell after Treatment with Cobrotoxin

Fig. 2. Effect of salicylic acid on the cobrotoxin-induced inhibition of cell growth in PC3 cells. Cell viability was determined by MTT assay as described under Materials and Methods. Values are mean ± S.D. of two experiments, with triplicate of each experiment.

# significantly different from normal group. * significantly different from cobrotoxin-treated group.

dose dependently. The percentages of the normal significantly decreased by cobrotoxin 4nM was 45 ± 4% and that of cobrotoxin 4nM significantly increased by salicylic acid 10, 20 and 30mM was 62 ± 6, 77 ± 14 and 93 ± 9% respectively (Fig. 2).

2. Expression of cell cycle and apoptosis regulatory proteins

Caspases are a family of cysteine proteases that are expressed as inactive pro-enzymes in normal cells, and upon activation, they are capable of cleaving structural and functional proteins involved in key cellular processes. The increase of apoptotic action was confirmed by the ability of
cobrotoxin to induce caspase-3 activation. Figure 3 reveals a western blot analysis of caspase-3 expression in PC cells before and after treatment with a different dose of cobrotoxin. Expression of active form of caspase 3 was increased in a dose-dependent manner in the cells treated by cobrotoxin for 24 hr. The density of the control significantly increased by cobrotoxin 1, 2, 4 and 8 nM was 109 ± 11, 99 ± 13, 143 ± 16 and 283 ± 9% respectively (Fig. 3).

3. Inhibition of NF-κB

It has been demonstrated that cobrotoxin negatively regulates nuclear transcription factor NF-κB by mean of protein-protein interaction.25 In addition, NF-κB is also known to inhibitory transcription factor of apoptosis. To confirm again whether p50 which has cysteine residue in their active sites, could react with cobrotoxin, surface plasmon resonance analyzer was performed. The analysis demonstrated that cobrotoxin binds to p50 subunit of NF-κB immobilized onto a surface of sensor chip, and increasing dose of cobrotoxin showed increased binding activity with p50 in a dose dependent manner and the maximum binding affinity (Kd) of cobrotoxin to p50 was 2.1×10^{-7} M in physiological buffer (pH:7.5) (Fig. 4). And then to investigate the hypothesis whether cobrotoxin can inactivate NF-κB, and thereby prevent anti-apoptotic ability of NF-κB causing the cells go apoptosis, we assessed NF-κB activity in the cells treated for different concentration with for 24 hr cobrotoxin. NF-κB was highly activated in these cells, however the activation of NF-κB was gradually decreased by the culture in the presence of cobrotoxin in the cells. The density of the normal significantly decreased by cobrotoxin 1, 2, 4 and 8 nM were 88 ± 23, 41 ± 21, 48 ± 14 and 39 ± 10 (Fig. 5). The inhibitory effect of cobrotoxin on the translocation into the nucleus of PC-3 cells and the expressions of proteins of the NF-κB subunit were also determined. The translocation of cobrotoxin into the nucleus of PC-3 cells and the expression of p50 were dose dependently inhibited by cobrotoxin in PC-3 cells. The density of the control significantly decreased by cobrotoxin 1, 2, 4 and 8 nM were 373 ± 98, 1763 ± 51, 453 ± 114 and 110 ± 19 respectively (Fig. 6). To further demonstrate the involvement of NF-κB in PC 3 cell death by cobrotoxin, We
employed Salicylic acid similar with GSH, DTT which were shown to abrogate interaction between cobrotoxin and NF-κB, thereby restore NF-κB activity from cobrotoxin-induced inactivation. Consistent with restore NF-κB activity, LY294002, specific inhibitors of Akt signal pathway, abrogated cobrotoxin-induced PC-3 cell death dose dependently. The percentages of the normal significantly decreased by cobrotoxin 4nM was 45 ± 8% and that of cobrotoxin 4nM significantly increased by LY294002 25 and 50μM was 67 ± 17 and 83 ± 16% respectively (Fig. 7, Fig. 8).

Fig. 4. Binding Affinity of Cobrotoxin and p50 in vitro Representative Grape of two Experiments

Fig. 5. Effect of Cobrotoxin on the DNA binding activity of NF-κB in PC3 cells. Nuclear extracts were prepared from PC3 cells, which were incubated with cobrotoxin. Gel mobility shift assay was done as described under Materials and Methods. Similar pattern of DNA binding activity was seen from three different sets of experiments.
Fig. 6. Inhibitory Effect of Cobroxin on the Translocation (A) and Expression (B) of p50 into Nucleus of PC3 Cells

Fig. 7. Effect of salicylic acid on the cobrotoxin-induced inhibition of p50 translocation in PC3 cells. The staining method for translocation of p50 was described under Materials and Methods

Fig. 8. Effect of LY294002 on the cobrotoxin-induced inhibition of cell growth in PC3 cells. Cell viability was determined by MTT assay as described under Materials and Methods. Values are mean ± S.D. of two experiments, with triplicate of each experiment.

