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Melittin Inhibits Vascular Smooth Muscle Cell Proliferation through Induction of Apoptosis via Suppression of Nuclear Factor-κB and Akt Activation and Enhancement of Apoptotic Protein Expression

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ABSTRACT

In the present study, we have investigated the bee venom (BV) and melittin (a major component of BV)-mediated antiproliferative effect and defined its mechanisms of action in cultured rat aortic vascular smooth muscle cell(s) (VSMC). BV and melittin (~0.4–0.8 μg/ml) effectively inhibited 5% fetal bovine serum-induced and 50 ng/ml platelet-derived growth factor BB (PDGF-BB)-induced VSMC proliferation. The regulation of apoptosis has attracted much attention as a possible means of eliminating excessively proliferating VSMC. In the present study, the treatment of BV and melittin strongly induced apoptosis of VSMC. To investigate the antiproliferative mechanism of BV and melittin, we examined the effect of melittin on nuclear factor κB (NF-κB) activation, the PDGF-BB-induced IκBα phosphorylation, and its degradation were potently inhibited by melittin and whether 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 and weakly inhibited phosphorylation of extracellular signal-regulated kinase 1/2, upstream signals of NF-κB. Treatment of melittin also potently induced proapoptotic protein p53, Bax, and caspase-3 expression but decreased antiapoptotic protein Bcl-2 expression. These results suggest the antiproliferative effects of BV and melittin in VSMC through induction of apoptosis via suppressions of NF-κB and Akt activation and enhancement of apoptotic signaling pathway.

The increased potential for growth of vascular smooth muscle cell(s) (VSMC) is a key abnormality in the development of atherosclerosis lesions and postangioplasty restenosis (Ross, 1993). Thus, inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the treatment of disease, such as atherosclerosis and restenosis (Jung et al., 2000). It is well known that, in response to a variety of stimuli, including many growth factors such as platelet-derived growth factor (PDGF), VSMC can initiate highly conserved signaling events, which lead to either cell migration or proliferation (Jung et al., 2000). However, the proliferative potential can be regulated by induction of VSMC apoptosis (Libby et al., 1988).

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 (Jacobson et al., 1997). 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

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ABBREVIATIONS: VSMC, vascular smooth muscle cell(s); BV, bee venom; PDGF, platelet-derived growth factor; FBS, fetal bovine serum; NF-κB, nuclear factor κB; ERK1/2, extracellular signal-regulated kinase; Bcl-2, B-cell leukemia/lymphoma-2; DMEM, Dulbecco's modified Eagle's medium; Bax, Bcl-2-associated X protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DAPI, 4,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; Z-DEVD-FMK, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase.

factor in atherogenesis (Bennett, 1999; Kockx and Herman, 2000; Kockx and Knaapen, 2000; Lesauskaite et al., 2003). 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 (Jung et al., 2000). 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 VSMC (Pollman et al., 1998; Wang et al., 1999). After vessel injury, diverse signaling mechanisms become activated in VSMC, leading to neointimal hyperplasia. Therefore, it is suggested that VSMC 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 (Perlman et al., 2000; Chen et al., 2003; Curcio et al., 2003; Hofmann and Sonenshein, 2003; Lesauskaite et al., 2003; Yang et al., 2004).

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 (MCDP) (Kwon et al., 2002). Many studies on the biological and pharmacological activities of BV have been carried out. The anti-inflammatory and antirheumatoid arthritis effect (Kwon et al., 2002), relief of pain (Kwon et al., 2001), and immune modulatory activity (Nam et al., 2005) of BV have been described. BV and melittin have been also reported to induce apoptosis in several cancer cells and rheumatoid arthritis synovial fibroblasts in vitro and in vivo (Liu et al., 2002; Jang et al., 2003; Orsolic et al., 2003; Hong et al., 2005). We recently also reported that target inactivation of nuclear factor κB (NF-κB) by directly binding to the p50 subunit is an important mechanism of the antiarthritic effect of BV (Park et al., 2004). NF-κB is an important regulator of gene expression in cell proliferation and apoptosis (Kucharczak et al., 2003), which is considered as a potential therapeutic target in atherosclerosis and restenosis (Brand et al., 1997; De Martin et al., 2000; Monaco and Paleolog, 2004; Mehrhof et al., 2005). Therefore, in this study, we evaluated the effect of BV and melittin on proliferation and apoptosis of VSMC and then investigated the NF-κB-associated apoptosis signal pathway as possible mechanisms in cultured rat aortic VSMC.

