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Melittin inhibits inflammatory target gene expression and mediator generation via interaction with IκB kinase

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ABSTRACT

We previously found that bee venom (BV) and melittin (a major component of BV) has anti-inflammatory effect by reacting with the sulfhydryl group of p50 of NF-κB. Since the sulfhydryl group is present in IκB kinase (IKKα and IKKβ), anti-inflammatory effect of melittin via interaction with IKKs was investigated. We first examined binding of melittin to IKKs using surface plasmon resonance analyzer. Melittin binds to IKKα ($K_d = 1.34 \times 10^{-9}$ M) and IKKβ ($K_d = 1.01 \times 10^{-9}$ M). Consistent with the high binding affinity, melittin (5 and 10 μg/ml) and BV (0.5, 1 and 5 μg/ml) suppressed sodium nitroprusside, TNF-α and LPS induced-IKKβ and IKKβ activities, IκB release, and NF-κB activity as well as the expressions of iNOS and COX-2, and the generation of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in Raw 264.7 mouse macrophages and synoviocytes obtained from rheumatoid arthritis patients. The binding affinities of melittin to mutant IKKs, was reduced, and the inhibitory effect of melittin on IKK and NF-κB activities, and NO and PGE_2 generation were abrogated by the reducing agents or in Raw 264.7 transfected with mutant plasmid IKKα (C178A) or IKKβ (C179A). These results suggest that melittin binding to the sulfhydryl group of IKKs resulted in reduced IKK activities, IκB release, NF-κB activity and generation of inflammatory mediators, indicating that IKKs may be also anti-inflammatory targets of BV.

1. Introduction

NF-κB has been suggested to play important roles in the regulations of inflammatory genes, such as, inducible nitric oxide synthetase (iNOS), cyclooxygenase-2 (COX-2), cytosolic phosphatase A_2 ($cPLA_2$), and tumor necrotic factor-α (TNF-α). Functionally active NF-κB exists mainly as a heterodimer consisting of subunits of the Rel family, and

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Abbreviations: BV, bee venom; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSH, glutathione; IκBs, inhibitors of κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PGs, prostaglandins; RA, rheumatoid arthritis; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-α

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this heterodimer is normally sequestered in the cytosol as an inactive complex by binding to inhibitory κB (IκBs) in unstimulated cells [1]. The mechanism of NF-κB activation involves the phosphorylation of IκBs via IκB kinase (IKK) activation [2]. Once IκBs are phosphorylated, they are targeted for ubiquitination and subsequent degradation by the 26s proteosome [3]. The resulting free NF-κB is translocated to the nucleus, where it binds to the κB binding sites in the promoter regions of target genes, thereby controls their expressions [4]. In several studies, potent inhibitors of IKKs preventing NF-κB activity through blockage of IκB release can be useful for the treatment of inflammatory diseases, such as, rheumatoid arthritis (RA) [5–8].

Bee venom (BV) therapy has been used to relieve pain and to treat inflammatory diseases including RA in humans [9] and in experimental animals [10]. BV contains melittin, a 26 amino acid peptide, which forms an amphipathic helix with a highly charged carboxyl terminus [11]. We previously found that melittin inhibits lipopolysaccharide (LPS)-induced NF-κB activation by preventing p50 translocation via a protein (melittin)-protein (sulfhydryl group of p50) interaction, and that this inhibits inflammatory reaction in the development of RA [12]. Structurally, IKK subunits have cysteine residues in the kinase domains of IKKα and IKKβ, and some of these are located at functionally important active sites [6,8]. In addition, several compounds that disrupt specific cysteine residues of IKKs, and thereby prevent NF-κB activation have been suggested to be useful agents for the treatment of rheumatoid arthritis (RA), a chronic inflammatory disease [7,13–15]. These compounds reduce large amounts of nitric oxide (NO) and prostaglandins (PGs), which are synthesized systemically in inflammatory diseases, as shown in animal models of arthritis and in patients with RA [16–20]. Moreover, IKKs are essential for NF-κB-mediated inflammatory responses [4]. In the present study, we therefore investigated whether BV or melittin inhibits NF-κB via disrupting IKKs through protein–protein (melittin-IKKs) interaction, and thereby inhibits the inflammatory response in Raw 264.7 macrophages and in the synoviocytes of RA patients.

2. Materials and methods

2.1. Chemicals

The expression plasmid encoding IKKβK44A-Flag (Lysine residue in the Mutant forms of IKKα (C178A) and IKKβ (C179A) is replaced with alanine) was obtained from Dr. Warner C. Greene (University of California, San Francisco, CA), and the expression plasmids encoding IKKαC178A-Flag and IKKβC179A-Flag (cysteine residue in the Mutant forms of IKKα (C178A) and IKKβ (C179A) is replaced with alanine) were obtained from Dr. Dae-Myung Jue (University of Catholic, Seoul, South Korea) [21]. Rabbit polyclonal antibodies to $cPLA_2$ (dilution 1:500), and goat polyclonal antibody to COX-2 (1:500), TNF-α (1:500), p50 (1:500), p65 (1:500), IκBβ (1:500), phospho-IκBα (1:200), IκBβ (1:500) and mouse polyclonal antibody to iNOS (1:500), IκB kinases (1:500), and all of the secondary antibodies used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). T4 polynucleotide kinase was obtained from Promega (Madison, WI). Poly (dI·dC), horseradish peroxidase-labeled donkey anti-rabbit second antibody, and the ECL detection reagent were obtained from Amersham Pharmacia Biotech (Centennial Ave., NJ, USA). Sodium nitroprusside, LPS, TNF-α, Griess reagent, monoclonal anti-β-actin antibody, 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). 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%), PLA_2 (12%), lyso PLA (1%), histidine (1–1.5%), minimine (2–3%), 6pp lipids (4–5%).

2.2. Cell culture

Raw 264.7, a mouse macrophage-like cell line and THP-1, a human monocytic cell line was 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 μg/ml streptomycin at 37 °C in 5% CO_2 humidified 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, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 humidified air.

2.3. Synoviocyte culture

Synovial tissues were obtained, with consent, from nine RA patients who were undergoing total knee replacement or arthroscopic synovectomy. All patients satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology [22]. The method of synoviocyte culture was described in elsewhere [12].

2.3.1. Determination of nitric oxide and prostaglandin E_2

The nitrite accumulation in the supernatant was assessed by Griess reaction [23], and the determination of prostaglandin E_2 (PGE_2) was performed as described by Akiba et al. [24].

2.4. DNA binding activity of NF-κB

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI) as described in previous study [12]. Briefly, nuclear extract was incubated with κB consensus oligonucleotides end-labeled using T4 polynucleotide kinase and [γ-^{32}P] 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 ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. For the competition assay, 100× or 200× excesses of unlabeled double-stranded oligonucleotide of the κB binding

site (or 100× irrelevant oligonucleotide of AP-1 or SP-1) were used as specific competitors. Supershift assay was done in the presence of p50 or p65 subunit of NF-κB (2 μg). Subsequently 1 μl 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 1 h 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).

