대한침구학회지 제22권 제2호(2005년 4월)
The Journal of Korean Acupuncture & Moxibustion Society

원저

The Effect of Cobrotoxin on NF-κB binding Activity in Raw264.7 cells

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Abstract

Cobrotoxin, a venom of Vipera lebetina turanica, is a group of basic peptidescomposed of 233 amino acids with six disulfide bonds formed by twelve cysteins. NF-kB is activated by subsequent release of inhibitory IkB and translocation of p50. Since sulfhydryl group is present in kinase domain of p50 subunit of NF-kB, cobrotoxin could modify NF-kB activity by protein-protein interaction. We therefore examined effect of cobrotoxin on NF-kB activities in lipopolysaccharide (LPS) and sodium nitroprusside (SNP)-stimulated Raw 264.7 mouse macrophages. Cobrotoxin suppressed the LPS and SNP-induced release of IkB and p50 translocation resulted in inhibition of DNA binding activity of NF-kB. Inhibition of NF-kB resulted in reduction of the LPS and SNP-induced production of inflammatory mediators NO and PGE2 generation. The inhibitory effect of cobrotoxin on the NF-kB activity were blocked by addition of reducing agents dithiothreitol and glutathione. These results demonstrate that cobrotoxin inhibits activation of NF-kB, and suggest that pico to nanomolar range of cobrotoxin could inhibit the expression of genes in the NF-kB signal pathway.

Key Words : Cobrotoxin, NF-κB, Raw264.7 cell

· **Acceptance** : 2005. 3. 2. · **Adjustment** : 2005. 3. 12. · **Adoption** : 2005. 3. 17.

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I. Introduction

Nuclear factor kappa B (NF-kB) is one of the most important transcription factor that regulates various immediately cellular genes involved in the processes of immune, acute phase, and inflammatory responses as well as genes involved in cell survival[1-2]. NF-kB is kept in the cytoplasm of nonstimulated cells through interaction with inhibitory proteins, including IkBs, but response to pro-inflammatory stimuli, IkBs are rapidly phosphrylated and degraded resulting in the release of free NF-kB dimmers (p50 and Rel A), which translocate to the nucleus to induce transcription of target genes. The protein kinase responsible for IkB phosphorylation and degradation in response to proinflammatory stimuli, is the IkB kinase (IKK)[3-6]. P50 possesses a critical cycteine residue (Cys-62) at the N-terminal region of its DNA-binding domain7). In several studies, potent inhibitors of NF-kB activation have showed to interact with a specific cysteine residue of p50 subunit of NF-kB resulting in target modified inactivation of NF-kB[5-7]. Moreover, this interaction was reversed by thiol-modifying agents[5-7], suggesting that cysteine in the proteins involving NF-kB signal can be targets of these compounds.

Currently, elucidating important molecular interfaces of specific toxin-receptor/ion channel complexes have been largely studied in drug discovery initiatives[8-12]. Cobrotoxin, a snake venom toxin of Vipera lebetina turanica, is a group of basic peptides, and important factor V activator composed of 235amino acids with six disulfide bonds formed by twelve cysteins[12-13]. It was reported that long chain snake toxin possessing five disulfide bonds has higher affinity to the nicotinic acetylcholine receptor (AchR) (Kd=1~12x10-9 M)than short chain snake toxin with four disulfide bonds (Kd=3~22x10-6 M), and reduced fifth disulfide bond lowers binding affinity to AchR (Kd=12x10-6 M[14]. Michalet et al reported that cys192-193 residue of ag 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[15-18]. These data suggest that cobrotoxin could bind to other molecular target possessing sulfhydryl group causing alteration of biological activity of the molecules.

In the present study, we investigated that whether cobrotoxin could bind with sulfhydryl group of p50 resulted in the inhibition of NF-kB activity, and its target gene expression in the Raw264.7 cells

II. Material and Method

Chemicals-Rabbit polyclonal antibodies to p50 (1:500), p65 (1:500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

T4 polynucleotide kinase obtained was from Promega (Madison, WI). Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Alfred Nobel Drive Hercules, CA, USA). LPS, monoclonal anti-b-actin antibody,was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were also purchased from Sigma-Aldrich unless otherwise stated. Cobrotoxin was purchased from Sigma-Aldrich or from Kyungwon University. The compositions are followings: protein (902%), phospolipase A2 (356U), coagulase (800008000U/mg).

Raw cell culture-Raw 264.7, a mouse macrophage-like cell line and THP-1, a human

monocytic cell line were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies (Rockville, MD, USA). Raw 264.7 cells were grown in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37C in 5% CO2humidified air. THP-1 cells were grown in RPMI 1640 with L-glutamine and 25 mM HEPES buffer (Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 mg/ml streptomycin at 37C in 5% CO2 humidified air.