Materials and Methods

Chemicals. Dried BV was purchased from You-Miel Bee Venom Ltd. (Hwasoon, Jeonnam, Korea). The composition of the BV was as follows: ~45 to 50% melittin, ~2.5 to 3% apamin, ~2 to 3% mast cell degranulating peptide, 12% phospholipase A_2, 1% lyso-phospholipase A, ~1 to 1.5% histidine, ~4 to 5% 6-pentyl-α-pyrone lipids, 0.5% secarpin, 0.1% tertiapin, 0.1% procamine, ~1.5 to 2% hyaluronidase, ~2 to 3% amine, ~4 to 5% carbohydrate, and ~19 to 27% of others, 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). PDGF-BB was obtained from Upstate Biotechnology (Lake Placid, NY). ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, and active-caspase-3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). IκBα, phospho-IκBα, NF-κB p50, p53, Bcl-2, Bax, and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [^{3}H]Thymidine was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). The cell culture materials were obtained from Invitrogen (Carlsbad, CA), and other chemical reagents were from Sigma Chemical Co.

Cell Culture. Rat aortic VSMC were isolated by enzymatic dispersion as described previously (Kim et al., 2002). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. The purity of VSMC culture was confirmed by immunocytochemical localization of α-smooth-muscle actin.

VSMC Proliferation Assays. The rat aortic VSMC proliferation was measured by cell counting and DNA synthesis as described previously (Kim et al., 2002). 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°C for 24 h. The cells were then cultured with serum-free medium containing BV and melittin (~0.4–0.8 μg/ml) or vehicle (dimethyl sulfoxide). Twenty-four hours later, the cells were stimulated by 5% FBS or 50 ng/ml PDGF-BB and then trypsinized with trypsin-EDTA and counted using a hemocytometer.

DNA synthesis was assayed by measurement of the [^{3}H]thymidine incorporation into cellular DNA. Cells were seeded in 24-well culture plates under the same conditions as above. The medium was then replaced by serum-free medium containing BV, melittin, or vehicle. Twenty-four hours later, cultures were then exposed to 5% FBS and 50 ng/ml PDGF-BB for 20 h before 2 μCi/ml [^{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 of scintillation cocktail (Ultimagold; Packard Bioscience Co., Meriden, CT) and quantified using a liquid scintillation counter (model LS3801; Beckman, Düsseldorf, Germany).

Apoptosis Assay. To determine whether apoptosis is induced by BV and melittin, TUNEL and DAPI staining were performed. Cells were plated, allowed to attach overnight, and then cultured with serum-free medium containing BV and melittin (~0.4–0.8 μg/ml) or vehicle (dimethyl sulfoxide). After treatment with BV or melittin (~0.4–0.8 μg/ml) for 24 h, the cells were stimulated by 50 ng/ml PDGF-BB for 24 h and then 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 Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. For the DAPI staining, slides were incubated for 30 min at room temperature in the dark with mounting medium for fluorescence with containing DAPI (Vector Laboratories, Inc., Burlingame, CA). 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.

Western Blot Analysis. Western blot analysis was performed as described previously (Park et al., 2004). VSMC were harvested and homogenized in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM phenylmethylsulfonyl 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; GE Healthcare). Blots were blocked for 2 h at room temperature with 5% (W/V) nonfat 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 MAPK

(ERK1/2), phospho-Akt, IκBα, phospho-IκBα, p53, Bcl-2, Bax, and active-caspase-3 antibodies at 1:500 dilution in bovine serum albumin/Tris-buffered saline with Tween 20 buffer overnight 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).