2.5. Transfection and assay of luciferase activity

Raw 264.7 or THP-1 cells were transfected with pNF-κB-Luc plasmid (5× NF-κB; Stratagene, CA, USA) or IKKα (C178A), IKKβ (C179A) and IKKβ(K44A) mutant plasmids 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 h, the cells were then co-treated with BV (or melittin) and sodium nitroprusside. Luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's instructions (WinGlow, Bad Wildbad, Germany). Cells carrying IKKα (C178A), IIKKβ (C179A) and IKKβ (K44A) plasmid were also used for determination of NO and PGE_2.

2.6. Western blot analysis

Cell lysates were prepared as described in the previous study [12]. Equal amount of lysate 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). The membrane was incubated for 5 h at room temperature with specific antibodies. 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).

2.7. In vitro kinase assays

Raw 264.7 cells and synoviocytes grown in 100-mm plates were treated with various agents. Cell lysate for in vitro assay of IKKα and IKKβ kinase activities was prepared as described in the Western blot analysis. In vitro kinase assay was performed with immune complexes and bacterially synthesized GST-IκBα proteins (2 μg) in 15 ml of kinase buffer containing 20 mM HEPES (pH 7.7), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, aprotinin (10 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and 1 mM DTT at 30 °C for 30–60 min. Phosphorylation of GST-IκBα and GST-IκBβ was separated in 12% SDS-PAGE, and then the phosphorylated GST-IκBβ and GST-IκBβ was scraped into 96-well and 100 μl cocktail were added each wells, and then IKKα and IKKβ kinase activity was quantitied by the Liquid Scintillation Counter (LSC: Beta Counter) (Beckman-LS 6500, Beckman Instrument Inc., Australia). The sample was also separated on SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane, and then the membrane probed with appropriate antibodies to detect the amount of IKKα and IKKβ as described in Western blot analysis.

2.8. Surface plasmon resonance analysis

Biacore 2000^{TM} and CM5 sensor chip were both supplied by Biacore AB (Uppsala, Sweden). Recombinant protein G, protein IKKs and melittin, a component of BV were purchased from Sigma–Aldrich (St. Louis, MO, USA). Activated CM-dextran matrix carried out by mixing ethyl-N-(dimethylaminopropyl) carbodiimide and N-hydroxysuccimide was surfaced on the sensor chip. Recombinant IKKα and IKKβ protein or melittin were then layered onto the CM-dextran sensor chip followed by blockage of the chip using 1 M ethanolamine, pH 8.5 as described in elsewhere [12]. Serial dilutions of BV, melittin or IKKα, and IKKβ protein were prepared using HEPES buffered saline buffer (pH 7.4), 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 pH 12. The Biacore 2000^{TM} system continuously monitors the change in mass at the sensor surface, and the kinetic of protein interaction was analyzed by BIAevaluation 3.0^{TM} software (Biacore AB, 5-75450, Uppsala, Sweden).

2.9. Statistical analysis

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

3. Results

3.1. Melittin interacts with IKKα and IKKβ, and inhibits the activities of IKKs

We previously found that the sulfhydryl group of p50 is a target of melittin. Since IKKα and IKKβ have cysteine residues in their active domains, we hypothesized that if these cysteine residues were also modified by melittin as in p50 [12], that IKKα and IKKβ activities might also be inhibited by reaction with melittin. To determine whether melittin interferes IKKs activity by reacting with IKKs, we studied the interaction between melittin and IKKs. The binding abilities of melittin to IKKα or IKKβ immobilized onto the surfaces of sensor chips were monitored using a Biacore analyzer. Increasing doses of melittin clearly increased binding affinity to IKKα and IKKβ, and the maximum binding affinities of K_d values were 1.34×10^{-9} M and 1.01×10^{-9} M, respectively (Fig. 1A and B). Next, the interaction between melittin immobilized on a sensor chip and immunoprecipitated IKKα or IKKβ extracted from a nuclear fraction of cells treated with either sodium nitroprusside alone or sodium nitroprusside with BV or melittin was studied. The interactions between melittin (or BV) immobilized onto sensor chips and immunoprecipitated IKKα or IKKβ extracted from Raw 264.7 cells treated with a

