원 저

The Apoptotic Effect of Bee Venom and Melittin on FBS-induced Vascular Smooth Muscle Cells Proliferation

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국문초록

봉약침액과 melittin의 세포고사 효과가 FBS에 의하여 유도되는 혈관 평활근 세포 증식에 미치는 영향

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목적 : 이 연구에서는 FBS에 의하여 유도되는 혈관 평활근 세포 증식에 대한 봉약침액과 Melittin의 세포 고사효과의 영향 및 작용 기전을 살펴보고자 하였다.

방법 : IκBα, p-IκBα, p-ERK1/2, p-Akt, p53, Bcl-2, Bax 및 active caspase-3는 Western blotting을, NF-κB는 EMSA와 immunofluorescence staining을 이용하여 측정하였다.

결과 : 1. Melittin은 NF-κB 활성에 대하여 IκBα의 인산화를 유의하게 억제하고 IκBα를 증가시켰으며, NF-κB의 DNA 결합과 NF-κB p50의 핵 내 유입을 유의하게 감소시켰다.

2. Melittin은 NF-κB 활성을 증가시키는 물질인 Akt의 인산화를 유의하게 억제하였고, ERK1/2의 인산화도 억제하였다.

3. Melittin은 세포사멸 전구 단백질인 p53, Bax 및 caspase-3의 발현을 유의하게 증가시켰고, 세포사멸 억제 단백질인 Bcl-2의 발현은 감소시켰다.

결론 : 이상의 결과는 NF-κB 와 Akt 활성을 억제함으로써 혈관평활근세포 증식을 억제하는 효과가 있음을 입증한 것이며, 향후 안전성 연구를 바탕으로 혈관성형술 후 재발성협착증과 동맥경화증의 치료제로 사용될 수 있을 것으로 기대된다.

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Ⅰ. Introduction

The increased potential for growth of vascular smooth muscle cells (VSMCs) is a key abnormality in the development of atherosclerosis lesions and post angioplasty restenosis[1]. Thus, inhibition of VSMCs proliferation represents a potentially important therapeutic strategy for the treatment of disease such as atherosclerosis and restenosis[2]. It is well known that in response to a variety of stimuli, including many growth factors such as platelet derived growth factor (PDGF) or Fetal Bovine Serum (FBS), VSMCs can initiate highly conserved signaling events, which lead to either cell migration or proliferation[2]. However, the proliferative potential can be regulated by induction VSMCs apoptosis[3].

Apoptosis(programmed cell death), which plays a critical role in both the normal development and pathology of a wide variety of tissues, is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation[4]. In recent years, apoptosis has been implicated in atherosclerosis, and numerous recent investigations on the development and morphology of atherosclerotic lesions have shown apoptosis to be an important factor in atherogenesis[5-8]. It has become more evident that the balance between changes in regulation of cell growth and cell death is an important determinant of vascular integrity and lesion formation[9]. Although the regulation of apoptosis in the vessel wall is complex and likely to consist of multiple interacting pathways within atherosclerotic plaques, the regulation of apoptosis has attracted much attention as a possible means of eliminating excessively proliferating VSMCs[10,11]. After vessel injury, diverse signaling mechanisms become activated in VSMCs, leading to neointimal hyperplasia. Therefore, it is suggested that VSMCs apoptosis is beneficial in that it offers protection to the walls of arteries against proliferative restenosis induced by arterial injury including arterial balloon angioplasty or stent implantation[8,12-16].

Bee venom (BV) is known to be a very complex mixture of active peptides, including melittin (a major component of BV), phospholipase A_2, apamin, adolapin, and mast cell degranulating peptide[17]. Many studies on the biological and pharmacological activities of BV have been carried out. The anti-inflammatory and anti-rheumatoid arthritis effect[17], relief of pain[18], and immune modulatory activity[19] of BV have been described. BV and melittin also have been reported to induce apoptosis in several cancer cells and rheumatoid arthritis synovial fibroblasts *in vitro* and *in vivo*[20-23]. It was also reported that target inactivation of nuclear factor-kappa B (NF-κB) by directly binding to the p50 subunit is an important mechanism of the anti-arthritic effect of BV[24], and that NF-κB is an important regulator of gene expression in cell proliferation and apoptosis[25], which is considered as a potential therapeutic target in atherosclerosis and restenosis[9,26-28].