Electrophoretic Mobility Shift Analysis. DNA binding activity of NF-κB was determined using an electrophoretic mobility shift assay (EMSA). Gel-shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI). In brief, cells were cultured in DMEM with 10% FBS at 37°C for 24 h and then cultured with serum-free medium containing melittin (~0.4 to 0.8 μg/ml) or vehicle. Twenty-four 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 ice-cold Eppendorf tubes 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, 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 P-40] was added to the pellet in a 2:1 ratio (v/v) and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2:1 ratio (v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000*g* for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [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. 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.

Immunofluorescence Staining. VSMC 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. Twenty-four hours later, the cells were stimulated by 50 ng/ml PDGF-BB for 30 (Akt phosphorylation) or 60 (NF-κB translocation) min 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 polyclonal antibodies for phospho-Akt, p50 or active-caspase-3 (1:100 dilution) overnight at 4°C. After washing slides with PBS, the cells were exposed to anti-rabbit- or anti-goat-biotinylated secondary antibodies Alexa Fluor 568 or Alexa Fluor 633 (Molecular Probes Inc., Eugene, OR) (1:200 dilution), for 4 h at room temperature. Upon nuclear stain and mount in antifade medium with DAPI (Vector Laboratories, 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.

Assay of Caspase-3 Activation. The activation of caspase-3 was examined using a caspase-3 inhibitor (DEVD-FMK) conjugated to FITC as the fluorescent in situ marker in living cells (catalog QIA91; Merck Biosciences, Darmstadt, Germany). FITC-DEVD-FMK is cell-permeable, nontoxic, and irreversibly binds to activated caspase-3 in apoptotic cells. At 24 h after treatment of VSMC to melittin (0.8 μg/ml), with or without a caspase-3 inhibitor Z-VAD-FMK, the cultures were stimulated by 50 ng/ml PDGF-BB and then trypsinized with trypsin-EDTA and transferred into microtubes. After the addition of FITC-DEVD-FMK (1 μl) to each tube and incubation for 1 h in a 37°C incubator with 5% CO_2, the cells were centrifuged at 3000 rpm for 5 min, and the supernatant was removed. The cells were suspended in 0.5 ml of wash buffer and centrifuged twice. The resuspended cells in 300 μl of wash buffer were examined immediately under flow cytometry using the FL-1 channel.

Statistical Analysis. The experimental results were expressed as mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnett's test. Differences with $P < 0.05$ were considered statistically significant.

Results

BV and Melittin Inhibit Proliferation of Rat Aortic VSMC. We first sought to determine whether BV and melittin can inhibit the proliferation of rat aortic VSMC. Inhibi-