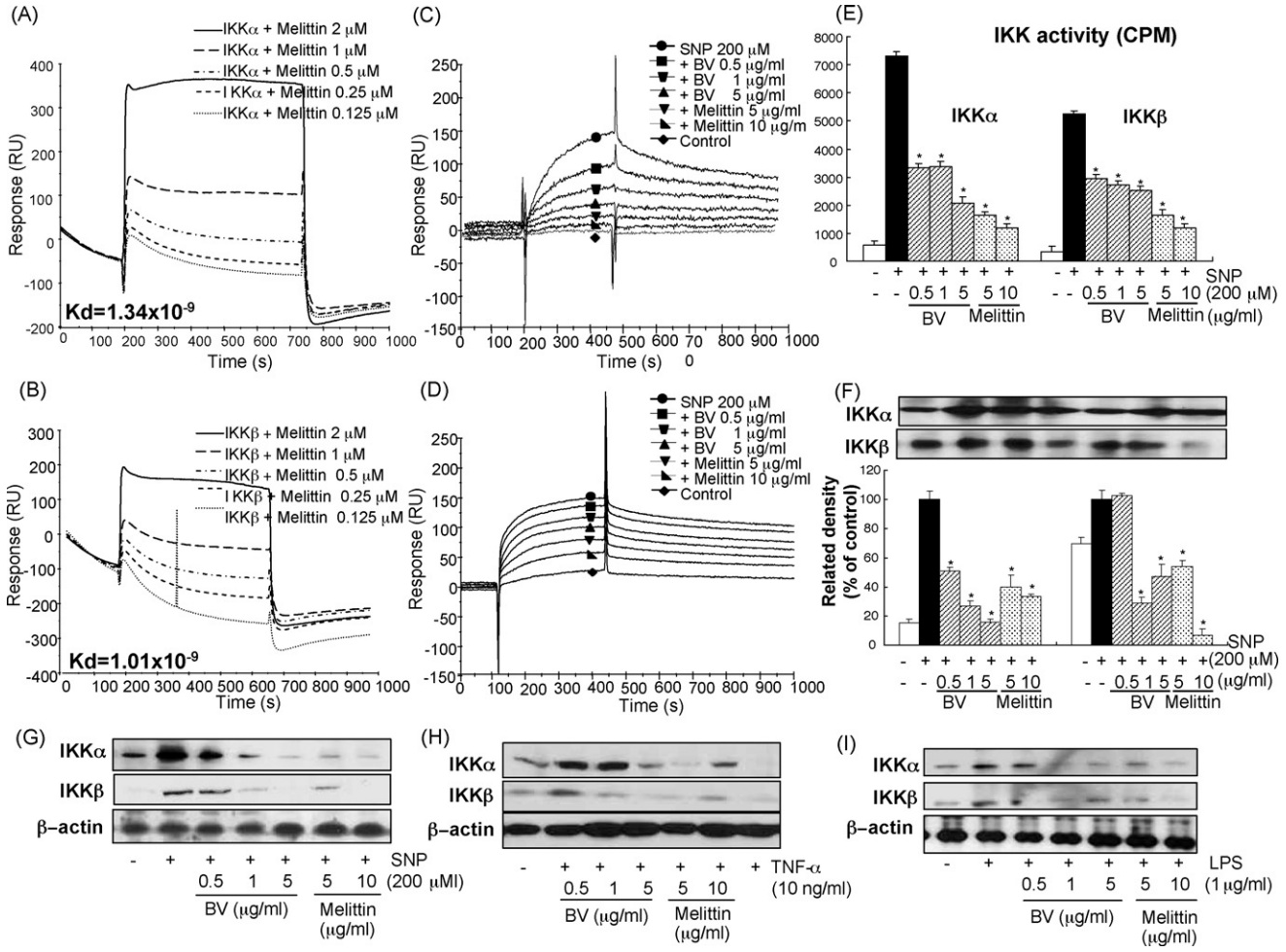

Fig. 1 – Melittin interacts with IKKs, and inhibits their activities. (A–D) Representative surface plasmon resonance binding kinetic trace. (A) Full kinetic dataset for the binding of melittin to IKKα immobilized on a sensor chip. Lines represent fits to a 1:1 binding model at the concentrations of melittin indicated. (B) Full kinetic dataset for the binding of melittin to immobilized IKKβ on a sensor chip. (C and D) Full kinetic dataset for the binding of melittin immobilized on a sensor chip and immunoprecipitated IKKα (C) and IKKβ (D) prepared from nuclear fraction of Raw 264.7 cells treated with sodium nitroprusside alone or with a combination of sodium nitroprusside and different doses of melittin or BV. Similar results were obtained from three different set of experiments. Raw 264.7 cells (E, F, H and I) or synoviocytes (G) were treated with 0.5–5 μg/ml BV or 5 or 10 μg/ml melittin in the presence of 200 μM sodium nitroprusside (E, F and G) or 10 ng/ml TNF-α (H) or 1 μg/ml LPS (I) at 37 °C for 24 h, and then cells were lysed. IKKs were immunoprecipitated with anti-IKKα or anti-IKKβ antibodies and used for in vitro kinase reactions with GST-IκBα, GST-IκBβ and [γ-^{32}P] ATP. GST-IκBα and GST-IκBβ phosphorylated by IKKs were determined by radioactivity (CPM) as IKKα and IKKβ activities (E) or visualized by SDS-PAGE followed with Western blotting (F, G, H and I). All values represent means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates significantly different from the sodium nitroprusside treated group.

combination of sodium nitroprusside and melittin or BV were lower than the corresponding interactions with immunoprecipitated IKKα and IKKβ extracted from cells treated with sodium nitroprusside alone, and these interactions were dose dependent (Fig. 1C and D). We also determined IKK activities to further investigate whether the interactions between melittin and IKKs can reduce the activities of IKKs. As expected, melittin dose dependently decreased sodium nitroprusside-induced IKKα and IKKβ activity as determined by biochemical assay (Fig. 1E) or by Western blotting (Fig. 1F) in Raw 264.7 cells, and in synoviocytes (Fig. 1G). These inhibitory effects of melittin or BV were also confirmed with physiological inflammatory stimuli (TNF-α or LPS)-treated in Raw 264.7 cells (Fig. 1H and I).

3.2. *Inhibitory effects of melittin on NF-κB-dependent luciferase and DNA binding activity*

We next investigated whether the binding of melittin to IKKs reduces NF-κB activity. Raw 264.7 and THP-1 cells transfected with a promoter reporter gene construct (a fusion gene containing SV40 promoter, five repeats of the consensus NF-