In this study, We therefore investigated the NF-κB-associated apoptosis signal pathway as possible anti-proliferative mechanisms in cultured rat aortic VSMCs.

Ⅱ. Materials and Methods

1. Chemicals

Dried BV was purchased from You-Miel Bee Venom Ltd. (Hwasoon, Jeonnam, Korea). The composition of the BV was as follow: 45~50% melittin, 2.5~3% apamin, 2~3% MCD peptide, 12% PLA_2, 1% lyso-PLA, 1~1.5% histidine, 4~5% 6pp lipids, 0.5% secarpin, 0.1% tertiapin, 0.1% procamine, 1.5~2% hyaluronidase, 2~3% amine, 4~5% carbohydrate, and 19~27% other, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephirne, and unknown amino acids, with >99.5% purity. Melittin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). FBS was obtained from Upstate Biotechnology (Lake Placid, NY, USA). ERK1/2, phospho-ERK1/2, Akt, phospho-Akt and active-caspase-3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). IκBα, phospho-IκBα, NF-κB p50, p53, Bcl-2, Bax and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [^{3}H]thymidine was from Amersham Pharmacia Biotech (Buckinghamshire, UK). The cell culture materials were obtained from Gibco-BRL (Rockville, MD, USA), and other chemical reagents were from Sigma Chemical Co.

2. Cell culture

Rat aortic VSMCs were isolated by enzymatic dispersion as previously described[23]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 g/ml streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. The purity of VSMCs culture was confirmed by immunocytochemical localization of smooth muscle actin.

3. Western blot analysis

Western blot analysis was performed as previously described[24]. VSMCs were harvested and homogenized lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM phenylmethylsufonyl fluoride, 10 μM aprotinin, 1% igapel 630 (Sigma Chemical Co.), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate]. The cell extracts were centrifuged at 23,000×g for 10 min. Equal amount of proteins (30 μg) were separated on a SDS polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc.). Blots were blocked for 2 h 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 washed and incubated with phospho-p44/42 MAP Kinase (ERK1/2), phospho-Akt, IκBα, phospho-IκBα, p53, Bcl-2, Bax, and active-caspase-3 antibodies at 1:500 dilution in BSA/TTBS-T buffer for over night at 4°C and horseradish peroxidase-conjugated IgG secondary antibody (Santa Cruz Biotechnology Inc.) at 4 °C over 3 h. 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, CA, USA).

4. Electrophoretic mobility shift analysis

DNA binding activity of NF-κB was determined using an electrophoretic mobility shift assay (EMSA). Gel shift assay was performed according to the manufacturer's recommendations (Promega, Madison, WI, USA). Briefly, cells were cultured in DMEM with 10% FBS at 37 °C for 24 h, then cultured with serum-free medium containing melittin (0.4~0.8 μg/ml) or vehicle. Twenty-four hours later, the cells were stimulated by 5% FBS for 1 h, and then washed twice with PBS followed by the addition of 1 ml of PBS. The cells were scraped into cold Eppendorf tube, and centrifuged 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 [DTT], 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet p40) 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^{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 counts per minute) of ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. Subsequently, 1 μl of gel-loading buffer was added to each reaction and placed on 4% nondenaturing gels 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 -80 °C.

5. Immunofluorescence staining

VSMCs were plated in chambered tissue culture slides at a density of 2×10^3 cells/well in DMEM. The cells were then cultured with serum-free medium containing melittin (0.8 μg/ml) or vehicle. 24 hours later, the cells were stimulated by 5% FBS for 1 h, and then 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 1 h. The cells were then exposed to primary goat polyclonal antibody for p50 (1:100 dilution) overnight at 4°C. After washes with ice-cold PBS followed by treatment with an anti-goat biotinylated secondary antibody Alexa Fluor 633 (Molecular Probes Inc., Eugene, OR, USA), 1:200 dilution, for 4 h at room temperature. Nuclear stain and mount in antifade medium with DAPI (Vector Laboratory Inc.), immunofluorescence images were acquired using a confocal laser scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany) equipped with a 630×oil immersion objective.