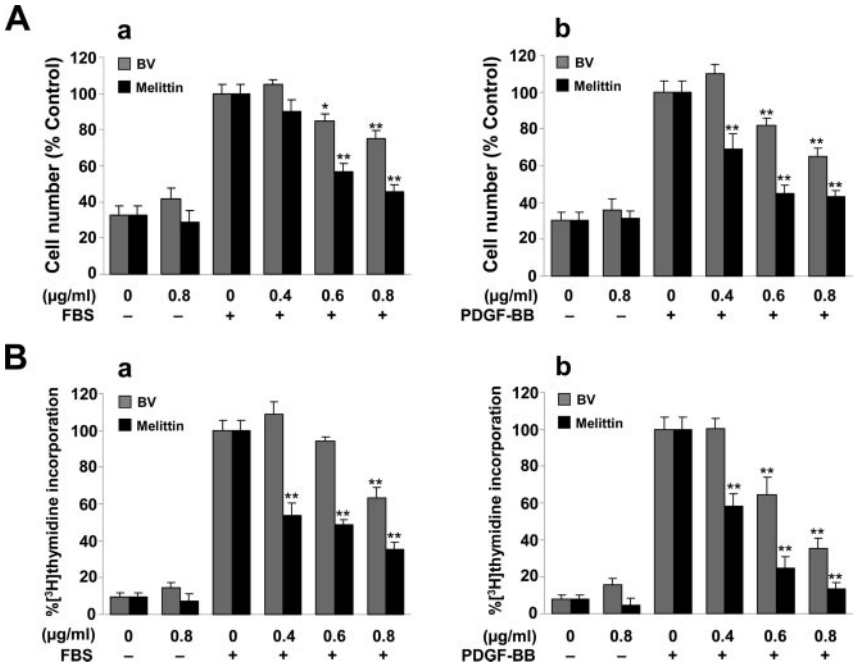

Fig. 1. Effect of BV and melittin on cell proliferation in VSMC. Cell proliferation was measured using by cell counting (A) and DNA synthesis assay (B) in rat aortic VSMC. Cells were pretreated with ~0.4 to 0.8 μg/ml BV and melittin for 24 h and then stimulated with 5% FBS (a) or 50 ng/ml PDGF-BB (b). Values are mean ± S.D. ($n = 3$). *, $P < 0.05$ and **, $P < 0.01$ indicate significantly different from the FBS- and PDGF-BB-treated cells.

TABLE 1
Inhibitory effect of BV and melittin on cell proliferation in VSMC
The results are expressed as mean ± S.D. (n = 9).

		Inhibition					
		BV			Melittin		
		0.4 μg/ml	0.6 μg/ml	0.8 μg/ml	0.4 μg/ml	0.6 μg/ml	0.8 μg/ml
		%					
Cell counting	FBS	0.0 ± 3.5	15.0 ± 2.9*	24.7 ± 3.6**	10.0 ± 1.6	43.0 ± 2.7**	54.2 ± 4.5**
	PDGF	0.0 ± 2.1	18.2 ± 1.9**	34.9 ± 2.7**	31.1 ± 3.2**	54.9 ± 4.1**	43.4 ± 2.2**
DNA synthesis	FBS	0.0 ± 1.8	5.8 ± 1.2	36.7 ± 3.4**	46.1 ± 2.8**	51.1 ± 1.9**	64.8 ± 3.5**
	PDGF	0.0 ± 3.6	35.4 ± 5.6**	64.4 ± 4.2**	45.9 ± 2.9**	75.2 ± 3.5**	86.6 ± 2.9**

* $P < 0.05$ and ** $P < 0.01$ indicate significantly different from the FBS- and PDGF-BB-treated cells.

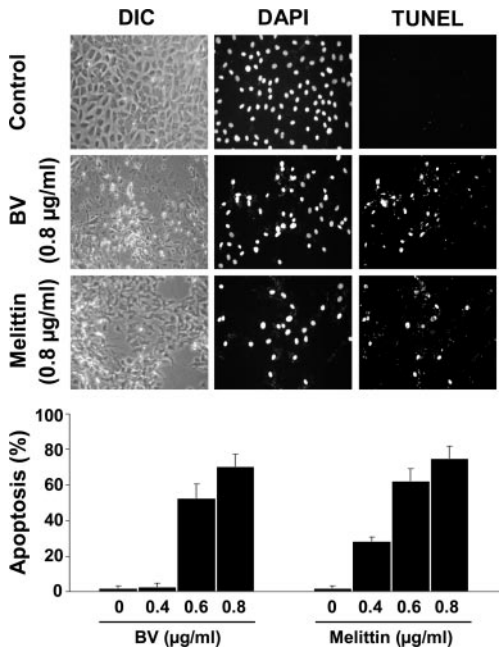

Fig. 2. Effect of BV and melittin on apoptosis induction of VSMC. The apoptotic cells were examined by morphologic analysis, DAPI staining, and TUNEL assay (top). Treatment of BV and melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, 200×). Total number of cells in a given area 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 (fluorescent microscopy magnification, 200×). The percentage of TUNEL-positive cells was calculated (bottom).

tory effects of BV and melittin on the proliferation of VSMC were examined by direct cell counting and DNA synthesis assay. The cell number was significantly increased by treatment with 5% FBS and 50 ng/ml PDGF-BB for 24 h and decreased significantly in a concentration-dependent manner by 24 h pretreatment with BV or melittin (Fig. 1A; Table 1). Effects of BV or melittin on DNA synthesis in rat aortic VSMC were tested using [^{3}H]thymidine incorporation. As shown in Fig. 1B, BV and melittin concentration-dependently inhibited [^{3}H]thymidine incorporation induced by FBS or PDGF-BB. The inhibitory effects were also dependent on concentration and corresponded with the inhibition of cell number (Table 1). Taken together, these results indicate that BV and melittin significantly inhibits rat aortic VSMC proliferation, and especially, melittin exhibited the strongest inhibition against PDGF-BB-induced VSMC proliferation.