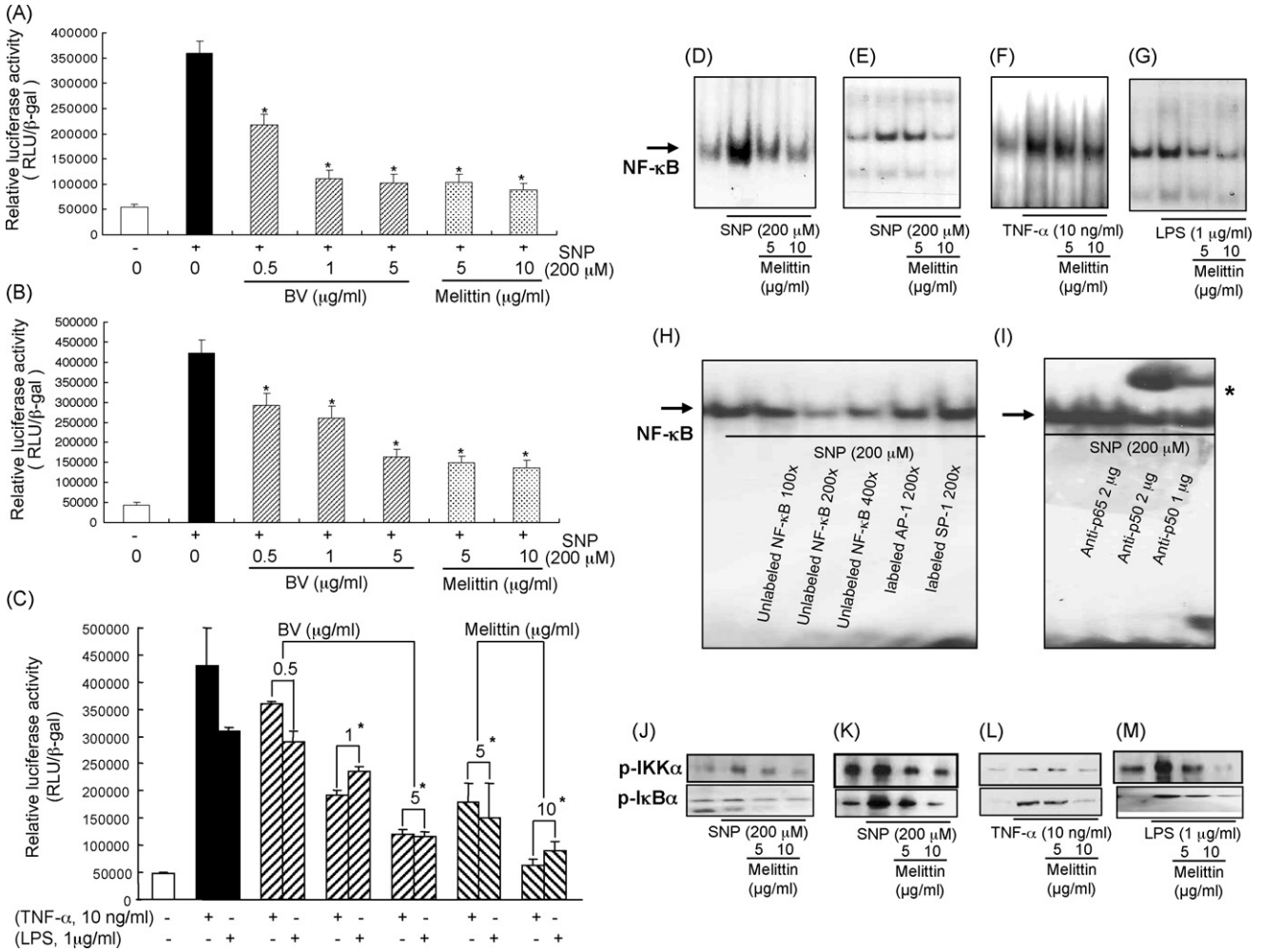

Fig. 2 – Effect of melittin and BV on NF-κB-dependent luciferase and DNA binding activities. Raw 264.7 cells (A and C) and THP-1 cells (B) were transfected with pNF-κB-Luc plasmid (5× NF-κB), and activated with sodium nitroprusside (200 μM), LPS (1 μg/ml) or TNF-α (10 ng/ml) in the absence or presence of 0.5–5 μg/ml BV or 5 or 10 μg/ml melittin for 2 h, and then luciferase activities were determined. All values represent means ± S.E. of three independent experiments performed in triplicate. *P < 0.05 indicates significantly different from the sodium nitroprusside or TNF-α or LPS treated groups. The DNA binding activation of NF-κB was investigated using EMSA. Nuclear extracts from Raw 264.7 cells (D, F and G) or synoviocytes (E) treated for 1 h with sodium nitroprusside (200 μM), TNF-κ (10 ng/ml) or LPS (1 μg/ml) with BV (0.5–5 μg/ml) or melittin (5 or 10 μg/ml) were incubated with ^{32}P-end-labeled oligonucleotide containing the κB sequence. H, For competition assays, nuclear extracts from cells treated with 200 μM sodium nitroprusside were incubated with unlabelled NF-κB oligonucleotide (100×, 200× or 400×) or with labeled SP-1 (200×) and AP-1 (200×) for 1 h before EMSA. I, For supershift assays, nuclear extracts from cells treated with 200 μM sodium nitroprusside were incubated with specific antibodies against the p50 and p65 NF-κB isoforms for 1 h before EMSA. (*) supershift by p50 antibody. (J–M) Effect of melittin on the nuclear translocation of the p50 subunit and the release of Iκ-B. Raw 264.7 cells (H and J) or synoviocytes (I and K) were treated with 5 μg/ml or 10 μg/ml melittin in the presence of sodium nitroprusside (200 μM) or TNF-α (10 ng/ml) or LPS (1 μg/ml) at 37 °C for 24 h. Eighty micrograms of cytosolic (p-IKKα and p-IκBα), or total protein extracted after treatment were used to determine of p-IκBα and p-KKα. Each panel is representative of three similar experiments.

κB binding sequence), and transcriptional activities were measured after stimulating the cells with sodium nitroprusside, LPS and TNF-α with or without melittin or BV. As shown in Fig. 2, co-treatment of transfected cells with melittin significantly inhibited the NF-κB luciferase activity induced by sodium nitroprusside in both cells (Fig. 2A and B). These inhibitory effects were also found in Raw 264.7 cells treated with TNF-α or LPS (Fig. 2C). Melittin also inhibited the NF-κB DNA binding activity induced by sodium nitroprusside in a dose dependent manner in Raw 264.7 cells (Fig. 2D) and in synoviocytes (Fig. 2E). Melittin also significantly reduced the NF-κB activation induced by TNF-α (Fig. 2F) and LPS (Fig. 2G) in Raw 264.7 cells. The specificity of DNA binding was examined by competition assay by adding an excessive amount of unlabeled/cold oligonucleotides to reaction mixtures containing Raw264.7 cell nuclear extract (Fig. 2H) or by supershift assay using antibodies for the p50 or p65 components of NF-κB (Fig. 2I).

One of the consequences of melittin binding to IKKs is the inhibition of the nuclear translocation of p50 and p65 through the blockage of IκB release. To study the translocation of subunits of NF-κB into the nucleus, we determined the appearance of the p50 and p65 subunits of NF-κB in the nucleus extracts. Melittin treatment dose dependently reduced the sodium nitroprusside-induced nuclear translocation of the p50 subunit, but their effects on p65 translocation were weak in Raw 264.7 cells and in synoviocytes (data not shown). Similar inhibitory effect of melittin on the p50 and p65 translocation was found in Raw 264.7 cells treated with LPS or TNF-α (data not shown). To elucidate the effects of melittin and BV on release of IκB, the kinetics of IκB release (IκBα phosphorylation and IKK activity (detected with phosphorylation of IKKα) in cytosol were studied by Western blot analysis. Sodium nitroprusside-induced IκBα and IKKα phosphorylation in Raw 264.7 cells (Fig. 2J) and synoviocytes (Fig. 2K) was reduced by melittin. As was observed in sodium nitroprusside-treated cells, melittin also inhibited IκBα and IKKα phosphorylation by TNF-α and LPS (Fig. 2L and M). By the inhibition of IκBα and IKKα phosphorylation, sodium nitroprusside-induced translocation of p50 into the nucleus of cells was significantly inhibited by melittin examined by confocal laser scaning microscopy (data not shown).

3.3. *Inhibitory effects of melittin on iNOS and COX-2 expression, and on NO and PGE_2 generation*

To investigate whether the inhibition of NF-κB activity suppresses inflammatory gene expression, iNOS and

