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

원저

The Study of NF-κB(P50) Suppression mechanism with main Component of Bee Venom and Melittin on Human Synoviocyte

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Abstract

Melittin,cationic 26-amino acid, is the principal component of the bee venom (BV) which has been used for treatment of inflammatory disease such as arthritis rheumatism. NF-kB is activated by subsequent release of inhibitory IkB via activation of a multisubunit IkB kinase (IKK). We previously found that melittin bind to the sulfhydryl group of p50, a subunit of NF-kB. Since sulfhydryl group is present in kinase domain of IKKa and IKKb, melittin could modify IKK activity by protein-protein interaction. We therefore examined effect of melittin on IKK activities in sodium nitroprusside (SNP)-stimulated synoviocyte obtained from RA patients. Melittin suppressed the SNP-induced release of IkB resulted in inhibition of DNA binding activity of NF-kB and NF-kB-dependent luciferase activity. Consistent with the inhibitory effect on NF-kB activation, IKKa and IKKb activities were also suppressed by melittin. Surface plasmon resonance analysis realized that melitin binds to IKKa (Kd = 1.34 x10-9 M) and IKKb(Kd= 1.01x10-9 M). Inhibition of IKKa and IKKb resulted in reduction of the SNP-induced production of inflammatory mediators NO and PGE2 generation. The inhibitory effect of melittin on the IKKs activities, binding affinity of melittin to IKKs, and NO and PGE2 generation were blocked by addition of reducing agents dithiothreitol and glutathione. In addition, melittin did not show inhibitory effect in the transfected Synoviocytes with plasmid carrying dominant negative mutant IKKa (C178A) and IKKb (C179A). These results demonstrate that melittin directly binds to sulfhydryl group of IKKs resulting in IkBrelease, thereby inhibits activation of NF-kB and expression of genes involving in the inflammatory responses.

Key words : Bee Venom, Melittin, IkB kinase, NF-kB, synoviocyte, NO, PGE2

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

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Ⅰ. Introduction

Bee venom (BV) Acupuncture has been used to relieve pain and to treat inflammatory diseases such as rheumatoid arthritis (RA) in humans[1] and experimental animals[2]. BV contains melittin, a 26 amino acid peptide, forms an amphipathic helix with a highly charged carboxyl terminus of protein[3]. We previously found that melittin inhibited NF-kB activation by prevention of p50 translocation through by the protein (p50)-protein (BV or melittin) interaction through modification of sulfhydryl group of p50[4].

Structural data of IKK subunits reveal that cysteine residues are present in the kinase domain of IKKa and IKKb, and some of them located at functionally important sites (Cys-179) such as activation of T loop and the catalytic site[5-6]. P50 also possesses a critical cycteine residue (Cys-62) at the N-terminal region ofits DNA-binding domain[7]. In several studies, potent inhibitors of NF-kB activation have showed to interact with a specific cysteine residue of the IKK catalytic subunits or p50 subunit of NF-kB resulting in target modified inactivation of NF-kB[5-7]. Moreover, this interaction was reversed by thiol-modifying agents, and expression of mutant IKKa, IKKb and p50, in which cyteine residues were replaced with other amino acids; alanine (IKK) or serine (p50) render the cells to become resistant to inhibitory effect of these inhibitors in NF-kB activation[5-7], suggesting that cysteine in the proteins involving NF-kB signal can be targets of these compounds.

In the present study, we investigated whether BV or melittin could bind with sulfhydryl group of p50 and IKKs resulted in the inhibition of NF-kB activity, IkB release via target disruption of IKK by protein-protein (IKK-melittin) interaction and SNP-induced production of inflammatory mediators such as NO in synoviocyte obtained from RA patients were focused.

Ⅱ. Materials and Methods

Goat polyclonal antibody to p50 (1:500), p65 (1:500), IkBa (1:500), phospho-IkB (1:200), IkB (1:500), mouse polyclonal antibody to iNOS (1:50) and all of the secondary antibodies used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA. USA). T4 polynucleotide kinase obtained was from Promega (Madison, WI). Poly (dIdC), horseradish peroxidase-labeled donkey anti-rabbit second antibody, and the ECL detection reagent were obtained from Amersham Pharmacia Biotech (Centennial Ave, NJ, USA). Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Alfred Nobel Drive Hercules, CA, USA). SNP, Griess reagent, monoclonal anti-β-actin antibody, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and melittin, a component of BV were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were also purchased from Sigma-Aldrich unless otherwise stated. BV was purchased from You-Miel BV Ltd (Hwasoon, Korea). The compositions are followings: melittin (45-50%), apamin (2.5-3%), MCD peptide (1-2%), PLA2 (12%), Lyso PLA (1%), Histidine (1-1.5%), Minimine (2-3%), 6pp lipids (4-5%).