Melittin Induces Apoptosis of VSMC. Given the potent inhibition on cell proliferation by BV and melittin, we evaluated VSMC apoptosis by cell-morphologic analysis and TUNEL assay. To observe the effect of BV and melittin on cell morphology, cells were examined via phase-contrast microscopy (ECLIPSE TE-300; Nikon Instech Co., Kawasaki, Kanagawa, Japan). Under the phase-contrast microscope, BV- and melittin-treated VSMC for 24 h had presented with cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 2, left). These morphological characteristics suggest that BV and melittin induce apoptotic cell death in VSMC. To further ascertain the induction of apoptosis by BV and melittin in VSMC, we 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 VSMC stained with DAPI (Fig. 2, middle). Indeed, TUNEL-positive cells were increased in BV and melittin-treated VSMC (Fig. 2, right). The treatments of BV and melittin strongly induced apoptosis of VSMC.

Melittin Suppress the NF-κB Activation. It has been well established that NF-κB activity is regulated by IκB proteins and that 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 and degradation (Fig. 3A). To further investigate, PDGF-BB-stimulated VSMC nuclear extract was prepared, and NF-κB DNA binding activity was assayed by EMSA. Cells were stimulated with 50 ng/ml PDGF-BB for 60 min in which PDGF-BB-induced strong NF-κB DNA binding activity was attenuated by melittin in a dose-dependent manner (Fig. 3B). 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, we 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 VSMC. Melittin (0.8 μg/ml) strongly attenuated this response in PDGF-BB-stimulated cells (Fig. 3C).

Melittin Inhibits Akt and ERK1/2 Activation. To investigate the mechanisms of the antiproliferative and proapoptotic effects exerted by melittin, we examined whether melittin could reduce the PDGF-BB-induced phosphorylation of Akt and ERK1/2. Pretreatment of 0.4, 0.6, and 0.8 μg/ml melittin significantly inhibited the PDGF-BB-induced phosphorylation of Akt in a concentration-dependent manner (Fig. 4A). Likewise, melittin (0.8 μg/ml) effectively inhibited PDGF-BB-induced phosphorylation of Akt in immunofluores-