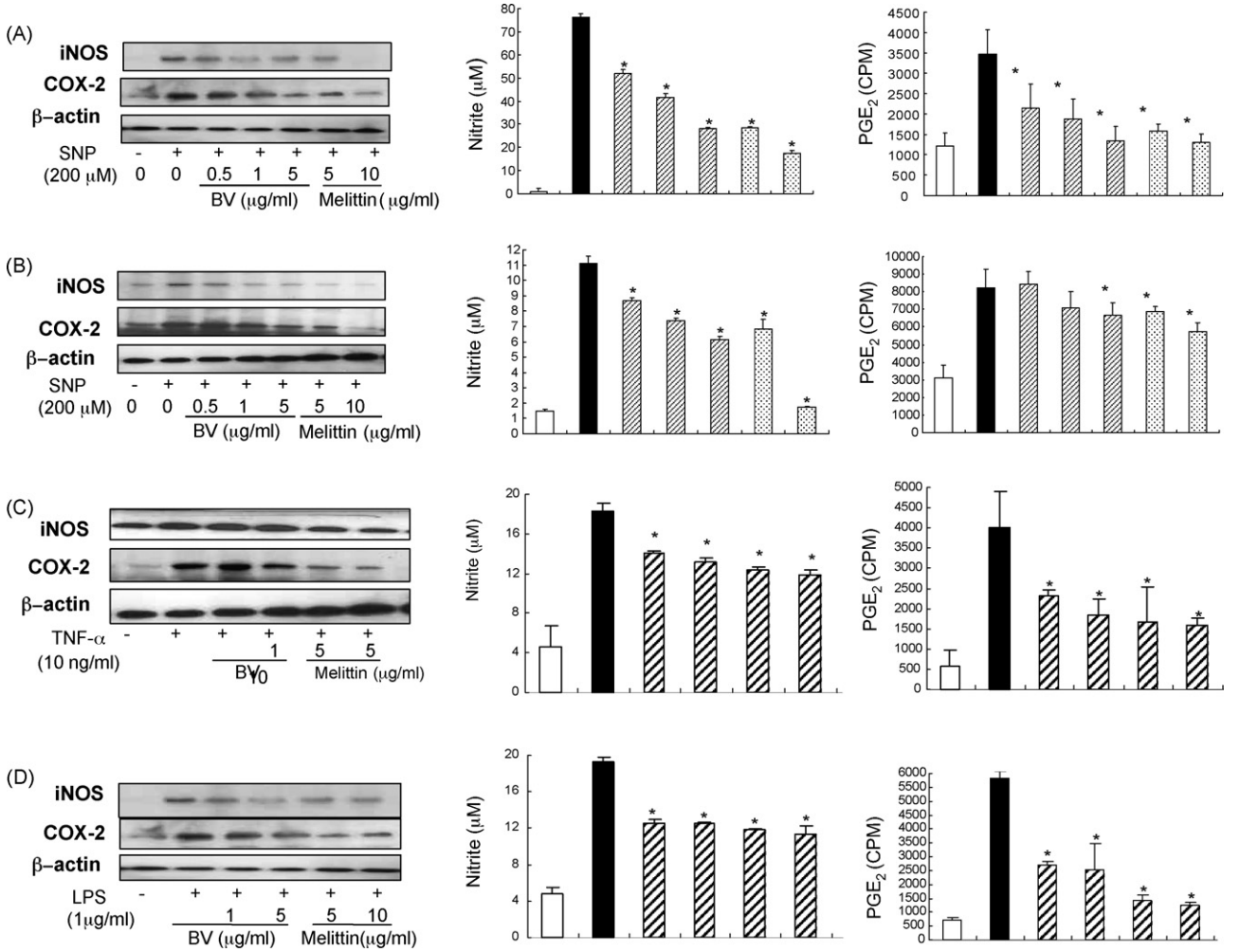

Fig. 3 – Effects of melittin and BV on inflammatory gene expression, and on NO and PGE_2 generation in Raw 264.7 cells and synoviocytes. (A, C and D) Raw 264.7 macrophages were treated with 0.5–5 μg/ml BV or 5 or 10 μg/ml melittin in the presence of sodium nitroprusside (200 μM) or TNF-α (10 ng/ml) or LPS (1 μg/ml) at 37 °C for 24 h. (B) Synoviocytes were treated with 0.5–5 μg/ml BV or 5 or 10 μg/ml melittin in the presence of 200 μM sodium nitroprusside at 37 °C for 24 h. Equal amounts of total proteins (80 μg/lane) were subjected to 10% SDS ± PAGE, and the expressions of iNOS, COX-2 and β-actin were detected by Western blotting using specific antibodies. Each panel representative of three independent experiments. The amounts of NO and PGE_2 in the medium of cultured Raw264.7 cells (A, C and D) or synoviocytes (B) treated with sodium nitroprusside (200 μM) or TNF-α (10 ng/ml) or LPS (1 μg/ml) with/without 0.5–5 μg/ml BV or 5 or 10 μg/ml melittin at 37 °C for 24 h were measured. Results are expressed as means ± S.E. of three independent experiments performed in triplicate. *$P < 0.05$ indicates significantly different from the sodium nitroprusside or TNF-α or LPS treated group.