6. 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 $P<0.05$ and $P<0.01$.

Ⅲ. Results

1. Melittin suppresses the NF-κB activation

It has been well established that NF-κB activity is regulated by IκB proteins, and the phosphorylation and degradation of IκB result in the activation of NF-κB. The exposure of quiescent cells to 5% FBS for 30 and 60 min stimulated a profound increase in IκBα phosphorylation and subsequent degradation, respectively. Melittin (0.4~0.8 μg/ml) strongly inhibited the FBS-induced IκBα phosphorylation (Fig. 1) and degradation (Fig. 2).

To further investigation, FBS-stimulated VSMCs nuclear extract was prepared and assayed NF-κB DNA binding by EMSA. Cells were stimulated with 5% FBS for 60 min which it is the time to activate NF-κB maximally (data not shown). FBS-induced strong NF-κB DNA binding activity was attenuated by melittin in a dose-dependent manner (Fig. 3).

Nuclear translocation of the p50 and p65 subunit is also involved in activation of NF-κB. To study the translocation of subunits of NF-κB into the nucleus during NF-κB activation, I determined the appearance of the p50 subunits of

NF-κB in the nucleus. FBS stimulation for 60 min increased NF-κB p50 translocation to the nucleus of the VSMCs. Melittin (0.8 μg/ml) strongly attenuated this response in FBS-stimulated cells (Fig. 4, 5).

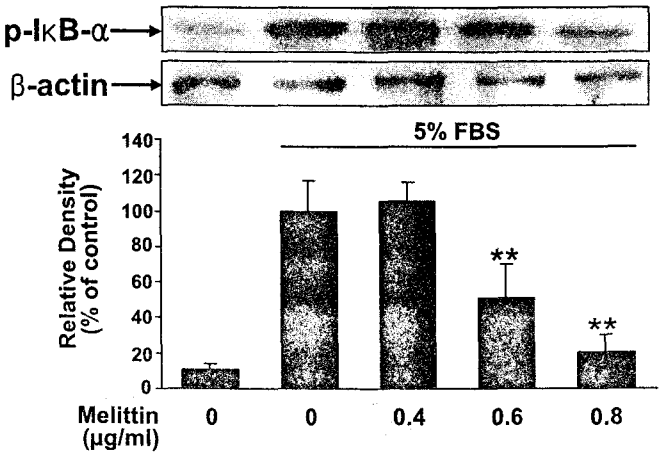

Fig. 1. The Effects of Melittin on Phosphorylation of IκBα in VSMCs
For examinations of IκBα phosphorylation, Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then 5% FBS was added to the cells for another 30 min. After the above treatment, cells were harvested and Western blot analysis was performed. Density of immunoblotting bands were measured as described under Materials and Methods.

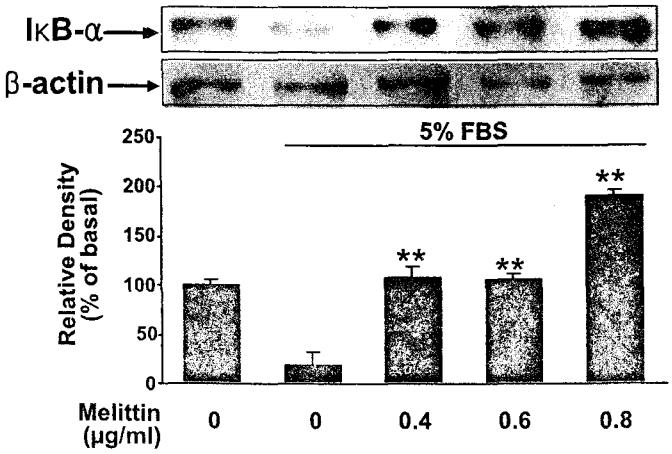

Fig. 2. The Effects of Mlittin on Degradation of IκBα in VSMCs
For examinations of IκBα degradation, Cells were pre-treated with 0.4~0.8 μg/ml Mlittin for 24 h, and then 5% FBS was added to the cells for another 60 min. After the above treatment, cells were harvested and Western blot analysis was performed. Density of immunoblotting bands were measured as described under Materials and Methods.