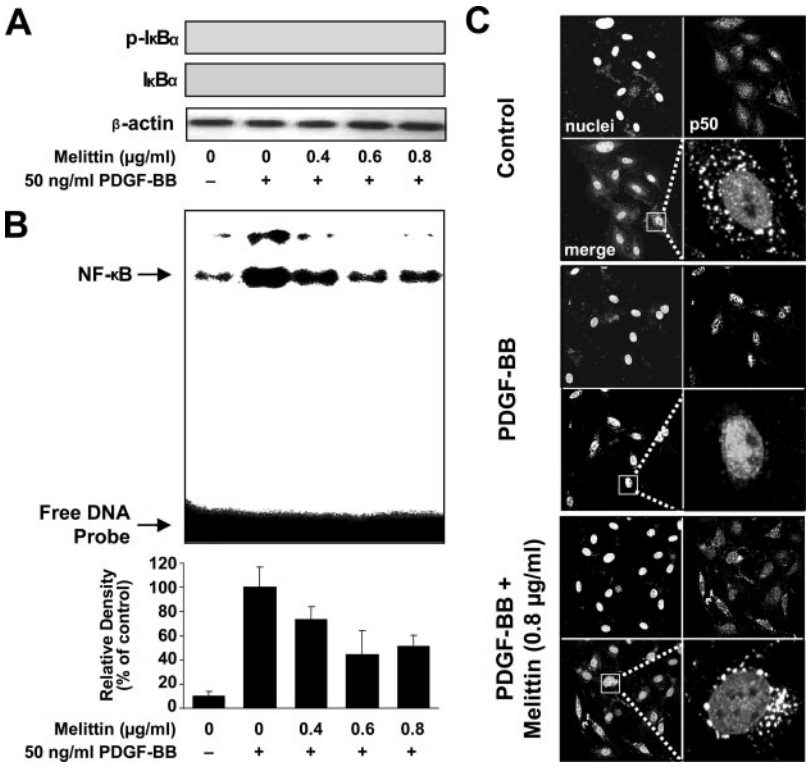

Fig. 3. Effects of melittin on phosphorylation and degradation of IκBα and NF-κB activation in VSMC. For examinations of IκBα phosphorylation and degradation (A), cells were pretreated with ~0.4 to 0.8 μg/ml melittin for 24 h, and then PDGF-BB was added to the cells for another 30 or 60 min. After treatment, cells were harvested, and Western blot analysis was performed. For examination of NF-κB DNA binding assay (B), cells were pretreated with ~0.4 to 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. The translocation of NF-κB p50 subunit (C) was determined by immunofluorescence confocal laser scanning microscopy (magnification, 630×). After stimulation with 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. The values are mean ± S.D. (n = 3).

