Original Article

The Inhibitory Effects of Bee Venom and Melittin on the Proliferation of Vascular Smooth Muscle Cells

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Abstract

In the present study, I have investigated the bee venom (BV) and melittin (a major component of BV) -mediated anti-proliferative effects, and defined its mechanisms of action in cultured rat aortic vascular smooth muscle cells (VSMCs). BV and melittin (0.4~0.8 μg/ml) effectively inhibited 50 ng/ml platelet derived growth factor BB (PDGF-BB)-induced VSMCs proliferations. The regulation of apoptosis has attracted much attention as a possible means of eliminating excessively proliferating VSMCs. In the present study, the treatment of BV and melittin strongly induced apoptosis of VSMCs. I examined the effects on NF-κB activation to investigate a possible mechanism for anti-proliferative effects of BV and melittin, the PDGF-BB-induced IκBα phosphorylation and its degradation were potently inhibited by melittin, and DNA binding activity and nuclear translocation of NF-κB p50 subunit in response to the action of PDGF-BB were potently attenuated by melittin. In further investigations, melittin markedly inhibited the PDGF-BB-induced phosphorylation of Akt but not ERK1/2, upstream signals of NF-κB. Treatment of melittin also potently induced pro-apoptotic protein p53, Bax, and caspase-3 expression, but decreased anti-apoptotic protein Bcl-2 expression. These results suggest that the anti-proliferative effects of BV and melittin in VSMCs through induction of apoptosis via suppressions of NF-κB and Akt activation, and enhancement of apoptotic signal pathway. Based on these results, BV acupuncture can be a candidate as a therapeutic method for restenosis and atherosclerosis.

Key words : *Bee venom, Melittin, VSMCs, Proliferation, Apoptosis, NF-κB, IκB, Akt, ERK1/2, p53, Bcl-2, Bax, Active Caspase-3*

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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), 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 A2, apamin, adolapin, and mast cell degranulating peptide, etc[17]. Many studies on the biological and pharmacological activities of BV have been carried out. The anti-inflammatory and anti-rheumatoid arthritis effects[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]. I recently 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]. 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, I therefore evaluated the effects of BV and melittin on proliferation and apoptosis of VSMCs, and then investigated the NF-κB-associated apoptosis signal pathway as possible 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). PDGF-BB 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[29]. 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. VSMCs proliferation assays

The rat aortic VSMCs proliferation was measured using by cell counting and DNA synthesis assay as previously described[29]. For cell counting, cells were seeded in 12-well culture plates at 1×10^5 cells/ml, and cultured in DMEM with 10% FBS at 37 ºC for 24 h. The cells were then cultured with serum-free medium containing BV and melittin (0.4~0.8 μg/ml) or vehicle (DMSO). 24 hours later, the cells were stimulated with 50 ng/ml PDGF-BB, and then trypsinized with trypsin-EDTA and counted using a hemocytometer under microscopy.

DNA synthesis was assayed by measurement of the [^{3}H]thymidine incorporation into cell DNA. Cells were seeded in 24-well culture plates under the same conditions. The medium was then replaced by serum-free medium containing BV and melittin, or vehicle. 24 hours later, cultures were then exposed to 50 ng/ml PDGF-BB for 20 h before 2 μCi/ml of [^{3}H]thymidine was added to the medium. Four hours later, labeling reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ ether (1:1, v/v). Acid-insoluble [^{3}H]thymidine was extracted into 300 μl of 0.5 M NaOH per well, and this solution was mixed with 3 ml scintillation cocktail (Ultimagold, Packard Bioscience Co., Meriden, CT, USA), and quantified using a liquid scintillation counter (model LS3801, Beckman, Düsseldorf, Germany).

4. Trypan blue

Cultures were incubated with 0.1% Trypan blue in PBS 5 min at 22 °C, rinsed with PBS, and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate. Trypan blue-positive and -negative cells were counted by an unbiased observer. More than 300 cells were evaluated in each experimental group (three cultures per group).

5. Apoptosis assays

In order to determine whether apoptosis is induced by BV and melittin, TUNEL and DAPI staining were performed. In short, cells were cultured on 8-chamber slides. After treatments with BV and melittin (0.4~0.8 μg/ml) for 24 h, the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at room temperature. TUNEL assays were performed by using the *in situ* Cell Death Detection Kit (Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. For the DAPI staining, slides were incubated 30 min at room temperature in the dark with mounting medium for fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

The cells were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany). Total number of cells in a given area was determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted × 100.

6. 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 ℃ and horseradish peroxidase-conjugated IgG secondary antibody (Santa Cruz Biotechnology Inc.) at 4 ℃ 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).

7. Electrophoretic mobility shift analysis

DNA binding activity of NF-κB was determind 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 ℃ for 24 h, then cultured with serum-free medium containing melittin (0.4~0.8 μg/ml) or vehicle. 24 hours later, the cells were stimulated by 50 ng/ml PDGF-BB 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 ℃. 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 ℃ for 1 h and exposed to film overnight at -80 ℃.

8. 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 50 ng/ml PDGF-BB 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.

Ⅲ. Results

1. BV and melittin inhibit proliferation of VSMCs

I first sought to determine whether BV and melittin can inhibit the proliferation of rat aortic VSMCs. The inhibitory effects of BV and melittin on the proliferation of VSMCs were examined by direct cell counting and DNA synthesis assay. The cell number was significantly increased by treatment with 50 ng/ml PDGF-BB for 24 h, and decreased significantly in a concentration-dependent manner by 24 h pre-treatment with BV or melittin. The percentages of the control cell number significantly decreased by BV 0.6 μg/ml and 0.8 μg/ml were 82.0 ± 3.9 and 54.1 ± 4.3% on PDGF-BB-stimulated cells (Fig. 1). The percentages of the control cell number significantly decreased by melittin 0.4 μg/ml, 0.6 μg/ml and 0.8 μg/ml were 68.9 ± 8.6, 45.1 ± 4.2 and 43.4 ± 2.9% on PDGF-BB-stimulated cells (Fig. 2).