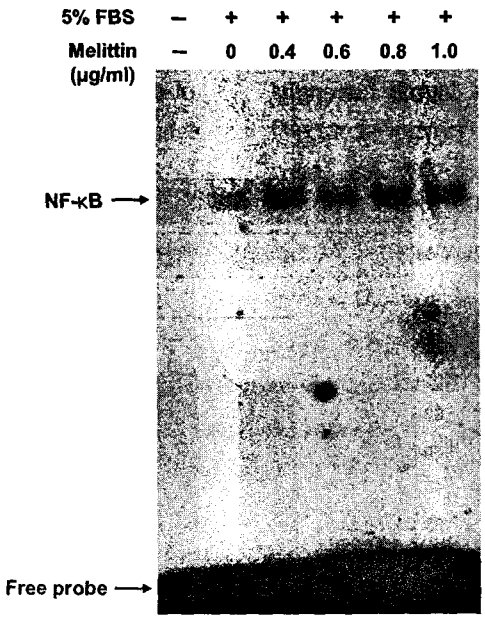

Fig. 3. The Effects of Melittin on NF-κB DNA Binding Activity in VSMCs
For examination of NF-κB DNA-binding assay, Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then stimulated with 5% FBS for 1 h. Nuclear extracts were subjected to NF-κB DNA binding assay by EMSA. Similar results were obtained in three independent experiments. Density of immunoblotting bands were measured as described under Materials and Methods.

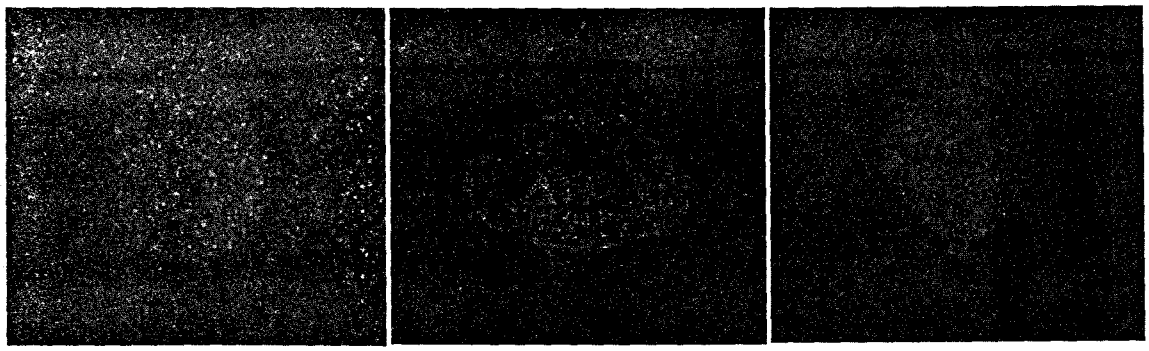

Fig. 4. The Effects of Melittin on NF-κB nuclear Translocation in VSMCs
The translocation of NF-κB p50 subunit was determined by immunofluorescence confocal laser scanning microscopy. In unstimulated cells, p50 was localized in the cytoplasm. After stimulation with 5% FBS, p50 was translocated into the nuclear in almost of the cells. Melittin (0.8 μg/ml) strongly attenuated this response. Similar results were obtained in three independent experiments.