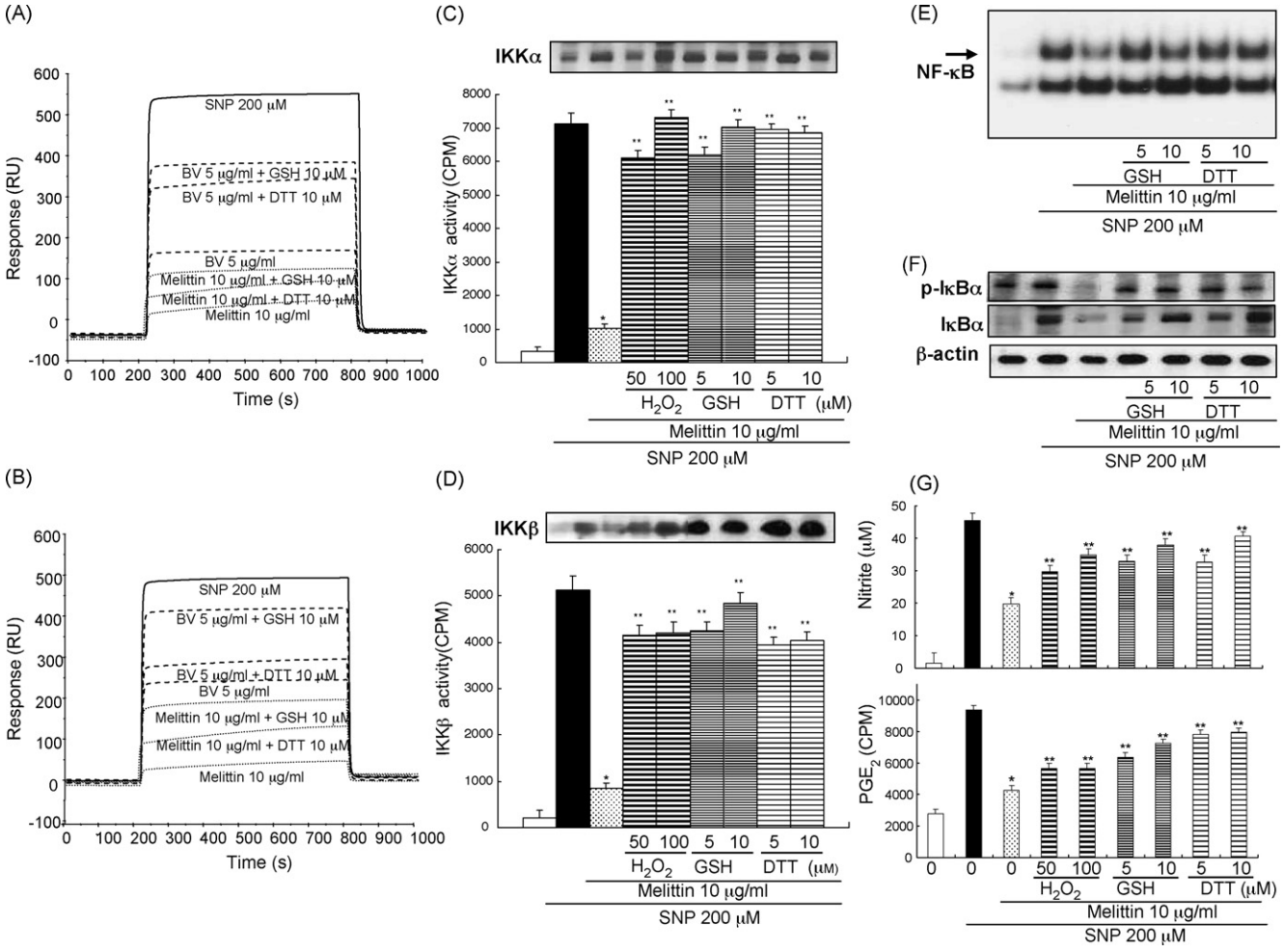

Fig. 4 – Abolition of the inhibitory effect of melittin or BV by DTT, GSH and H_2O_2. (A and B) Full kinetic dataset for the binding between melittin immobilized on a sensor chip and immunoprecipitated IKKα (A) and IKKβ (B) prepared from nuclear fraction of Raw 264.7 cells treated with sodium nitroprusside alone or with a combination of sodium nitroprusside and melittin or BV in the presence of DTT or GSH. Similar results were obtained from three different set of experiments. C and D, Raw 264.7 macrophages were treated with 0.5–5 μg/ml BV or 5 or 10 μg/ml of melittin in the presence of 200 μM sodium nitroprusside with/without DTT or GSH or H_2O_2 at 37 °C for 24 h. IKKs were immunoprecipitated with anti-IKKα or anti-IKKβ antibodies and used for in vitro kinase reactions with GST-IκBα, GST-IκBβ and [γ-^{32}P] ATP. GST-IκBα and GST-IκBβ phosphorylated by IKKs were determined by radioactivity (CPM) as IKKα and IKKβ activities or visualized by SDS-PAGE followed with Western blotting (C and D). All values represent means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates significantly different from the sodium nitroprusside treated group. $^{**}P < 0.05$ indicates significantly different from the sodium nitroprusside with melittin treated group. (E) NF-κB DNA binding activity (EMSA) in Raw 264.7 cells treated with 10 μg/ml melittin in the presence of 200 μM sodium nitroprusside with/without DTT or GSH at 37 °C for 1 h. (F) Release of IκBα (phosphorylation of IκBα) Similar results were obtained from three independent experiments performed in triplicate. (G) The amounts of NO and PGE_2 in the medium of cultured Raw264.7 cells treated with 10 μg/ml melittin in the presence of 200 μM sodium nitroprusside with/without DTT or GSH at 37 °C for 24 h were measured. Results are expressed as means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates significantly different from the sodium nitroprusside treated group. $^{**}P < 0.05$ indicates significantly different from the sodium nitroprusside with melittin treated group.

COX-2 expressions were determined. Correlated well with the inhibitory effect of melitin on NF-κB activity, sodium nitroprusside-induced iNOS and COX-2 protein expression in Raw 264.7 cells (Fig. 3A) and in synoviocytes (Fig. 4B) was dose dependently inhibited by melittin or BV. Similarly in sodium nitroprusside-treated cells, melittin and BV also inhibited the expressions of iNOS and COX-2 in Raw 264.7 cells induced by TNF-α (Fig. 4C) and by LPS (Fig. 3D). The effect of melittin or BV on inflammatory mediator generation was then examined. Significant dose-dependent inhibition of NO and PGE_2 generation was also observed in Raw 264.7 cells (Fig. 3A, right two panels) and synoviocytes

(Fig. 3B, right two panels) treated with melittin or BV in combination with sodium nitroprusside. Treatment with TNF-α on NO and PGE_2 generation (Fig. 3C, right two panels), or treatment of LPS on NO and PGE_2 generation (Fig. 3D, right two panels) was also inhibited by melittin and BV.

3.4. *Abolition of the melittin effects by DTT and GSH, and in IKKα and IKKβ mutant cells*

To further demonstrate the sulfhydryl group of IKKα and IKKβ is a target of melittin as in p50 [12], the interactions between melittin and immunoprecipitated IKKα and IKKβ extracted