Effects of BV or melittin on DNA synthesis in rat aortic VSMCs were tested using [^{3}H]thymidine incorporation. As shown in Figure 3 and 4, BV and melittin concentration-dependently inhibited [^{3}H]thymidine incorporation induced by PDGF-BB. The percentages of the control significantly decreased by BV 0.6 μg/ml and 0.8 μg/ml were 94.2 ± 2.5 and 63.3 ± 5.4% on PDGF-BB-stimulated

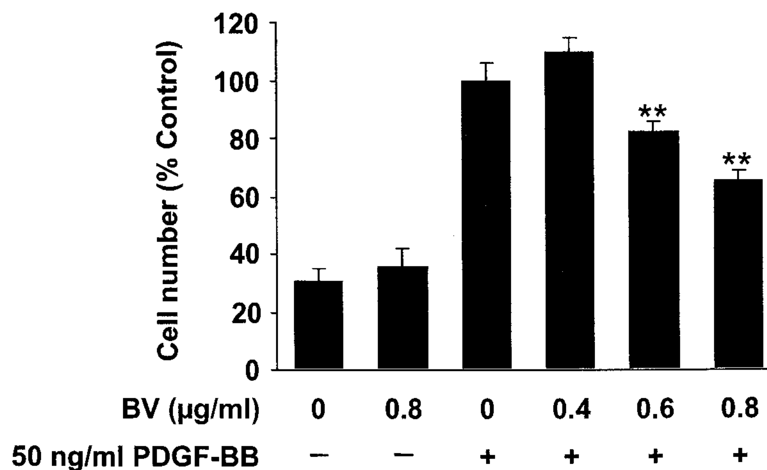


Fig. 1. Effect of BV on Cell Number in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml BV for 24 h, and then stimulated with 50 ng/ml PDGF-BB.

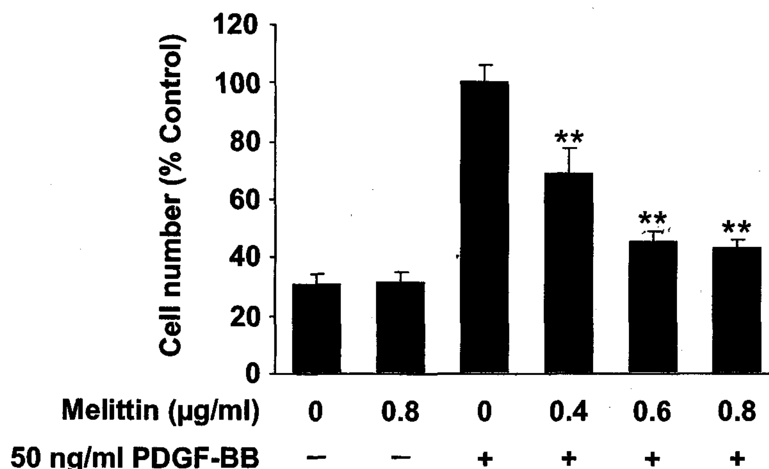


Fig. 2. Effect of Melittin on Cell Number in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then stimulated with 50 ng/ml PDGF-BB.

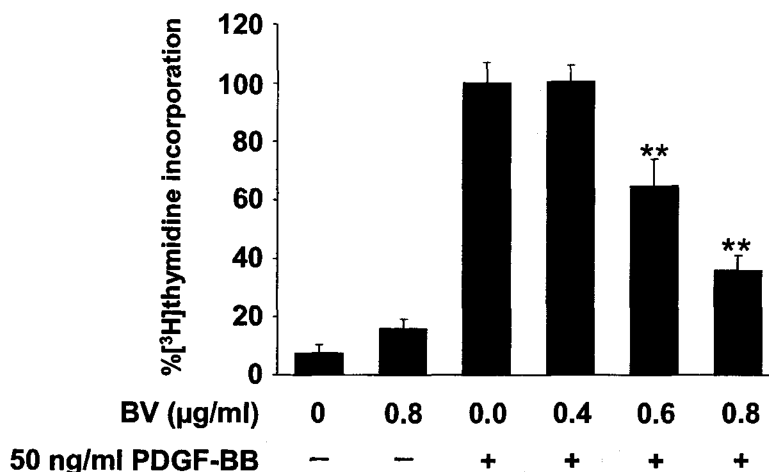


Fig. 3. Effect of BV on DNA Synthesis in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml BV for 24 h, and then stimulated with 50 ng/ml PDGF-BB.

cells (Fig. 3). The percentages of the control significantly decreased by melittin 0.4 μg/ml, 0.6 μg/ml and 0.8 μg/ml were 53.9 ± 6.5, 48.9 ± 2.5 and 35.2 ± 4.2% on PDGF-BB-stimulated cells (Fig. 4). The inhibitory effects were also dependent on concentration and corresponded with the inhibition of cell number. Taken together, these results indicate that BV and melittin significantly inhibit rat aortic VSMCs proliferation, and melittin especially exhibited the strongest inhibition against PDGF-BB-induced VSMCs proliferation.

2. BV and melittin induce apoptosis of VSMCs

Given the potent inhibition on cell proliferation by BV and melittin, I evaluated VSMCs apoptosis by cell morphologic analysis and TUNEL assay. Cytotoxicity effect of BV in VSMCs was first measured by Trypan blue exclusion assay to confirm that the inhibitory effects of BV and melittin mentioned above should not be the resulted from toxic effect (cell death by BV and melittin) (Fig. 5, 6).

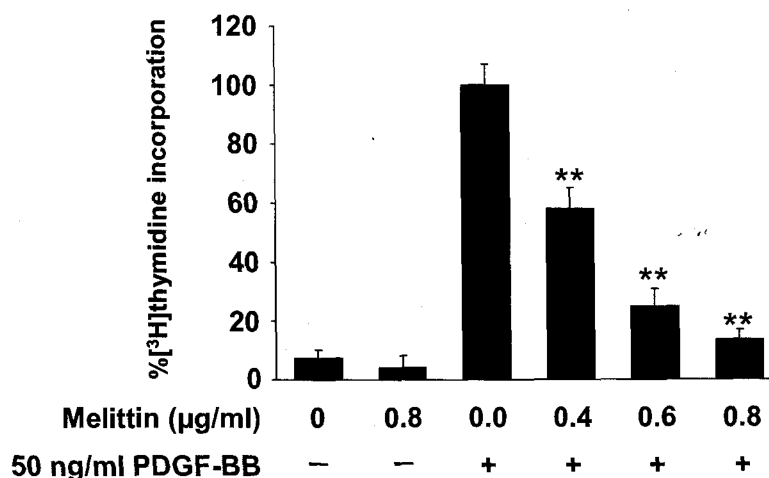


Fig. 4. Effect of Melittin on DNA Synthesis in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then stimulated with 50 ng/ml PDGF-BB.