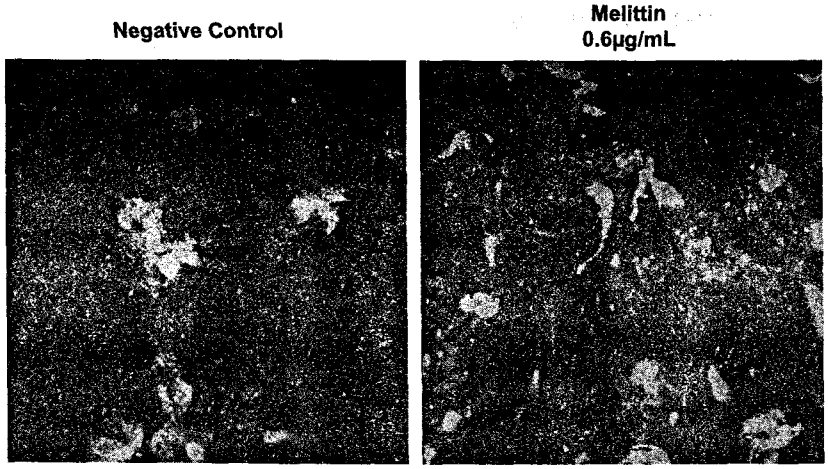

Fig. 5. The Uptake of Melittin into VSMCs
Uptake of Melittin into the membrane and nuclear of VSMCs (original magnification × 360). Negative control cells were treated Alexa Fluor 488 alone, Positive cells were treated with Melittin labeled with Alexa Fluor 488

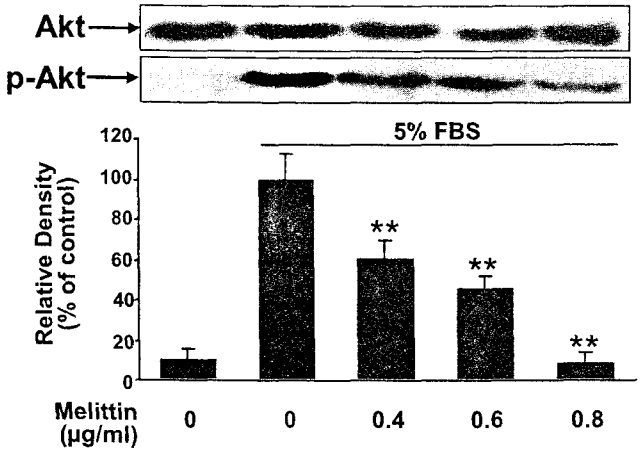

Fig. 6. The Effect of Melittin on Expression of Akt in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then stimulated with 5% FBS. Equal amounts of whole cell lysate (30 μg) were subjected to electrophoresis and analysis by Western blot for total and phosphorylated Akt. Density of immunoblotting bands of phosphorylted Akt were measured as described under Materials and Methods.

2. Melittin inhibits Akt and ERK1/2 activation

To investigate the mechanisms of the anti-proliferative and pro-apoptotic effects exerted by melittin, I examined whether melittin could reduce the FBS-induced phosphorylation of Akt and ERK1/2. Pre-treatment of 0.4, 0.6 and 0.8 μg/ml melittin significantly inhibited the FBS-induced phosphorylation of Akt in a concentration-dependent manner. The percentages of the control significantly decreased by melittin 0.4, 0.6 and 0.8 μg/ml were 60.5 ± 9.6, 45.1 ± 6.9 and 8.7 ± 5.6% (Fig. 6), respectively.

Melittin also inhibited 5% FBS-induced phosphorylation of ERK1/2 in a concentration- dependent manner, though it is not potently like in Akt phosphorylation. The percentages of the control decreased by melittin 0.4, 0.6 and 0.8 μg/ml were 69.3 ± 16.3, 61.1 ± 9.3 and 38.1 ± 10.3% (Fig. 7), respectively.

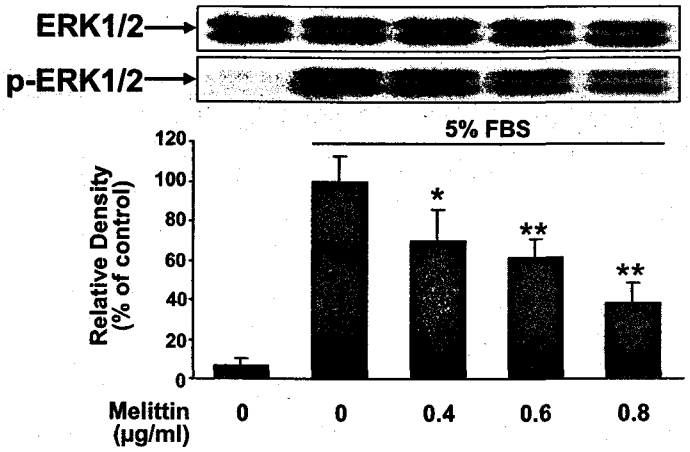

Fig. 7. The Effect of Melittin on Expression of ERK1/2 in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then stimulated with 5% FBS. Equal amounts of whole cell lysate (30 μg) were subjected to electrophoresis and analysis by Western blot for total and phosphorylated ERK1/2. Density of immunoblotting bands of phosphorylted EKR1/2 were measured as described under Materials and Methods.