III. Synoviocyte culture

Synovial tissues were obtained, with consent, from nine RA patients who were undergoing total knee replacement or arthroscopic synovectomy. From these samples, three cell lines were studied. To avoid the effect of steroids on the synovial cells, the patients were instructedto cease steroid medication for 2 months before the operation. All patients satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (31). Synovial tissues were minced and treated for 4 hr with 4 mg/ml collagenase (type 1; Worthington Biochemical, Freehold. NJ, USA) in DMEM at 37C in 5% carbon dioxide. Dissociated cells were plated in DMEM supplemented with 10% fetal bovine serum (Gibco Life Technologies, Rockville, MD, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were used between the third and fifth passages

IV. Determination of nitric oxide

Cells were grown in 24-well plates and then incubated with or without SNP (200 mM) in the absence or presence of various concentrations of BV (0.5, 1, 5 μg/ml), or melittin (5,10 μg/ml) for 72 hr. The nitrite accumulation in the supernatant was assessed by Griess reaction (32). Each 100 ml of culture supernatant was mixed with an equal volume of Griess reagent (0.1% N (1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phophoric acid) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader, and a series of known concentrations of sodium nitrite was used as a standard.

V. 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 ml/ml aprotinin, 1% igapel 630 (Sigma Chem. Co.), 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 mg) 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 0.05% tween-20. The membrane was incubated for 4 hr at room temperature with specific antibodies: rabbit polyclonal antibodies against p50, p65, IkBa, IkBb (1:500) and phospho-IkBa (1:200)(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).

VI. Preparation of nuclear extracts and EMSA

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI). Briefly, 1 106cells/ml were 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 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 37 C. 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% non denaturing gel, and then 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 protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

VII. Transfection assay

Synoviocyte were transfected with pNF-kB-Luc plasmid (5x NF-kB; Stratagene, CA, USA) using a mixture of plasmid and lipofectamine plus in OPTI-MEN according to manufacture's specification (Invitrogen, Carlsbad, CA, USA). The control pCMV (Clontech, CA, USA) was co-transfected to monitor the transfection efficiency. After 24 hr, the cells were then co-treated with BV (or melittin) and SNP.

VIII. Surface plasmon resonance analysis

Activated CM-dextran matrix carried out by mixing ethyl-N-(dimethylaminopropyl) carbodiimide and N-hydroxysuccimide 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 neuclear 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 2000TM system continuously monitors the change in mass at the sensor surface, and the protein

interaction kinetics were analyzed by BIAevaluation 3.0software (BIACORE AB, 5-75450, Uppsala, Sweden).

IX. 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 $p<0.05$.

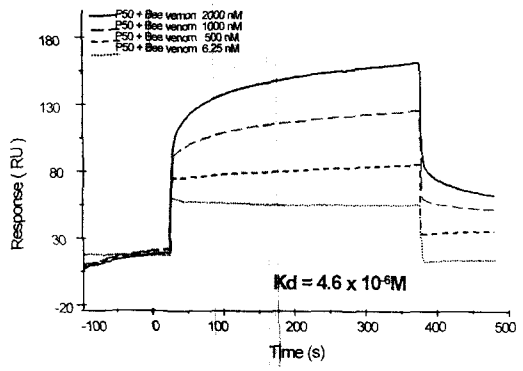

Fig. 1. Binding Affinity of Be Venom and p50 in Vtro Representative grape of two experiments

X. Results

1. Interaction between p50 and BV or melittin

we studied the interaction between p50 and melittin or BV. Binding ability of the immunoprecipitates against p50 antibodies to melittin immobilized onto a surface of sensor chip was monitored by Biacore analyzer. Increasing dose of immunoprecipitates against p50 antibodies clearly showed increased binding activity with BV or melittin.

The maximum Kd value of binding affinity of p50 with BV or melittin is 4.6×10-6, 1.2×10-8M respectively(fig. 1, fig.2).