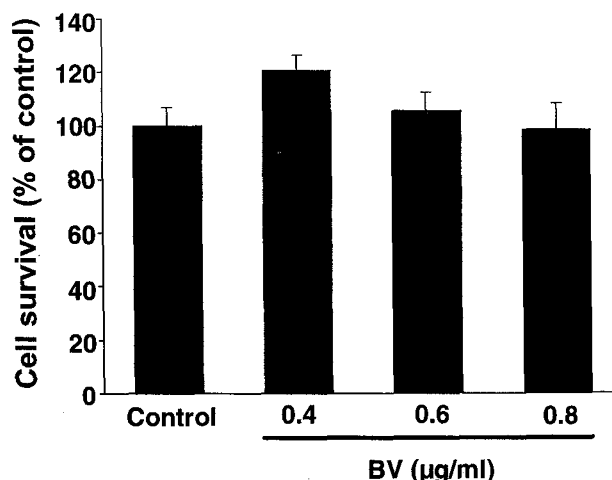


Fig. 5. Cytotoxicity Effect of BV in VSMCs
VSMCs were pre-cultured in serum-free medium at the different (0.4~0.8 μg/ml) concentrations of BV. After 24 h, cells were transferred to hemocytometer. Trypan blue exclusion assay was performed as described in materials and methods. Data are expressed as mean ± S.E.M. from three different sets of experiments.

To observe the effects of BV and melittin on cell morphology, cells were examined via phase-contrast microscopy (ECLIPSE TE-300, Nikon Instech Co., Kawasaki, Kanagawa, Japan). In the morphologic analysis, BV and melittin were seen to cause characteristic changes of VSMCs. Under the phase-contrast microscope, BV and melittin-treated VSMCs for 24 h presented with cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 7, 8). These morphological characteristics suggest that BV and melittin induce apoptotic cell death in VSMCs.

To further ascertain the induction of apoptosis by BV and melittin in VSMCs, I evaluated the TUNEL assay with DAPI staining. Apoptotic bodies, the presence of which is stringent morphological criteria for apoptosis, were observed in BV and melittin-treated VSMCs stained with DAPI (Fig. 9, 10). Indeed, TUNEL-positive cells (stained green) were increased in BV and melittin-treated

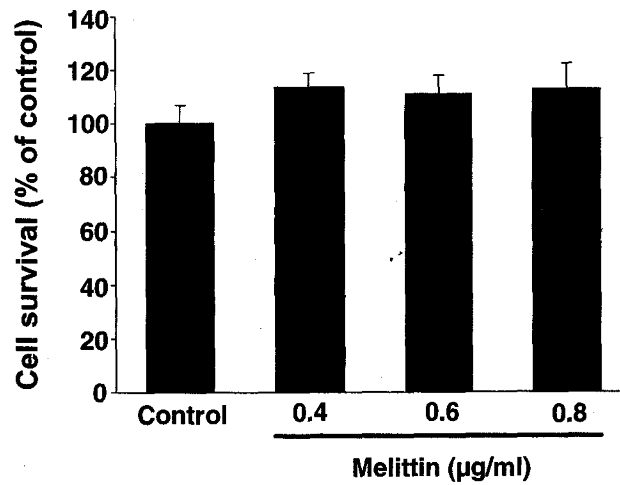


Fig. 6. Cytotoxicity Effect of Melittin in VSMCs
VSMCs were pre-cultured in serum-free medium at the different (0.4~0.8 μg/ml) concentrations of melittin. After 24 h, cells were transferred to hemocytometer. Trypan blue exclusion assay was performed as described in materials and methods. Data are expressed as mean ± S.E.M. from three different sets of experiments.

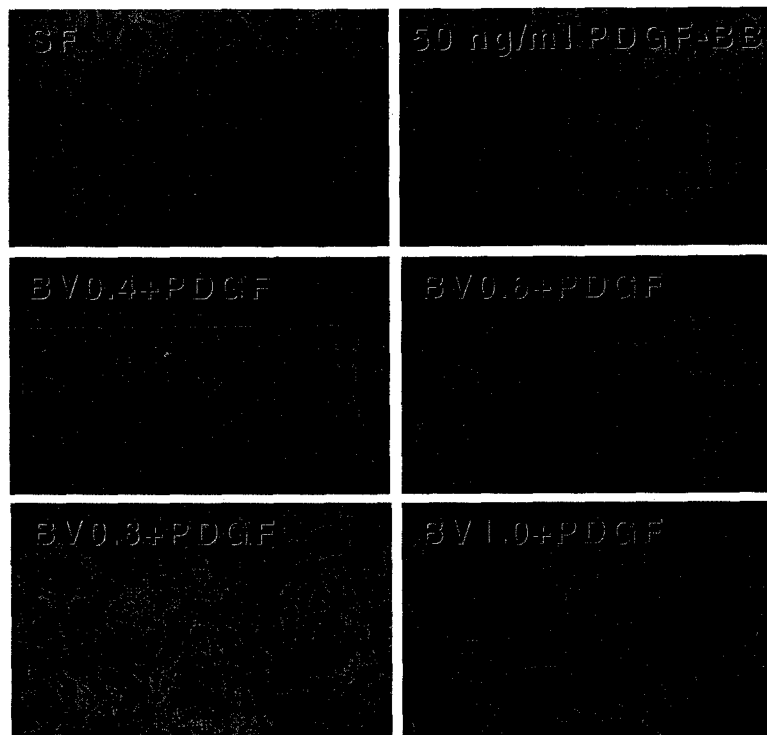


Fig. 7. Effect of BV on Cell Morphological Changes in VSMCs
Treatment of BV for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200).

Fig. 8. Effect of Melittin on Cell Morphological Changes in VSMCs
Treatment of melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200).