Preparation of nuclear extracts and EMSA-Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI). Briefly, 1 106cells/ml was washed twice with 1PBS, 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 g/ml phenylmethylsulfonyl fluoride, 1 g/ml pepstatin A, 1 g/ml leupeptin, 10 g/ml soybean trypsin inhibitor, 10 g/ml aprotinin, 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 [g-32P] ATP for 10 min at 37C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 l (50,000-200,000 cpm) of 32P-labeled oligonucleotide and another 20 min of incubation at room temperature. Subsequently 1 l of gel loading buffer was added to each reaction and loaded onto a 4% nondenaturing gel and electrophoresed until the dye was three-fourths of the way down the gel. The gel was dried at 80 C for 1hr and exposed to film overnight at 70C. 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).

Immunocytochemistry-To determine whether cobrottoxin could be uptaken into the cells, astrocytes and cells (1x105 cells/cm2) were cultured on the chamber slide (Lab-Tak II chamber slider system, Nalge Nunc Int., Napervill, IL, USA) and then treated by melittin 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-Red, Hercules, CA, USA) with a 60 X oil immersion objective.

III. Results

1. Inhibition of NF-kB activation

Direct binding of cobrotoxin with p50 could

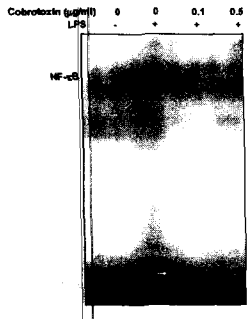

Fig. 1. Inhibitory Effects of Cobrotoxin in NF-kB DNA Binding Activity in Raw 264.7 cells after Treatment with LPS
Representative photography of three separated experiments with duplicates

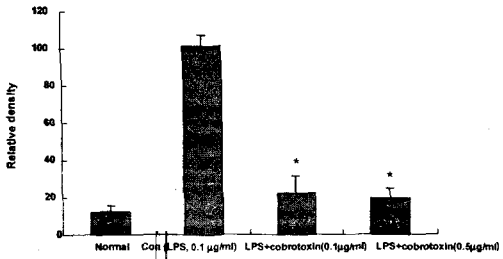

Fig. 2. Inhibitory effect of cobrotoxin in NF-kB DNA binding activity in Raw264.7 cells after treatment with LPS
Values are mean±SD of three experiment with duplicates
*: P<0.05, significant different from control group

also result in in prevention of p50 translocation into nucleus, which finally inactivate NF-kB DNA binding activity. We therefore studied the consequence of interaction between molecules of NF-kB signals and cobrotoxin. Protein p50 was incubated with NF-kB DNA binding element in the absence or presence of cobrotoxin in vitro, and then EMSA was performed to determine binding ability of p50 to NF-kB binding element. Inhibitory effect of corotoxin on the binding ability of p50 to NF-kB binding element was observed. NF-kB DNA binding activity was then performed by EMSA in nuclear extracts of Raw 264.7cells treated with LPS alone, or combination with cobrotoxin for 1.5 hr that was the time to induce NF-kB activation maximally. DNA binding activity of p50 extracted from the combination of LPS and cobrotoxin- treated nucleus of Raw 264.7cells was much lowered than the NF-kB DNA binding activity of LPS-treated cells in a dose dependent manner(Fig.1, Fig.2). This effect was also observed in Raw 264.7 cells treated by SNP with cobrotoxin(Fig.3, Fig.4). These effects suggest that physical interaction of cobrotoxin with molecules in the NF-kB signals could change biological effect of NF-kB.

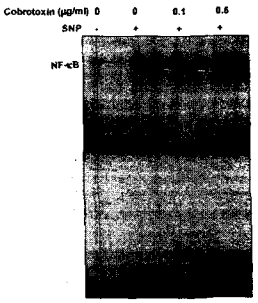

Fig. 3. Inhibitory Effects of Cobrotoxin in NF-kB DNA Binding Activity in Raw 264.7 cells after Treatment with SNP
Representative photography of three separated experiments with duplicates

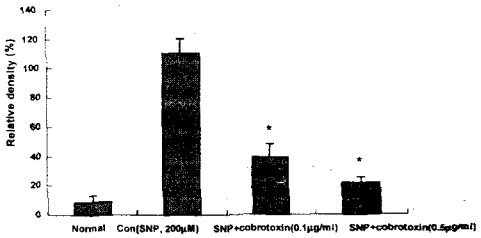

Fig. 4. Inhibitory Effects of Cobrotoxin in NF-kB DNA Binding Activity in Raw 264.7 cells after Treatment with SNP
Values are means±S.D. of three experiments with duplicates
*: P<0.05, Statistical Significance Compared with Control

Cysteine residues are involved in interaction between cobrotoxin with molecules of NF-kB signals-The x-ray analysis has defined the presence of cysteine residues in the structure of p50 of DNA-binding domain(Fig.5).