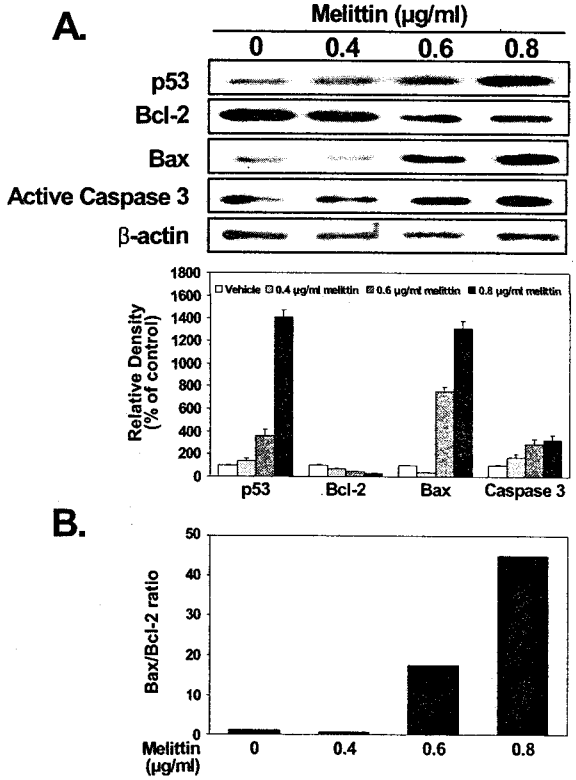

Fig. 8. The Effect of Melittin on Apoptosis regulatory Protein Expression
Cells were incubated for 24 h with or without 0.4~0.8 μg/ml Melittin, and the expression of p53, Bcl-2, Bax, and active-caspase-3 determined by Western blot analysis. Density of immunoblotting bands were measured as described under Materials and Methods.

3. Melittin induces expression of apoptosis regulatory proteins

I investigated the involvement of apoptosis regulatory proteins on melittin-induced apoptosis in VSMCs. Cell cytosol extracts were prepared from VSMCs in exponential growth and following treatment for 24 h with melittin and were subjected to immunoblot analysis for expression of apoptosis regulatory proteins. Expression of pro-apoptotic proteins, p53, Bax, and active form of caspase-3 was up-regulated in a dose-dependent manner in the VSMCs treated by melittin (0.4~0.8 μg/ml), whereas the expression of anti-apoptotic protein Bcl-2 was down-regulated (Fig. 8).

Ⅳ. Discussion

The purposes of the present study were to define Bee Venom and Melittin related anti-proliferative mechanisms in 5% FBS-induced rat aortic VSMCs proliferation. It demonstrated the anti-proliferative effect of BV and melittin in VSMCs through induction of apoptosis via

suppressions of NF-κB and Akt activation, and enhancement of apoptotic signal pathway.

The abnormal growth of VSMCs is prominent features of vascular disease, including atherosclerosis, post-angioplasty restenosis[1]. Neointimal thickening is mainly due to VSMCs, which proliferate and migrate from the media. Excessive proliferative potential can be regulated by apoptosis[15]. In the previous study, We found that BV and melittin significantly inhibited FBS-induced proliferations of VSMCs. Since our results also demonstrated that BV- and melittin-induced apoptosis of VSMCs, We therefore believe that the inhibitory effect of BV and melittin on cell proliferation of VSMCs results from pro-apoptotic properties.