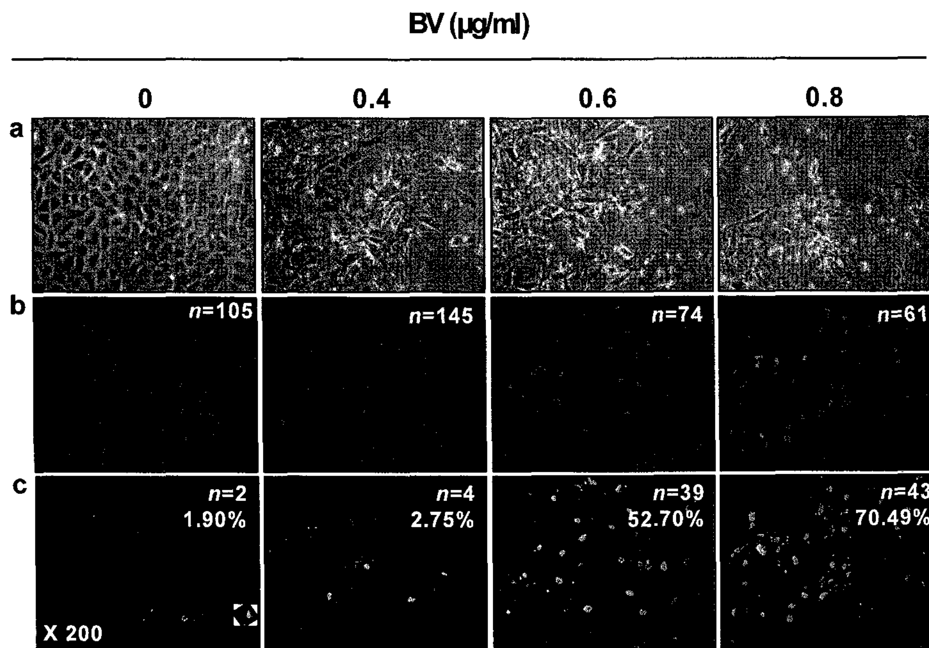


Fig. 9. Effect of BV on Apoptosis Induction of VSMCs
The apoptotic cells were examined by morphologic analysis (a), DAPI staining (b) and TUNEL assay (c). Treatment of BV and melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200). Total number of cells in a given area were determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted (fluorescent microscopy magnification, ×100). The percentage of TUNEL-positive cells calculated (low panel).

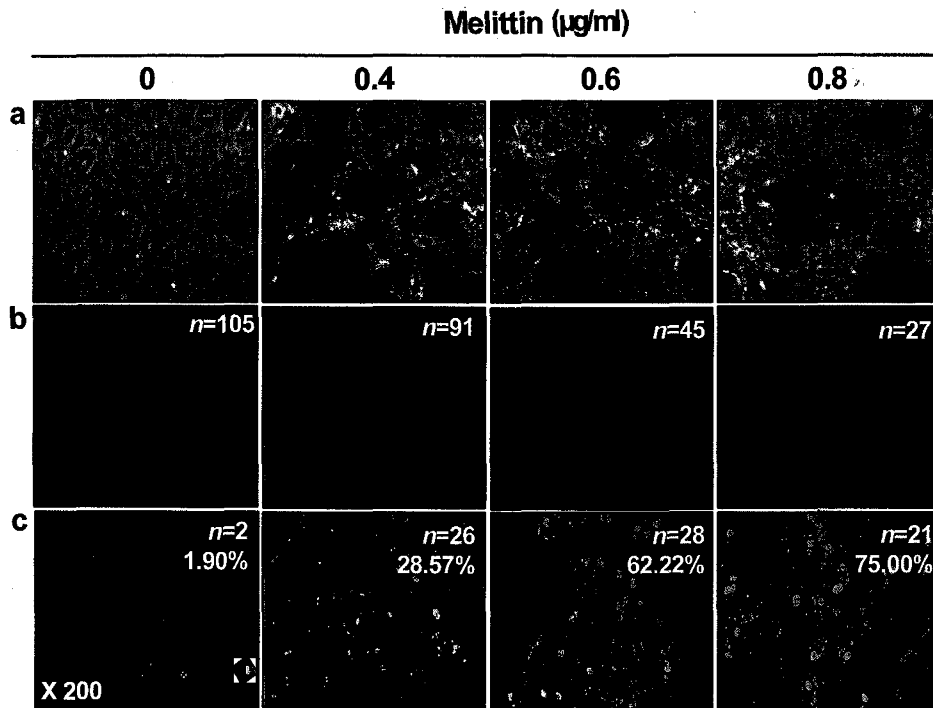


Fig. 10. Effect of Melittin on Apoptosis Induction of VSMCs

The apoptotic cells were examined by morphologic analysis (a), DAPI staining (b) and TUNEL assay (c). Treatment of BV and melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200). Total number of cells in a given area were determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted (fluorescent microscopy magnification, ×100). The percentage of TUNEL-positive cells calculated (low panel).

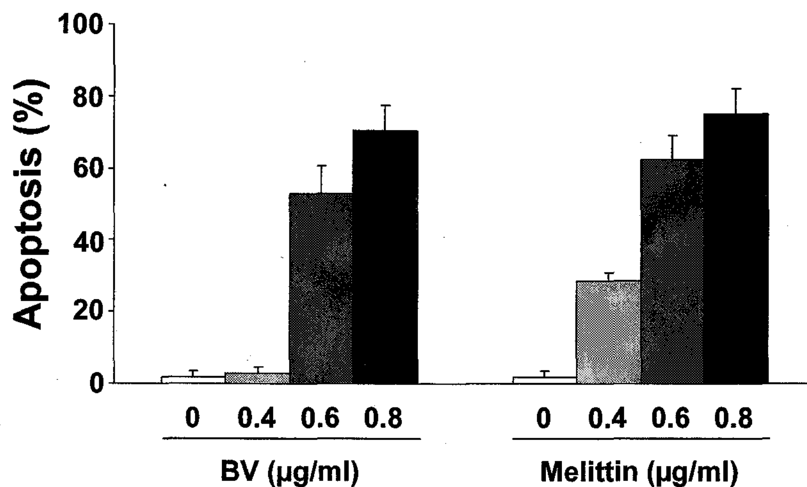


Fig. 11. Effect of BV and Melittin on Apoptosis Induction of VSMCs

VSMCs (Fig. 11). The treatments of BV and melittin (0.4~0.8 μg/ml) strongly induced apoptosis of VSMCs with percentage values of 2.75 ± 1.9, 52.70 ± 7.9 and 70.49 ± 6.8% by BV, and 28.57 ± 2.34, 62.22 ± 6.97 and 75.00 ± 6.98% by melittin, respectively.

3. 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 50 ng/ml PDGF-BB 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 PDGF-BB- induced IκBα phosphorylation (Fig. 12) and degradation (Fig. 13).

To further investigate, PDGF-BB-stimulated VSMCs nuclear extract was prepared and assayed NF-κB DNA binding by EMSA. Cells were stimulated with 50 ng/ml PDGF-BB for 60 min which it is the time to activate NF-κB maximally (data not shown). PDGF-BB-induced strong NF-κB DNA binding activity was attenuated by melittin in a dose-dependent manner, The percentages of the control significantly decreased by melittin 0.4 μg/ml, 0.6 μg/ml and 0.8 μg/ml were 73.6 ± 10.6, 44.7 ± 19.3 and 51.1 ± 9.6%(Fig. 14), respectively .

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. PDGF-BB 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 PDGF-BB-stimulated cells (Fig. 15, 16).