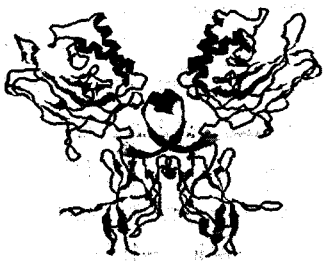

Fig.5. structure of p50

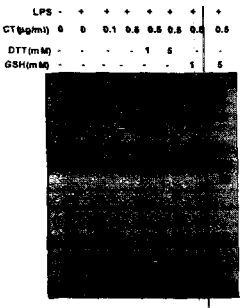

Fig.6. Reverse Effects of DTT and GSH on the inhibitory Effects of Cobrotox -in in NF-kB DNA Binding Activity in Raw 264.7 cells after Treatment with LPS

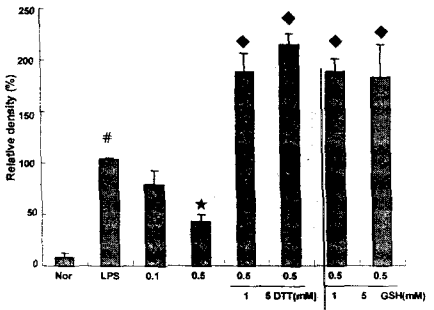

Fig. 7. Reverse Effects of DTT and GSH on the inhibitory Effects of Cobrotox -in in NF-kB DNA Binding Activity in Raw 264.7 cells after Treatment with LPS

Values are means±S.D. of two experiments with triplicates
#: P<0.05 Statistical Significance Compared with Normal
★: P<0.05 Statistical Significance Compared with Control
◆: P<0.05 Statistical Significance Compared with 0.5 μg/ml of Cobrotoxin treated group

Several investigations have been shown that target disruption of cysteine residues of these molecules can inhibit NF-kB activation (28). To explore this possibility, NF-kB DNA binding element mixture were incubated with cobrotoxin in the presence of the disulfide reducing agent dithiothreitol (DTT) and GSH, and then DNA binding activity was determined by EMSA. DTT did reverse the inhibitory effect of cobrotoxin on p50 DNA binding ability dose dependently. This reversing effect of DDT and GSH on the inhibitory effect of cobrotoxin was also observed in DNA binding activity of p50 extracted from nucleus of astrocytes and Raw 264.7 cells treated with LPS or SNP(Fig.6, Fig.7) . We also further investigated whether cobrotoxin can be uptaken into cells, thereby interacts with NF-kB signal molecules. We labeled cobrotoxin with Alexa Fluor 488, a photostable dye using a protein labeling kit, and the conjugated cobrotoxin with Alexa Fluor was treated in incubated Raw 264.7 cells. The uptake of the labeled cobrotoxin into the cells was shown under a confocal laser scanning microscope.

cobrotoxin was uptaken into the membrane and nucleus of the cells(Fig.8).

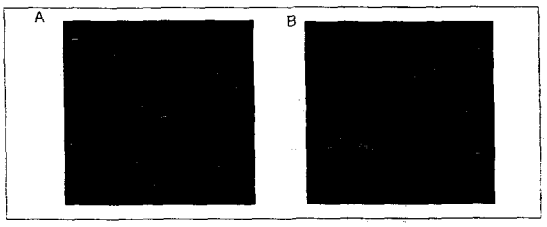

Fig. 8. Uptake of Cobrotoxin in Raw 264.7 cells
Figure representative from two experiments

Uptake of Cobrotoxin into cytosolic and nucleus of Raw 264.7 cells demonstrated by confocal laser scanning microscope (x 360).
A: cells were treated with Cobrotoxin unlabelled.
B: cells were treated with Cobrotoxin labelled with Alexa Fluor 488 d

Ⅳ. Discussion

In the present study demonstrate that The interaction between cobrotoxin and these NF-kB signal molecules resulted in inhibition of DNA binding of NF-kB, consequently the expression of IkB release and p50 translocation. Using reducing agents, we showed that cobrotoxin may interact with the cysteine residue of active sites of these molecules.