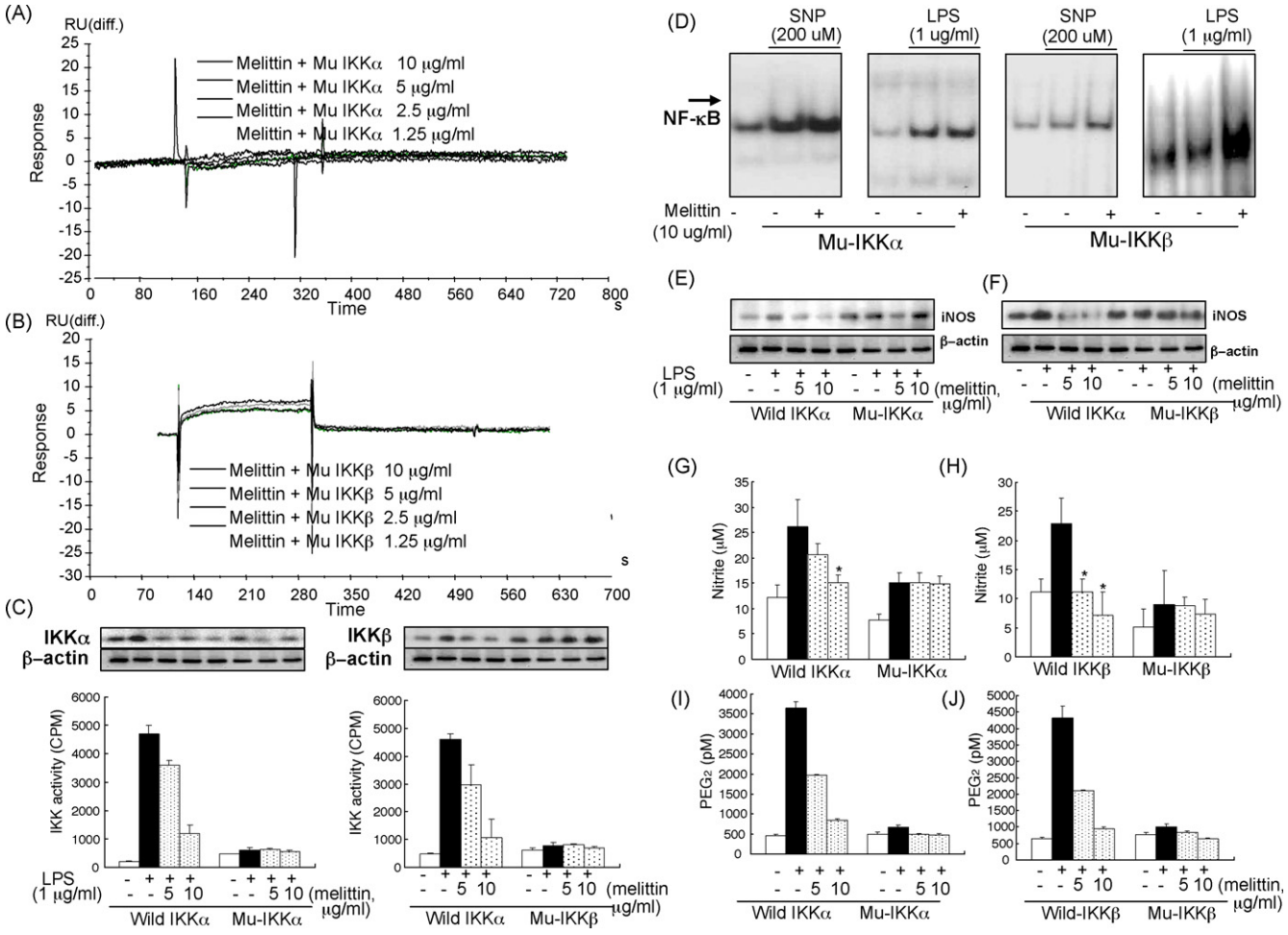

Fig. 5 – Abolition of the inhibitory effect of melittin in the cells harboring mutant IKKα or IKKβ. (A and B) Full kinetic datasets for the binding of melittin to mutant IKKs prepared by peptide synthesis (Bioneer, Cheongju, Korea). Similar results were obtained from three different sets of experiments. (C) Raw 264. 7 cells were transiently transfected with wild or mutant types of IKKs for 24 h, and then the cells were treated with LPS (1 μg/ml) in the absence or presence of melittin for 24 h to determine IKK activity or Western blotting or NO and PGE_2 assay, or for 1 h to determine NF-κB activity (EMSA). (C) IKKs were immunoprecipitated with anti-IKKα or anti-IKKβ antibodies and used for in vitro kinase reactions with GST-IκBα, GST-IκBβ and [γ-^{32}P] ATP. GST-IκBα and GST-IκBβ phosphorylated by IKKs were determined by radioactivity (CPM) as IKKα and IKKβ activities or visualized by SDS-PAGE followed with Western blotting. All values represent means ± S.E. of three independent experiments performed in triplicate. (D) The DNA binding activation of NF-κB was investigated using EMSA. Nuclear extracts from transfected Raw 264.7 cells treated for 1 h with sodium nitroprusside (200 μM) or LPS (1 μg/ml) with melittin (10 μg/ml) were incubated with ^{32}P-end-labeled oligonucleotide containing the κB sequence. (E–F) Effect of melittin on the expression of iNOS. Transfected Raw 264.7 cells were treated with 10 μg/ml melittin in the presence of LPS (1 μg/ml) at 37 °C for 24 h. Eighty micrograms of cytosolic or total protein extracted after treatments were used to determine of iNOS; β-actin protein was used as an internal control. Each panel is representative of three similar experiments. (G–J) Amounts of NO (G and H) and PGE_2 (I and J) in medium were measured in the Raw 264.7 cells transiently transfected with wild or mutant IKKs after treatment with LPS (1 μg/ml) with or without melittin (5 or 10 μg/ml) for 24 h. Results are expressed as means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates significantly different from the sodium nitroprusside or LPS treated group.

from cells treated with sodium nitroprusside with/without melittin in the presence of the reducing agents DTT or GSH was studied. As shown in Fig. 4A, the interactions between melittin and immunoprecipitated IKKα and IKKβ extracted from cells treated with sodium nitroprusside and melittin in the presence of the reducing agents DTT or GSH was higher than that in the Raw 264.7 cells and synoviocytes treated without DDT or GSH (Fig. 4A and B). These effects of DTT or GSH on the binding affinity were consistent with the effects of DTT and GSH on IKKα and IKKβ activity as determined by Western blotting and by biochemical analysis (Fig. 4C and D) induced by sodium nitroprusside in Raw 264.7 cells. Since redox status is critical for sulfhydryl group modification in the cysteine residues of IKKs, and we also found that melittin reduces sodium nitroprusside-induced H_2O_2 generation (data not shown), we therefore examined whether the inhibitory effect of melittin on sodium nitroprusside-induced IKK activity was partially involved with the scavenging of H_2O_2 by melittin. The addition of H_2O_2 dose dependently blocked the inhibitory effect of melittin on IKKα and IKKβ activity (Fig. 4C and D). In addition, the inhibitory effect of melittin on the DNA binding activity of NF-κB was also abrogated by adding DTT or GSH in a reaction mixture containing a nuclear extract of Raw 264.7 cells (Fig. 5E). DTT or GSH also dose dependently prevented the melittin-induced prevention of IκBα release (determined with phosphorylation of IκBα) induced by sodium nitroprusside in Raw 264.7 cells (Fig. 5F). A similar reducing effect was observed in TNF-α or LPS treated Raw 264.7 cells (data not shown). Proportional to abrogation of the activities of IKKs, the reverse effects of DTT, GSH and of H_2O_2 on the sodium nitroprusside-induced release of NO and PGE_2 were also found in Raw 264.7 cells (Fig. 4G) and synoviocytes (data not shown).

The interaction between melittin and mutant IKKα and mutant IKKβ was next analyzed with mutant IKKs (cysteine was replaced with alanine) prepared by synthesized peptide. The interaction between melittin and mutant IKKα (Fig. 5A), and mutant IKKβ (Fig. 5B) was significantly reduced compared with interaction between melittin and IKKs (see Fig. 1). We also conducted a transient transfection assay using a fusion gene containing pCMV promoter and IKK mutants. Raw 264.7 cells were transfected with these promoter reporter genes constructs, and then determined IKKs activity by Western blotting and by biochemical assay. Consistent with the lower binding affinity, IKKs were not elevated by LPS treatment, and the inhibitory effect of melittin was not observed (Fig. 5C). This abolishment of the inhibitory effects of melittin on NF-κB activity was also found after treating Raw 264.7 cells transfected with mutant IKKα and IKKβ (Fig. 5D) by treatment with sodium nitroprusside or LPS. Expression of iNOS, and generation of NO and PGE_2 were then measured after stimulating the cells for 24 h with LPS with/without melittin in cell supernatant. In contrast to significant inhibition of iNOS expression, and NO and PGE_2 generation in cells transfected with wild type of IKKS, melittin did not inhibit LPS-induced iNOS expression (Fig. 5E and F) in Raw 264.7 transfected with IKKα (C178A) or IKKβ (C179A) mutants. NO (Fig. 5G and H) and PGE_2 (Fig. 5I and J) generation in Raw 264.7 transfected with IKKα (C178A) or IKKβ (C179A) mutants.