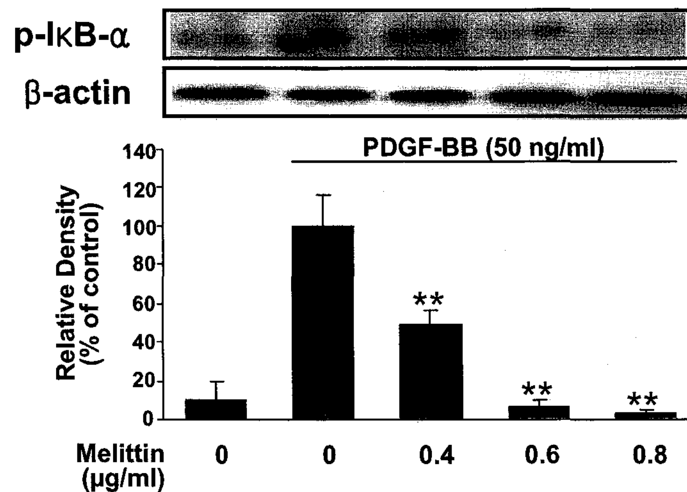


Fig. 12. Effect of Melittin on Phosphorylation of IκBα in VSMCs
For examinations of IκBα phosphorylation, cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then 50 ng/ml PDGF-BB was added to the cells for another 30 min. After the above treatment, cells were harvested and Western blot analysis was performed. Density of immunoblotting bands was measured as described under Materials and Methods.

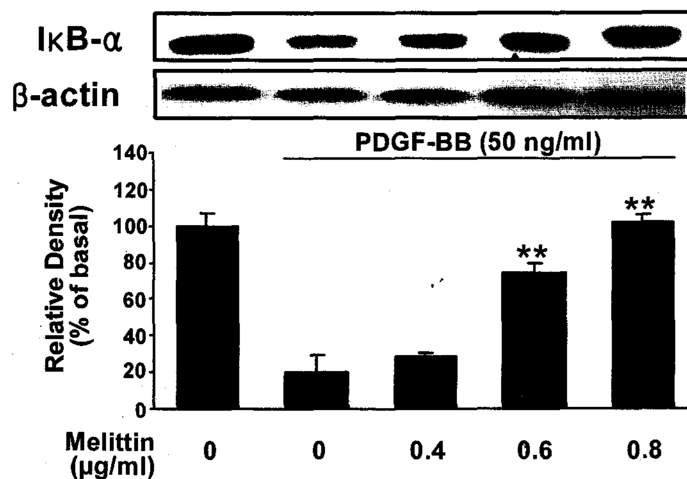


Fig. 13. Effect of Melittin on Degradation of IκBα in VSMCs

For examinations of IκBα degradation, cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then 50 ng/ml PDGF-BB 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 was measured as described under Materials and Methods.

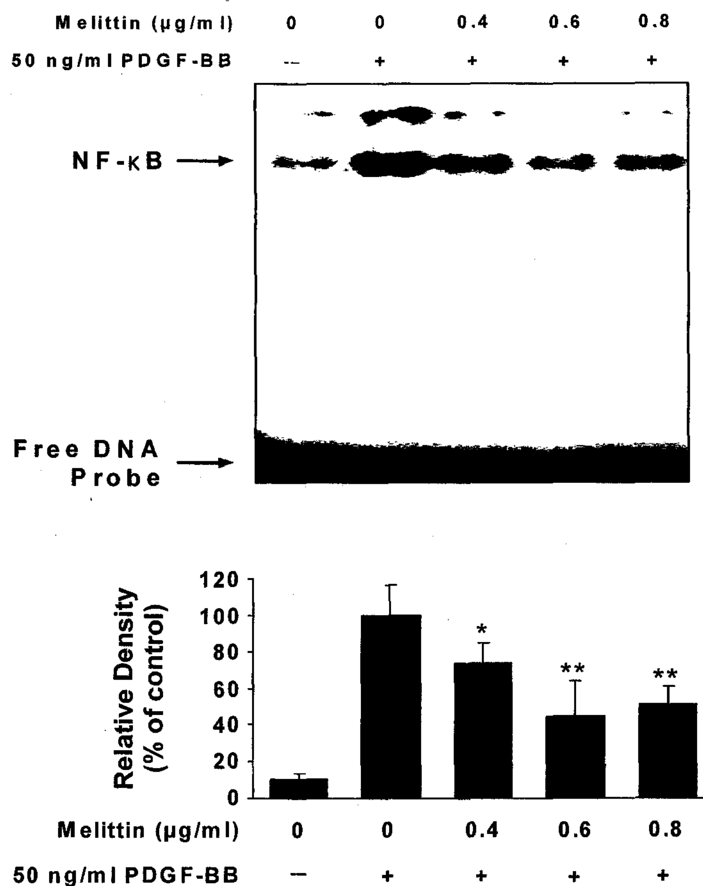


Fig. 14. Effect of Melittin on NF-κB DNA Binding Activity in VSMCs

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 50 ng/ml PDGF-BB 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 was measured as described under Materials and Methods.