# significant difference from normal group, * significant difference from cobrotoxin treated group

4. Uptake of cobrotoxin into nucleus

To investigate whether cobrotoxin can be uptaken into nucleus, and thereby inactivation of NF-κB, we determined the location of cobrotoxin after treatment of cells with fluorescent dye labeled cobrotoxin. The uptake of the labeled cobrotoxin into the cells was shown under a
confocal laser scanning microscope. As seen in Figure 9, cobrotoxin was uptaken into the membrane and nucleus of cells, translocation into the nucleus was evidenced by the merging of PI staining of nucleus and labeled cobrotoxins.

IV. Discussion

The central and novel finding in the present study is the identification of in vitro anticancer efficacy of cobrotoxin against advanced human prostate carcinoma PC-3 cells. Most of the present available cytotoxic anticancer drugs mediate their effect via induction of apoptosis in cancer cells, and apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including prostate cancer. In case of advanced prostate cancer, cancer cells become resistant to apoptosis and do not respond to cytotoxic chemotherapeutic agents. Therefore, the agents that induce apoptotic death of prostate cancer cells could be useful in controlling this malignancy. Consistent with this approach, my data showing an induction of apoptotic death in prostate cancer control suggest that extremely low concentration of nanomolar natural toxic agent could be useful as a certain anti-cancer agent.

It has been well established that Akt promotes prostate cancer cell survival and growth through phosphorylation and inactivation of downstream pro-apoptotic targets. Nuclear factor-κB (NF-κB) is another important element in regulating growth or apoptosis of tumors, including prostate cancers. Several studies have shown that Akt enhances survival signals via activation of NF-κB-dependent expression of anti-apoptotic genes. Thus, both Akt/NF-κB-mediated signals and their cross talk strongly affect prostate tumorigenesis through regulating apoptosis. Li et al showed that Akt is located upstream of NF-κB, regulating its activity in PC-3 cells. In the present study, we found that cobrotoxin inhibited phosphatidylinositol 3-kinase/Akt pathways and a pronounced down-regulation of nuclear localized protein levels of nuclear factor-B family members. Down regulated Akt and NF-κB signals by cobrotoxin is consistent with cell growth inhibition. Moreover, specific inhibitors such as LY294002 of Akt abrogated cobrotoxin-induced cell growth inhibition.
and NF-κB inactivation. These data suggest that Akt and NF-κB signal may be significant contributor in cobrotoxin-induced PC-3 cell death. At the molecular level cobrotoxin inhibits constitutively activated NF-κB signaling by impairing phosphorylation of p65 and p50 with inhibition of IkBα phosphorylation. We previously found that cobrotoxin binds with NF-κB, IKKα and IKKβ resulting in down regulation of NF-κB activity in Raw 264.7 cells and astrocytes. Moreover, DTT and GSH treatment reduced cobrotoxin-induced apoptotic cell death. Therefore, cobrotoxin act in a very similar mechanism in PC-3 cells. In fact, by an confocal microscopic analysis, in the present study found that cobrotoxin translocated into nucleus in which p50 could bind to IkB binding elements of target genes. Consequently, the reduced nuclear translocations of NF-κB proteins may be associated with down-regulation of the constitutively overexpressed and NF-κB-dependent anti-apoptotic proteins Bcl-2, and/or upregulated apoptotic genes such as Bax, caspase and p53. In the present study, consistent with the increase of the induction of apoptosis, the expression of apoptotic proteins; active caspase 3 was dose dependently increased. These data suggest that cobrotoxin induced apoptosis of PC 3 cells, and the alternation of the expression of apoptosis regulatory protein (active caspase 3) resulting in a shift the cells favoring apoptosis.

In conclusion, present findings showing the in vitro anticancer efficacy of cobrotoxin, with mechanistic rationale (apoptosis induction), against advanced human prostate cancer cells, warrant its further in vivo efficacy studies in preclinical human prostate cancer models. The positive outcomes of such an in vivo study could form a basis for the development of cobrotoxin acupuncture as a novel method for human prostate cancer prevention.

V. References