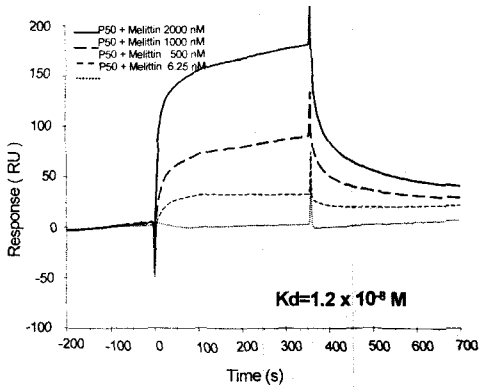

Fig. 2. Binding Affinity of Melittin and p50 in Vtro Representative grape of two experiments Representative grape of two experiments

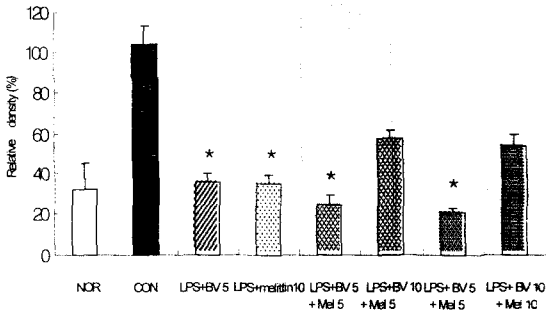

Fig. 3. Inhibitory Effect of BV or Melittin on the LPS-induced Release of p-IkB in Synovicytes
*: $P<0.05$. Statistical significance compared with control

2. Inhibitory effect of BV and melittin on SNP-induced IkB release

Release of the inhibitory IkB subunit is involved in activation of NF-kB. To elucidate the effect of melittin and BV on this step, the kinetics of IkB release in cytosol were studied by western blot analysis. SNP-induced IkBs release was markedly inhibited by melittin and BV in synoviocytes (Fig. 3, Fig.4). The inhibitory effect of melittin on release of IkB was similar to BV. The synergic effect of

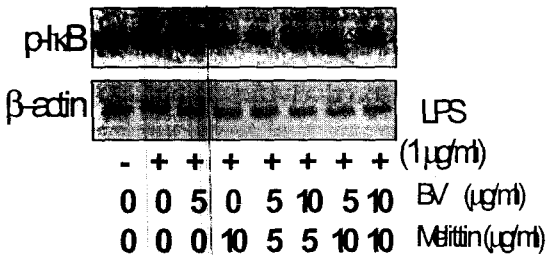

Fig. 4. Inhibitory Effect of BV or Melittin on the LPS-induced Release of p-IkB in SynoviocytesRepresentative photography of three separated experiments with duplicates both is also found.

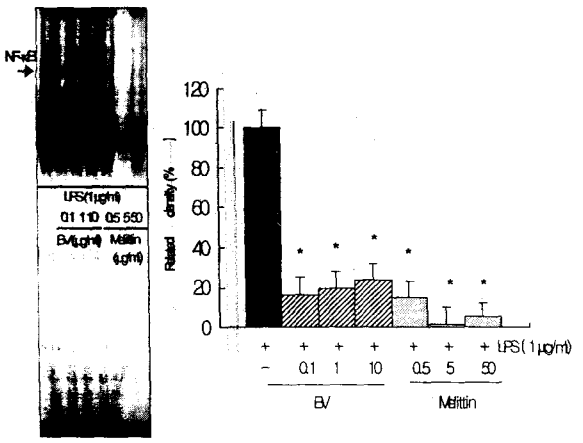

Fig. 5. Inhibitory Effect of BV and Melittin on the LPS-induced p50-DNA Binding Ativity Values are mean±S.D. of three experiments with triplicates

* : P<0.05. Statistical significance compared with control

Values are mean±S.D. of three experiments with triplicates

* : P<0.05. Statistical significance compared with control

3. Inhibitory effect of BV and melittin on DNA binding activity

NF-kB DNA binding activity was performed by EMSA in nuclear extracts of synoviocytes treated with SNP alone, or combination with melittin or BV for 1 hr that was the time to induce NF-kB activation maximally(data not shown). BV and Melittin inhibited NF-kB DNA binding activity by SNP in a dose dependent manner in synoviocytes (Fig. 5).