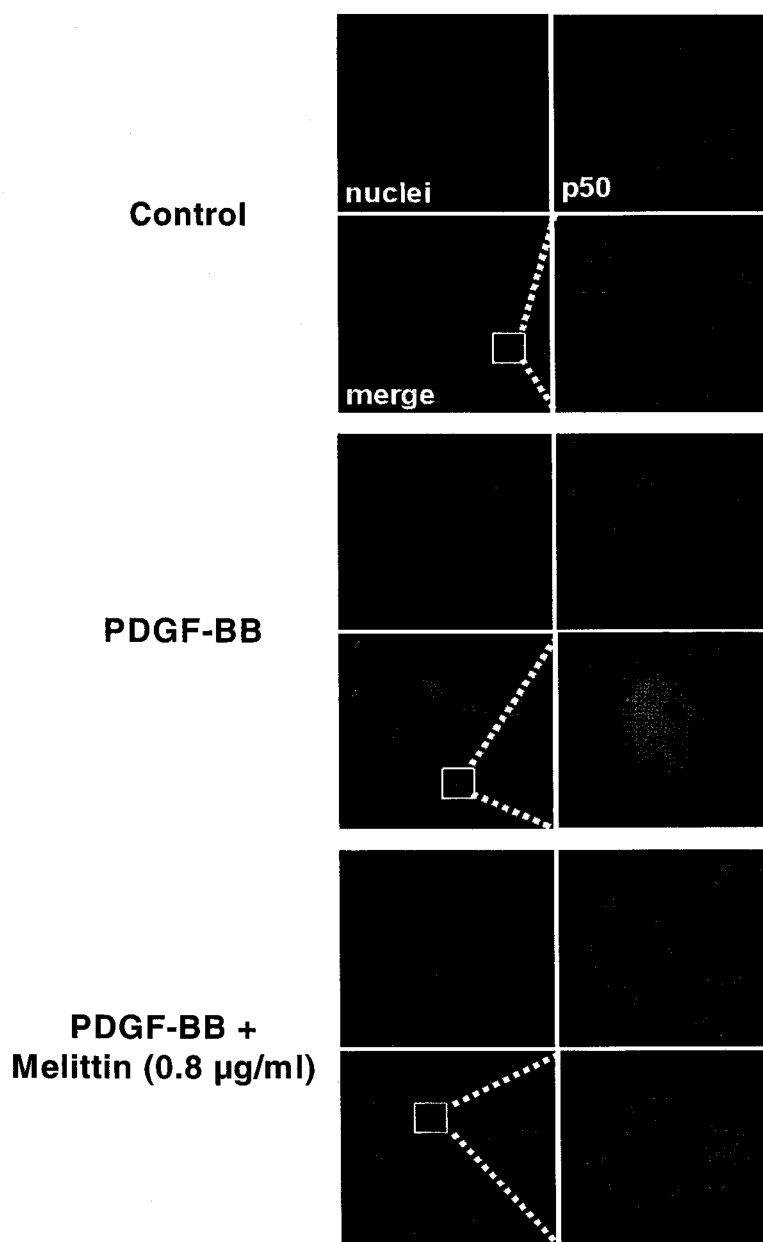


Fig. 15. Effect of Melittin on NF-κB Nuclear Translocation in VSMCs
The translocation of NF-κB p50 subunit was determined by immunofluorescence confocal laser scanning microscopy. In unstimulated cells, p50 was localized in the cytoplasm. After stimulation with 50 ng/ml PDGF-BB, p50 was translocated 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.

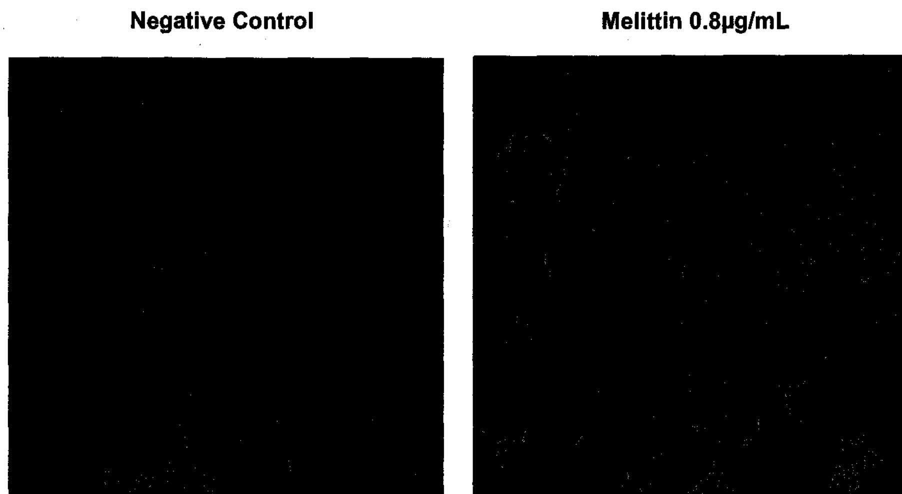


Fig. 16. Uptake of Melittin into VSMCs
Uptake of melittin into the membrane and nucleus of VSMCs (original magnification × 360). Negative control cells were treated with Alexa Fluor 488 alone, Positive cells were treated with melittin labeled with Alexa Fluor 488

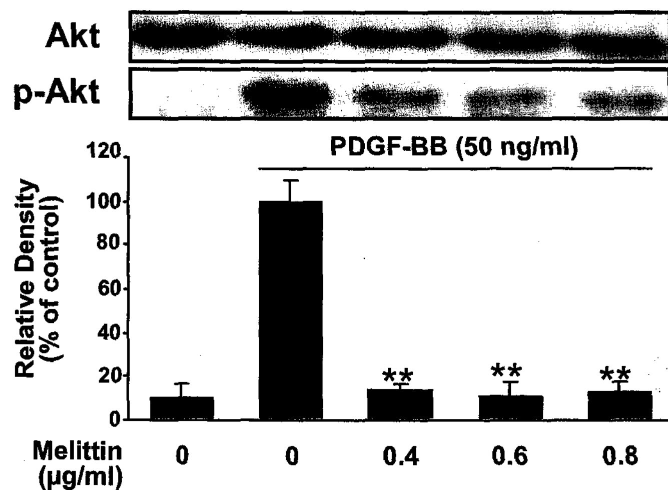


Fig. 17. Effect of Melittin on Expression of Akt in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then stimulated with 50 ng/ml PDGF-BB. Equal amounts of whole cell lysate (30 μg) were subjected to electrophoresis and analysis by Western blot for total and phosphorylated Akt. Density of immunoblotting bands of phosphorylated Akt was measured as described under Materials and Methods.

4. 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 PDGF-BB-induced phosphorylation of Akt and ERK1/2. Pre-treatment of 0.4 μg/ml, 0.6 μg/ml and 0.8 μg/ml melittin significantly inhibited the PDGF-BB-induced phosphorylation of Akt in a concentration-dependent manner. The percentages of the control significantly decreased by melittin 0.4 μg/ml, 0.6 μg/ml and 0.8 μg/ml were 14.1 ± 2.4, 11.1±6.3 and 12.5±5.1% (Fig. 17), respectively.

Melittin also inhibited 50 ng/ml PDGF-BB-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 μg/ml, 0.6 μg/ml and 0.8 μg/ml were 80.8 ± 10.8, 66.4 ± 9.8 and 55.2 ± 15.6% (Fig. 18), respectively.