NF-κB and its inhibitory proteins (IκB) form an autoregulatory system that has been linked to vascular disease. Involvement of NF-κB in the process of atherosclerosis and restenosis has become evident in a variety of studies. Activated nuclear NF-κB has been detected in VSMCs after balloon injury of carotid arteries and atherosclerosis lesions[9,27]. In contrast, little activated NF-κB is detected in normal healthy vessels. After balloon injury of the rat carotid artery, the levels of IκB are rapidly reduced in medial VSMCs and NF-κB activation correlates with VSMCs proliferation and induced expression of NF-κB-dependent genes[37]. These strongly suggest a causative role for NF-κB in development and maintenance of atherosclerosis and neointimal hyperplasia. We therefore investigated the effects of melittin on NF-κB because NF-κB is important regulator of cell proliferation and apoptosis. To investigate whether melittin inhibits NF- κB activation, We first examined the effects on IκBα phosphorylation and degradation as these two events are essential for the nuclear translocation and activation of NF-κB[31]. We found that melittin significantly inhibited the FBS-induced IκBα phosphorylation (Fig. 1) and its degradation (Fig. 2). To further demonstrate the inhibitory effect of melittin in NF-κB activation, We examined the NF-κB DNA-binding activity and nuclear translocation and found that melittin potently attenuated the DNA-binding activity (Fig. 3) and NF-κB p50 subunit nuclear translocation (Fig. 4) in response to the action of FBS. *Park et al.* (2004)[24] recently demonstrated that BV and melittin strongly reduced NF-κB activation through directly binding to the p50 subunit. Our results, therefore, strongly suggest that melittin suppresses the NF-κB activation, leading to an inhibition of VSMCs proliferation and an increase in VSMCs apoptosis.

ERK1/2 MAPK and Akt (also called protein kinase B), upstream signals of NF-κB, are major signal transduction molecules regulating cell proliferation, differentiation, and apoptosis. Many studies indicate that the MAPK pathway and the Akt pathway appear to lead to two distinct end effectors and that they are regulated independently by various stimulators and intermediate signal transduction molecules[32,33]. Thus, We examined whether melittin inhibits ERK1/2 and Akt activation, because suppression of ERK1/2 or Akt could inhibit proliferation and induce apoptosis in VSMCs. In our study, melittin markedly inhibited the FBS-induced phosphorylation of Akt (Fig. 6). However, melittin weakly affected the ERK1/2 phosphorylation induced by FBS for 5 min (Fig. 7). It is recently reported that Akt activation induces cell proliferation and enhances resistance to apoptosis signaling through regulation of NF-κB[34,35]. Our results therefore suggested that the Akt pathway was involved in melittin-induced pro-apoptotic effect through suppression of NF-κB.

Recently, *Jang et al.* (2003)[21] and *Hong et al.* (2005)[20] reported that BV induces apoptosis in human lung cancer cell line NCI-H1299 cell and human rheumatoid synovial fibroblast through an increase in Bax and caspase-3 expression and a decrease in Bcl-2 expression. We were interested in investigating whether melittin induces expressions of apoptosis regulatory proteins in VSMCs. It was found that consistent with the increase of the induction of apoptosis, the expression of pro-apoptotic proteins p53, Bax and

active-caspase-3 was dose dependently increased but that anti-apoptotic protein Bcl-2 was decreased (Fig. 8). Based on these results, melittin appears to active specific intracellular death-related pathway, leading to a down-regulation of Bcl-2, up-regulation of p53, Bax and caspase-3 activation, and induction of apoptosis in VSMCs.

In summary, We have demonstrated that BV and melittin inhibit cell proliferation and induce apoptosis in rat aortic VSMCs. In particular, melittin potently inhibited FBS-induced phosphorylation and degradation of IκB, and markedly suppressed activation of NF-κB and phosphorylation of Akt but not ERK1/2. Melittin also increased expression of pro-apoptotic protein p53, Bax and caspase-3, decreased anti-apoptotic protein Bcl-2. The anti-proliferative effect of BV and melittin on VSMCs may be due to induction of apoptosis via suppressions of NF-κB and Akt activation, and enhancement of pro-apoptotic signals. These findings suggest the possibility that BV acupuncture can be candidate as an useful therapeutic method for restenosis and atherosclerosis.

V. References

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