4. DTT and GSH reversed inhibitory effect of BV and melittin.

We previously found that BV and melittin bind to p50 subunit of NF-kB and IKK which prevent the release of IκB and the translocation of p50 into nucleus. This Inhibitory effect was reversed by thiol reducing agents such as DTT, GSH and H2O2, suggesting that sulfhydryl group of p50

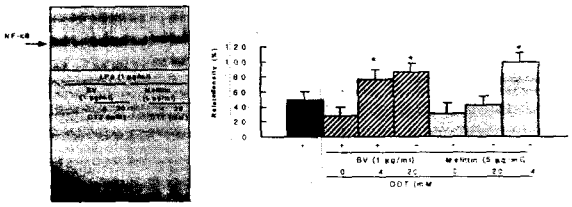

Fig. 6. Reverse Effect of DTT on the inhibitory Effect of BV and Melittin in the LPS-induced p50-DNA Binding Activity in cultured Synoviocytes Values are mean±S.D. of three experiments with triplicates

* : P<0.05. Statistical significance compared with LPS+BV (or Melittin) treated group

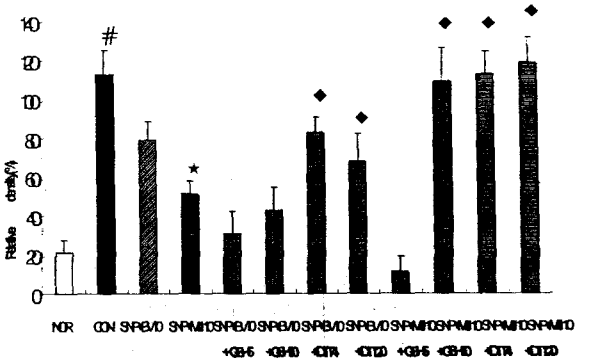

Fig. 7. Reversed inhibitory Effect of BV and Melittin on p-IkB Expression and Synoviocytes after Reducing Agents DTT and GSH Treatment after Treatment by SNP

: P<0.05, Statistical significance compared with normal (p<0.05)

★ : P<0.05, Statistical significance compared with control (SNP treated) group

□□ : P<0.05, Statistical significance compared with BV or Melittin treated group

may be a target molecule binding to BV and melittin.

To demonstrate this possibility, we first performed DNA binding activity of NF-kB by EMSA and then did Release of IκB by western blotting with DTT and GSH in Synoviocyte and found inhibitory effect of BV and melittin on NF-kB activity(fig.6) and Release of IκB was reversed by treatment of reducing agents DTT and GSH dose dependently(fig.7)..

The preventive effect of the SNP-induced release of NO was reversed by DTT, GSH and H2O2 in proportion to the reversed effect on DNA binding activities of NF-κB in synoviocytes virtually

5. Abolition of the inhibitory effects of melittin on NO generation in p50 mutant cells

We conducted a transient transfection assay with a fusion gene containing NF-kB-luc plasmid DNA (0.8g), NF-kB-gal plasmid DNA (0.2g) promoter. Synoviocytes were transfected with these promoter reporter gene constructs. NO was then measured after stimulating the cells by SNP with or without melittin or BV in cell supernatant. Contrast to a significant inhibition of NO generation in the cells transfected vector alone(data not shown), melittin and BV did not inhibit SNP-induced NO generation(fig.8) and iNOS expression (fig.9,10) in the transfected with p50 dominant negative mutants in synoviocytes.

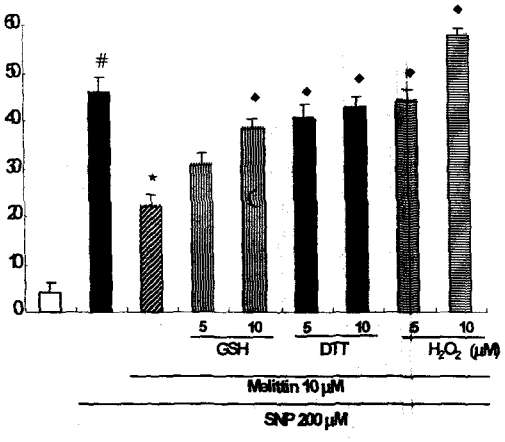

Fig. 8. Inhibitory Effect of Melittin on the SNP-induced NO Levels and reverse Effect of GSH, DTT and H2O2 in Synoviocytes Values are mean±S.D. of three experiments with triplicates

: P<0.05, Statistical significance compared with normal

* : P<0.05, Statistical significance compared with control (SNP treated) group □□ : P<0.05, Statistical significance compared with melittin treated group