4. Discussion

In the present study, we found that melittin interacts with IKKα and IKKβ high affinity. The binding affinities of melittin to IKKα and IKKβ were 1.34×10^{-9} M and 1.01×10^{-9} M, respectively. Moreover, these protein–protein interactions were associated with the suppressions of the activities of IKKα and IKKβ, the blockade of inflammatory stimulus-induced IκB release, and the inhibition of NF-κB activity in Raw 264.7 macrophages and synoviocytes. Consistent with the inhibitions of the activities of IKKα, IKKβ, and NF-κB, the generation of the inflammatory mediators NO and PGE_2 and the expressions of iNOS and COX-2 were also suppressed. By using reducing agents and transfection assays with plasmids containing mutant forms of IKKα and IKKβ whereby cysteine residues were replaced by other amino acids), we found that melittin probably interacts with the cysteine residues of active cites in IKKs.

The promoter region of the murine gene encoding iNOS and COX-2 contains NF-κB binding sites [5,25], which suggests that the inhibitory effect of inflammatory gene expression is related with the inhibition of the DNA binding activity of NF-κB. The binding affinities of melittin to IKKα and IKKβ were 1.34×10^{-9} M and 1.01×10^{-9} M, respectively, and these binding affinities of melittin with IKKs are much stronger than normal binding affinities between proteins and their receptors under physiological conditions. For example, the estrogen receptor-ligand interaction has a binding affinity about $K_d = 10^{-2}$ to 10^{-3} M [26], and the binding affinity of major histocompatibility complex (MHC)-peptide complex to T cell receptors (TCRs) is about $K_d = 1 \times 10^{-7}$ M [27]. In addition, the interactions between melittin and immunoprecipitated IKKα and IKKβ extracted from a combination of inflammatory stimulant and melittin treated cells, were reduced much more than its interaction with immunoprecipitated IKKα and IKKβ extracted from cells treated with stimulant alone. Therefore, this strong protein–protein interaction is likely to modify the activities of IKKα and IKKβ, and thereby inhibit IκBα and IκBβ release and reduce the DNA binding activity of NF-κB. The binding affinity of melittin to IKKs is much stronger (10-fold) than that to p50 of NF-κB ($K_d = 1.2 \times 10^{-8}$ M) as described in previous report [12]. Thus, the inhibitory effect of melittin or BV on IKKα and IKKβ activities may be more critical for the anti-inflmmatory effect of by melittin or BV than NF-κB itself.

Structural data on IKK subunits reveals that cysteine residues are present in the kinase domains of IKKα and IKKβ, and that some are located at functionally important sites (Cys-179), such as at the activating T loop and at the catalytic site [8,13]. We found that the addition of the thiol reducing agents; DTT and GSH as well as H_2O_2 abrogated the melittin-induced inhibition of the activities of IKKs. Furthermore, in the presence of H_2O_2, this inhibitory effect was absent as similar to the findings by Jeon et al. [6]. Therefore, a redox reaction could underlie the binding of melittin and IKKs. This notion was further reinforced by the observations that the inhibitory

effect of melittin on the activities of IKKα and IKKβ was abolished in cells transfected with IKKα or IKKβ mutant plasmid where cysteine residues were replaced by alanine. This resistance of cells transfected with IKKα or IKKβ mutants to the inhibitory effect of melittin or BV on the activities of IKKα and IKKβ was associated with the abolishment of the inhibitory effects of melittin or BV on NO and PGE_2 generation. Moreover, the interaction between melittin and mutant IKKs was significantly lowered. These findings suggest that melittin may bind sulfhydryl groups on IKKα and IKKβ, and thereby hinder the activities of IKKα and IKKβ which blocks IκB release. A similar thiol redox mechanism has been shown to be involved in the thiol-reactive metal-induced inhibition of IKKs. Arsenite, a potent inhibitor of NF-κB activation was shown to interact with a specific cysteine residue (Cys-179) of IKK catalytic subunits [21], and this interaction was inhibited by thiol-modifying agents [6]. Other modifications of the activations of IKKα and IKKβ have been reported. That is, peroxisome proliferators activated receptor gamma agonist 15-deoxy-delta12,14-prostaglandin J_2 (15-deoxy PGJ_2) inhibited IκBα degradation via direct covalent modification of cysteine 179 of IKKβ via a Michael-type reaction [28]. Although we did not map precisely how or which residue(s) of these IKKs bind to melittin, and verse, it is likely that melittin could bind to cysteine residues in IKKα and IKKβ through redox mechanism since these are not exist any amino acid residue which could bind covalently. Even though the exact binding mechanism is not clear, but it is noteworthy that melittin modifies phospholipase A_2 activity through peptide–enzyme complex formation [29,30].

The induction of COX-2 was found to result in enhanced prostaglandin formation, and to contribute to the pathophysiology of local and chronic inflammation [31], and selective inhibitors of COX-2 were found to exert potent anti-inflammatory effects [32]. In inflammatory arthritis, NO also behave as a pro-inflammatory and causes tissue injury [33,34]. NO can modulate COX-2 expression [35], and react with reactive oxygen species to form another highly reactive intermediate, peroxinitrate, which destroys tissue [36]. Therefore, the therapeutic potential of the combined inhibition of prostanoid and nitric oxide synthesis systems via the inhibition of IKK activity has been recognized as an effective anti-inflammatory treatment strategy against the progression of arthritis [37]. The inhibition of NO and PGE_2 generation by melittin was observed at 5–10 μg/ml (1.79–3.57 μM) in both Raw 264.7 cells and synoviocytes. The dose of melittin (or BV) used was the same as that used in a previous study [12], where melittin and BV inhibited LPS-induced NF-κB activity and the generation of inflammatory mediators. Thus, the target disruption of IKKs could also (or greater than NF-κB itself) contributes to the anti-inflammatory effect of melittin or BV. The present data with previous data show that BV (melittin) inhibits NF-κB activity in several inflammatory cells such as Raw 264.7 cells, THP-1 cells, a monocyte cell line as well as synoviocytes from RA patients, suggesting that the BV (melittin) may be also useful for the treatment of other inflammatory diseases. The present study also demonstrates that in addition to the target disrupting effect of melittin or BV on the p50 of NF-κB, the interfering effects of melittin or BV on the IKKs, upstream of NF-κB regulatory targets through protein–protein reaction may be also significant aspects of its anti-inflammatory effects.

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