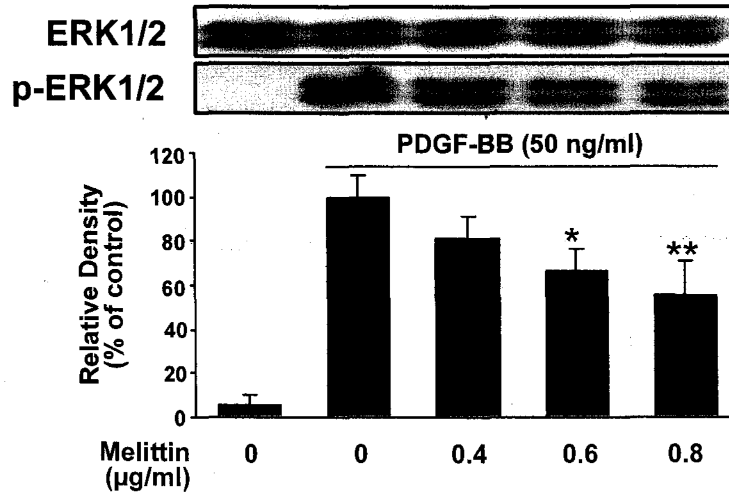
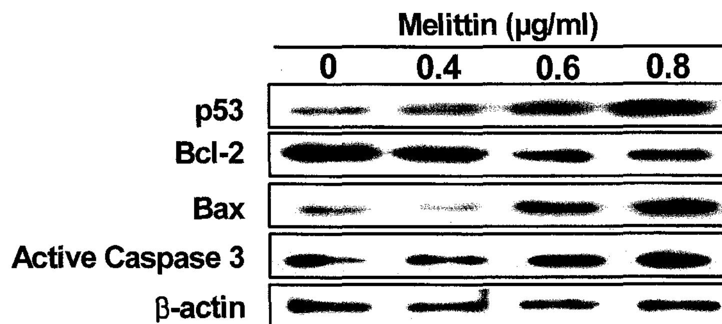


Fig. 18. Effect of Melittin on Expression of ERK1/2 in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml melittin for 24 h, and then stimulated with 50 ng/ml PDGF-BB. Equal amounts of whole cell lysate (30 μg) were subjected to electrophoresis and analysis by Western blot for total and phosphorylated ERK1/2. Density of immunoblotting bands of phosphorylated EKR1/2 was measured as described under Materials and Methods.



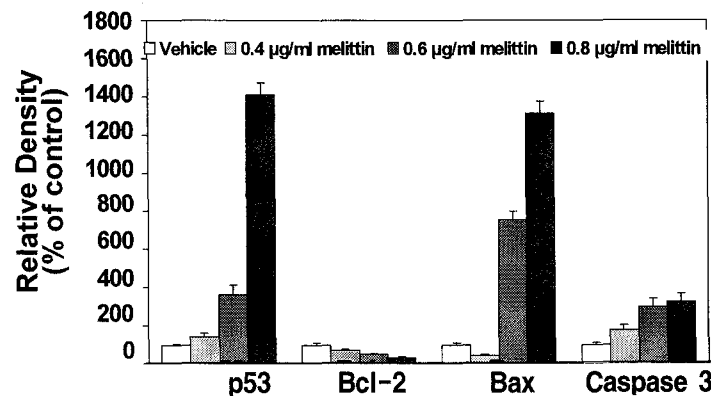


Fig. 19. Effect of Melittin on Apoptosis Regulatory Protein Expression
Cells were incubated for 24 h with or without 0.4~0.8 μg/ml melittin, and the expression of p53, Bcl-2, Bax, and active caspase-3 determined by Western blot analysis. Density of immunoblotting bands were measured as described under Materials and Methods.

5. 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. 19).

Ⅳ. Discussion

The purposes of the present study were to find out whether BV and melittin possess an inhibitory effect, and to define its mechanisms of action on rat aortic VSMCs proliferation. It demonstrated the anti-proliferative effects 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 present study, I showed that BV and melittin significantly inhibited PDGF-BB-induced proliferations of VSMCs (Fig. 1−4). Since my results also demonstrated that BV and melittin-induced apoptosis of VSMCs, I therefore believe that the inhibitory effects of BV and melittin on cell proliferation of VSMCs results from pro-apoptotic properties.

Various studies have implicated a role for PDGF in VSMCs proliferation, atherosclerosis, and neointimal hyperplasia. PDGF-BB, which is expressed in multiple cell types including VSMCs, is a potent mitogen for VSMCs *in vitro* and *in vivo*[30-32]. It is well known that PDGF-BB activates NF-κB in cultured cells, which lead to cell proliferation[33-34]. 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 various 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[35]. These strongly suggest a causative role for NF- κB in development and maintenance of atherosclerosis and neointimal hyperplasia. I 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, I first examined the effects on IκBα phosphorylation and degradation as these two events are essential for the nuclear translocation and activation of NF-κB[34]. I found that melittin significantly inhibited the PDGF-BB-induced IκBα phosphorylation (Fig. 12) and its degradation (Fig. 13). To further demonstrate the inhibitory effects of melittin in NF-κB activation, I examined the NF-κB DNA binding activity and nuclear translocation and found that melittin potently attenuated the DNA binding activity (Fig. 14) and NF-κB p50 subunit nuclear translocation (Fig. 15) in response to the action of PDGF-BB. *Park et al.* (2004)[24] recently demonstrated that BV and melittin strongly reduced NF-κB activation through directly binding to the p50 subunit. My results, therefore, strongly suggest that melittin suppress 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[36-37]. Thus, I 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 my study, melittin markedly inhibited the PDGF-BB-induced phosphorylation of Akt (Fig. 17). However, melittin weakly affected the ERK1/2 phosphorylation induced by PDGF-BB for 5 min (Fig. 18). It is recently reported that Akt activation induces cell proliferation and enhances resistance to apoptosis signaling through regulation of NF-κB[38-39]. My results 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. I was 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. 19). Based on these results, melittin appears to activate 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, I have demonstrated that BV and melittin inhibit cell proliferation and induce apoptosis in rat aortic VSMCs. In particular, melittin potently inhibited PDGF-BB-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 effects 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 a candidate as a therapeutic method for restenosis and atherosclerosis.

V. References

1. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature.* 1993 ; 362 : 801-809.
2. Jung F, Haendeler J, Goebel C, Zeiher AM, Dimmeler S. Growth factor-induced phosphoinositide 3-OH kinase/Akt phosphorylation in smooth muscle cells: induction of cell proliferation and inhibition of cell death. *Cardiovasc Res.* 2000 ; 48 : 148-157.
3. Libby P, Warner SJ, Friedman GB. Interleukin 1: a mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanoids. *J Clin Invest.* 1998 ; 81 : 487-498.
4. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell.* 1997 ; 88 : 347-354.
5. Bennett MR. Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res.* 1999 ; 41:361-368.
6. Kockx MM, Knaapen MW. The role of apoptosis in vascular disease. J Pathol. 2000 ; 190 : 267-280.