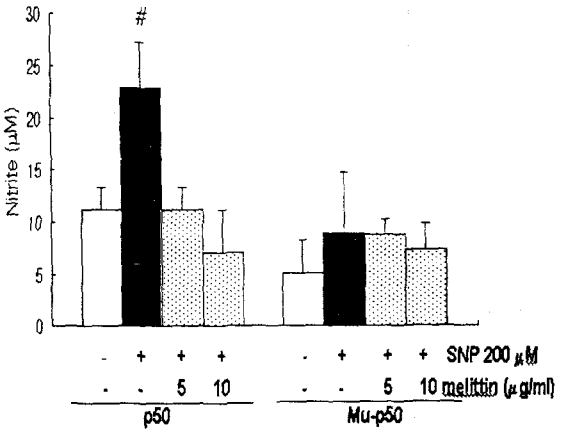

Fig. 9. Reversed inhibitory Effect of Melittin on NO Synthesis in Mutant p50 Plasmid transfected Synoviocytes after Treatment by SNP Values are mean±S.D. of three experiments with triplicates

: P<0.05, Statistical significance compared with wild type of cells

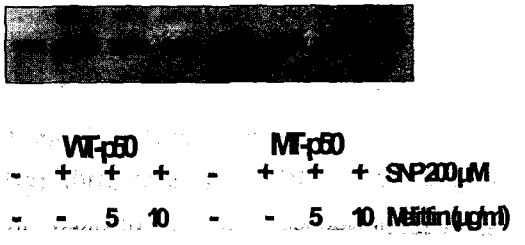

Fig. 10. Reversed inhibitory Effect of Melittin on iNOS Expression in Mutant p50 Plasmid transfected Synoviocytes after Treatment by SNP

XI. Discussion

In the present study, the binding affinity of p50 proteins with BV or melittin immobilized on the chip was much greatly lower in the cells treated with SNP in combination of melittin compared to that in the cells treated by SNP alone. 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 IKKaand IKKb molecules[8-9]. 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[10], and this interaction was further inhibited by thiol-modifying agents[11]. The direct interaction between BV or melittin with p50 was evidenced by SPR analysis in the present study. The binding affinity of BV or melittin to p50 is about 4.6×10-6, 1.2×10-8M respectively. The binding affinity of melittin with p50 is much stronger than the binding affinity of other biological protein-protein (receptor) interaction in physiological condition. For examples, estrogen receptor-ligand interaction has affinity about (Kd = 10-2~10-3 M)[12], and the binding affinity of MHC-peptide complex to T cell receptors (TCRs) is about Kd = 1x10-7 M[13]. The binding affinity of melittin with p50 is about 1000 times stronger than the binding affinity of of MHC-peptide complex to TCRs binding affinity[14]. Therefore, our data suggest that the binding of BV or melittin with p50 could directly influence on p50 resulted in inhibition of DNA binding activity of NF-κB and IkB release in physiological condition of body. The inhibitory effect of BV or melittin was associated with suppression of IkBa and IkBb release through suppression of IKKa and IKKb activation. The promoter region of the murine gene encoding iNOS and COX-2 contains NF-kB binding sites[15]. This result therefore shows that the inhibitory effect of inflammatory gene expression could be related with inhibition of DNA binding activity of NF-kB. The inhibitory effect on the IkBa and IkBb activity may be critical mechanism of inactiviation of NF-kB by BV or melittin. This notion was supported with the inhibitory effect on IkBa and IkBb release by melittin. Structural data of IKK subunits reveal that cysteine residues are present in the kinase domain of IKKa and IKKb, and some of them located at functionally important sites such as activation of T loop and the catalytic site[10,16]. We showed that BV or melittin directly binds p50. This data suggest that melittin may modify a sulfhydryl group of p50 protein, thereby hinder IkB release and NF-kB activation. Exogenous addition of a thiol reducing agents; DTT and GSH reversed the melittin-induced inhibition of the DNA binding activity of NF-kB. Furthermore in the presence of H2O2, the effect was also reversed supporting this explanation[11].

Our data showed that DTT, GSH and H2O2 reversed the Inhibitory effect of BV or Melittin on IkB release and DNA binding activity of NF-kB, and that inhibitory effect of BV or melittin on DNA binding activity of NF-kB(p50), iNOS expression and NO generation was abolished in the cells tansfected with NF-kB-luc plasmid DNA (0.8g), NF-kB-gal plasmid DNA (0.2g) which cysteine residue replace by alanine.

we think that BV or Melittin might also inhibit IKKa and IKKb activities to release IkB because of their structure with cystein residue. If this hypothesis were true, BV or melittin could compete with complex protein

involving in multiple step of NF-kB activation and exert the therapeutic potential of combined inhibition of the prostanoid and nitric oxide synthesis systems by inhibition of IKK and NF-kB.

XII. References

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