Structural data of p50 subunit reveals that cysteine residues are present in the catalytic domain of p50. With the structural data, several studies have been demonstrated that cysteine residue of this molecules are target of NF-kB inactivating compounds. For example, kamebakaurin and other three kaurane diterpenes, the extract of Isodon japonicus, selectively inhibit activation of NF-kB by

directly interaction with p50[19]. In other report, the synthetic quinone derivative, (2E)-3-[5-(2, 3-dimethoxy-6-methyl-1, 4-benzoquinoyl)] -2-nonyl-2-propenoic acid (E3330), is a novel anti-NF-kB drug that specifically also suppresses DNA binding activity of NF-kB by interaction with p50[20]. In other report, mercuric ion (Hg2+), a potent thiol inhibitor, prevents expression of NF-kB by mercaptide bond formation with the p50 subunit[21]. It was also shown that NF-kB inhibiting natural compounds and other thiol metals inhibit NF-kB activation through interacts with the IKK catalytic subunits[5-6]. We recently also found that melittin, cationic 26-amino acid and the principal component of the bee venom toxin and bee venom itself directly binds to sulfhydryl group of IKKs and p50 resulting in IkB release, thereby inhibits activation of NF-kB and expression of genes involving in the inflammatory responses[22]. Cobrotoxin has twelve cysteine residues and forms six disulfide bonds. By the reference of structural data and other studies showing binding of cobrotoxin with biological receptor such as AchR14-18)(fig.9), we questioned that cobrotoxin may bind to cysteine residues in p50 of NF-kB signals. Although we have been not map the accurate site to bind,

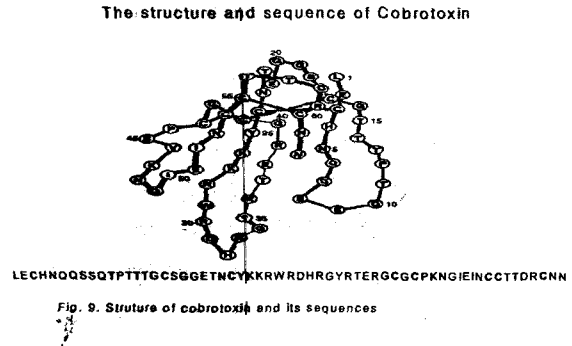

Fig. 9. Struture of cobrotoxin and its sequences

Regarding this perspectives, we demonstrated the inhibitory effect of cobrotoxin on the DNA binding activity in vivo and in vitro (cell free system) in Raw 264.7 cells induced by SNP or LPS. This data suggested that the binding of cobrotoxin with NF-kB signal molecules should alter signal pathway of NF-kB, and certain level of cobrotoxin may use as at least a therapeutic compound to control pathophysiological condition such as inflammatory reaction and cancer development in vivo.

We further demonstrated the possible reactive sites of NF-kB molecules to cobrotoxin. Exogenous addition of thiol reducing agents; DTT and GSH reversed inhibitory effect of cobrotoxin on the p50 DNA binding activity. These data suggest that cobrotoxin modify a sulfhydryl group of p50 protein.

Similar study was reported with the peroxisome proliferators activated receptor gamma agonist 15-deoxy-delta12,14 -prostaglandin J2 (15-deoxy PGJ2) which inhibited IkBa degradation in a way of direct modification IKKa and IKKb molecules[23]. In other study, arsenite, a potent inhibitor of NF-kB activation was showed to interact with a specific cysteine residue (Cys-179) of the IKK catalytic subunits, and this interaction was inhibited by thiol-modifying agents[6]. It is suggested that cyclopentanon 15-deoxy- PGJ2 covalently modified cystein 62 in p50 and 179 in IKKb through a Michal-type reaction[24]. A thiol redox mechanism has been shown to be involved in thiol-reactive metal-induced inhibition of IKKs[25]. Therefore, it is likely that cobrotoxincould bind to cysteine residue in the different target molecules such as p50, IKKa and IKKb. Although we did not map precisely how and what residue of p50 could bind to cobrotoxin, however, it is the most interesting to note that reduced cobrotoxin with DDT or modified the cysteine residue of cobrotoxin with 2,2-diyhiopyridine lowered the binding

affinity of cobrotoxin to AchR[26]. Identifications of binding the sites of cobrotoxin, and involvement of disulfide bond in the cobrotoxin binding to the NF-kB signal molecules are being investigated. In summary, the present results demonstrate that cobrotoxin directly binds to sulfhydryl group of p50 resulting in translocation of p50 and IkB release, thereby inhibits activation of NF-kB, and suggest that pico to nanomolar range of cobrotoxin could inhibit the expression of genes in the NF-kB signal pathway.

V. Reference

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