7. Kockx MM, Herman AG. Apoptosis in atherosclerosis : beneficial or detrimental *Cardiovasc Res.* 2000 ; 45 : 736-746.
8. Lesauskaite V, Ivanoviene L, Valanciute A. Programmed cellular death and atherogenesis : from molecular mechanisms to clinical aspects. *Medicina.* 2003 ; 39 : 529-534.
9. Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA. Role of nuclear factor-kappa B in atherosclerosis. *Exp Physiol.* 1997 ; 82 : 297-304.
10. Pollman MJ, Hall JL, Mann MJ, Zhang L, Gibbons GH. Inhibition of neointimal cell bcl-x expression induces apoptosis and regression of vascular disease. *Nat Med.* 1998 ; 4 : 222-227.
11. Wang BY, Ho HK, Lin PS, Schwarzacher SP, Pollman MJ, Gibbons GH, Tsao PS, Cooke JP. Regression of atherosclerosis : role of nitric oxide and apoptosis. *Circulation.* 1999 ; 99 : 1236-1241.
12. Chen JH, Wu CC, Hsiao G, Yen MH. Magnolol induces apoptosis in vascular smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol.* 2003 ; 368 : 127-133.
13. Curcio A, Torella D, Cuda G, Coppola C, Faniello MC, Achille F, Russo VG, Chiariello M, Indolfi C. Effect of stent coating alone on *in vitro* vascular smooth muscle cell proliferation and apoptosis. *Am J Physiol Heart Circ Physiol.* 2003 ; 286 : H902-H908.
14. Hofmann CS, Sonenshein GE. Green tea polyphenol epigallocatechin-3 gallate induces apoptosis of proliferating vascular smooth muscle cells via activation of p53. *FASEB J.* 2003 ; 17 : 702-704.
15. Perlman H, Sata M, Krasinski K, Dorai T, Buttyan R, Walsh K. Adenovirus-encoded hammerhead ribozyme to Bcl-2 inhibits neointimal hyperplasia and induces vascular smooth muscle cell apoptosis. *Cardiovasc Res.* 2000 ; 45 : 570-578.
16. Yang HM, Kim HS, Park KW, You HJ, Jeon SI, Youn SW, Kim SH, Oh BH, Lee MM, Park YB, Walsh K. Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. *Circulation.* 2004 ; 110 : 301-308.
17. Kwon YB, Lee HJ, Han HJ, Mar WC, Kang SK, Yoon OB, Beitz AJ, Lee JH. The water-soluble fraction of bee venom produces antinociceptive and anti-inflammatory effects on rheumatoid arthritis in rats. *Life Sci.* 2002 ; 71 : 191-204.
18. Kwon YB, Lee JD, Lee HJ, Han HJ, Mar WC, Kang SK, Beitz AJ, Lee JH. Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. *Pain.* 2001 ; 90 : 271-280.
19. Nam S, Ko E, Park SK, Ko S, Jun CY, Shin MK, Hong MC, Bae H. Bee venom modulates murine Th1/Th2 lineage development. *Int Immunopharmacol.* 2005 ; 5 : 1406-1614.
20. Hong SJ, Rim GS, Yang HI, Yin CS, Koh HG, Jang MH, Kim CJ, Choe BK, Chung JH. Bee venom induces apoptosis through caspase-3 activation in synovial fibroblasts of patients with rheumatoid arthritis. *Toxicon.* 2005 ; 46 : 39-45.
21. Jang MH, Shin MC, Lim S, Han SM, Park HJ, Shin I, Lee JS, Kim KA, Kim EH, Kim CJ. Bee venom induces apoptosis and inhibits expression of cyclooxygenase-2 mRNA in human lung cancer cell line NCI-H1299. *J Pharmacol Sc.* 2003 ; 91 : 95-104.
22. Liu X, Chen D, Xie L, Zhang R. Effect of honey bee venom on proliferation of K1735M2 mouse melanomacells *in vitro* and growth of murine B16 melanomas *in vivo.* *J Pharm Pharmacol.* 2002 ; 54 : 1083-1089.
23. Orsolic N, Sver L, Verstovsek S, Terzic S, Basic I. Inhibition of mammary carcinoma cell proliferation *in vitro* and tumor growth *in vivo* by bee venom. *Toxicon.* 2003 ; 41 :

861–870.

24. Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song HS, Kim GJ, Oh GT, Yoon do Y, Hong JT. Antiarthritic effect of bee venom : inhibition of inflammation mediator generation by suppression of NF-κB through interaction with the p50 subunit. *Arthritis Rheum.* 2004 ; 50 : 3504–3515.
25. Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be : NF-κB is the answer-role of Rel/NF-κB in the regulation of apoptosis. *Oncogene.* 2003 ; 22 : 8961–8982.
26. De Martin R, Hoeth M, Hofer-Warbinek R, Schmid JA. The transcription factor NF-κB and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol.* 2000 ; 20 : E83–E88.
27. Mehrhof FB, Schmidt Ullrich R, Dietz R, Scheidereit C. Regulation of vascular smooth muscle cell proliferation : role of NF-κB revisited. *Circ Res.* 2005 ; 96 : 958–964.
28. Monaco C, Paleolog E. Nuclear factor-kappa B : a potential therapeutic target in atherosclerosis and thrombosis. *Cardiovasc Res.* 2004 ; 61 : 671–682.
29. Kim TJ, Zang YH, Kim YS, Lee CK, Hong JT, Yun YP. Effect of apigenin on the serum and platelet-derived growth factor-BB-induced ERK1/2, c-fos mRNA and proliferation of rat aortic vascular smooth muscle cells. *Planta Med.* 2002 ; 68 : 605–609.
30. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science.* 1991 ; 253 : 1129–1132.
31. Ikeda U, Ikeda M, Oohara T, Kano S, Yaginuma T. Mitogenic action of interleukin-1 alpha on vascular smooth muscle cells mediated by PDGF. *Atherosclerosis.* 1990 ; 84 : 183–188.
32. Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev.* 2004 ; 15 : 237–254.
33. Obata H, Biro S, Arima N, Kaieda H, KiharaT, Eto H, Miyata M, Tanaka H. NF-κB is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Commun.* 1996 ; 224 : 27–32.
34. Romashkova JA, Makarov SS. NF-κB is a target of Akt in anti-apoptotic PDGF signalling. *Nature.* 1999 ; 401 : 86–90.
35. Landry DB, Couper LL, Bryant SR, Linder V. Activation of the NF-κB and IκB system in smooth muscle cells after rat arterial injury : induction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. *Am J Pathol.* 1997 ; 151 : 1085–1095.
36. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD and Glass DJ. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science.* 1999 ; 286 : 1738–1741.
37. Zimmermann S and Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science.* 1999 ; 286 : 1741–1744.
38. Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-κB, Mapkinase and p53 pathways. *Leukemia.* 2005 ; 19 : 586–594.
39. Jeong SJ, Pise-Masison CA, Radonovich MF, Park HU, Brady JN. Activated Akt regulates NF-κB activation, p53 inhibition and cell survival in HTLV-1-transformed cells. *Oncogene*, in press. 2005.