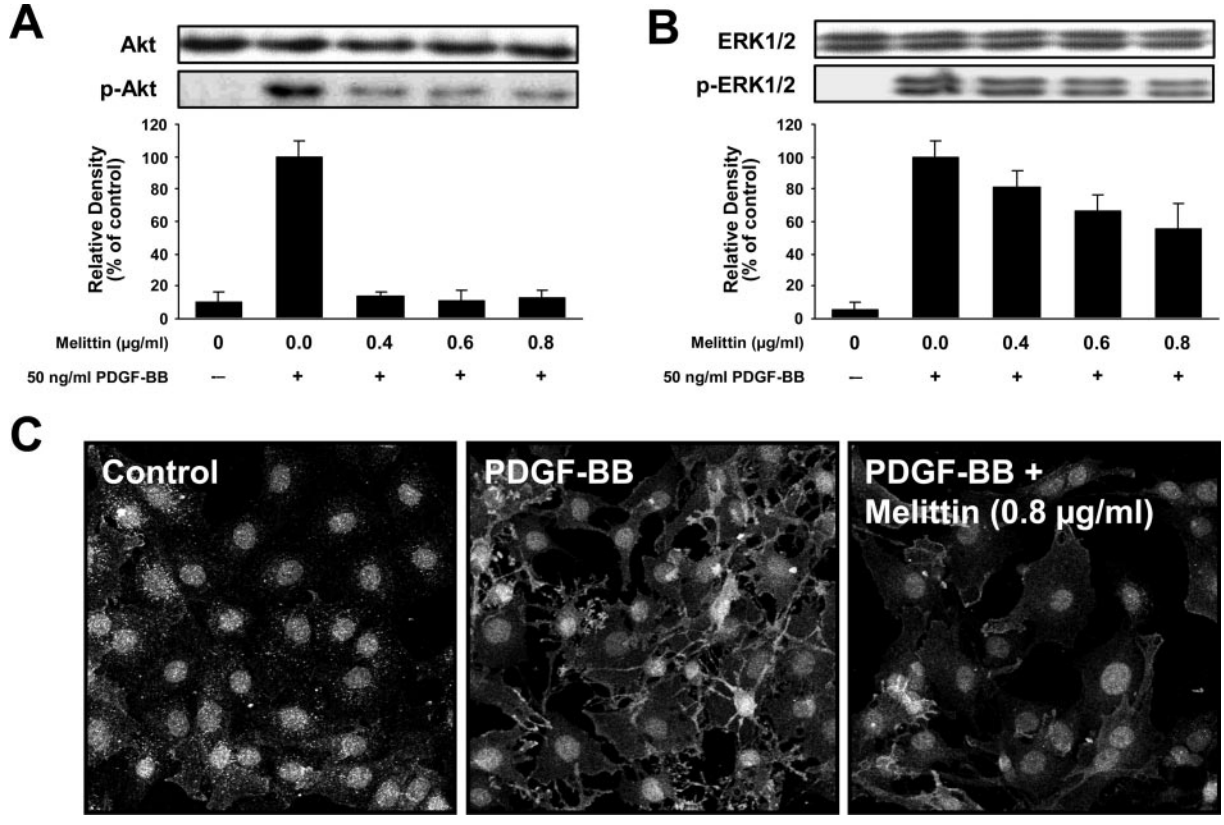

Fig. 4. Effect of melittin on phosphorylation of Akt and ERK1/2 in rat aortic VSMC. Cells were pretreated with ~0.4 to 0.8 μg/ml melittin for 24 h and then stimulated with 50 ng/ml PDGF-BB. Equal amounts of whole-cell lysate (30 μg) were subjected to electrophoresis and analysis by Western blot for total and phosphorylated Akt (A) and ERK1/2 (B). The inhibitory effect of melittin on phosphorylation of Akt was also confirmed immunofluorescence confocal laser-scanning microscopy (magnification, 630×) (C). Cells were examined under control condition (left) after being stimulated by 50 ng/ml PDGF-BB (middle) or pretreatment with 0.8 μg/ml melittin for 24 h (right). VSMC were specifically stained for phospho-Akt (red) and nuclear (blue). The values are mean ± S.D. (n = 3).

cence assay (Fig. 4C). Melittin also inhibited 50 ng/ml PDGF-BB-induced phosphorylation of ERK1/2 in a concentration-dependent manner, but not as potently as Akt (Fig. 4B).

Melittin Induces Expression of Apoptosis Regulatory Proteins. We investigated the involvement of apoptosis regulatory proteins on melittin-induced apoptosis in VSMC. Cell cytosol extracts were prepared from VSMC 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 proapoptotic proteins p53, Bax, and active form of caspase-3 was up-regulated in a dose-dependent manner in the VSMC treated by melittin (~0.4–0.8 μg/ml), whereas the expression of antiapoptotic protein Bcl-2 was down-regulated (Fig. 5A). We also performed the flow cytometry and confocal laser-scanning microscopy analysis to demonstrate clearly the effect of melittin on caspase-3 activation in situ in VSMC. Treatment of melittin (0.8 μg/ml) increased the number of caspase-3-activated cells (right-shifted), which was inhibited (reversed) by pretreatment of Z-VAD-FMK, a caspase-3 inhibitor (Fig. 5B, top). Moreover, melittin strongly increased active-caspase-3 in VSMC (Fig. 5B, bottom).

Discussion

The purposes of the present study were to find out whether BV and melittin possesses an inhibitory effect and to define its mechanisms of action on rat aortic VSMC proliferation. In the present study, it was demonstrated that antiproliferative mechanism of BV and melittin on VSMC proliferation was induction of apoptosis via suppressions of NF-κB and Akt activation and enhancement of apoptotic signal pathway.

The abnormal growth of VSMC is a prominent feature of vascular disease, including atherosclerosis and postangioplasty restenosis (Ross, 1993). Neointimal thickening is mainly due to VSMC, which proliferate and migrate from the vascular media. Excessive proliferative potential can be regulated by apoptosis (Perlman et al., 2000). In the present study, we showed that BV and melittin significantly inhibited FBS- and PDGF-BB-induced proliferations of VSMC (Fig. 1). Because our results also demonstrated that BV and melittin induced apoptosis of VSMC, we believe that the inhibitory effect of BV and melittin on cell proliferation of VSMC results from proapoptotic properties.

Various studies have implicated a role for PDGF in VSMC proliferation, atherosclerosis, and neointima hyperplasia. PDGF-BB, which is expressed in multiple cell types, including VSMC, is a potent mitogen for VSMC in vitro and in vivo (Ikeda et al., 1990; Ferns et al., 1991; Raines, 2004). It is well known that PDGF-BB activates NF-κB in cultured cells, which lead to cell proliferation (Obata et al., 1996; Romashkova et al., 1999). 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 VSMC after balloon injury of carotid arteries and atherosclerosis lesions (Brand et al., 1997; Mehrhof et al., 2005). 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 VSMC, and NF-κB activation correlates with VSMC proliferation and induced expression of NF-κB-dependent genes (Landry et al., 1997). These strongly suggest a causative role for NF-κB in the development and maintenance of atherosclerosis and neointimal hyperplasia. Therefore, we investigated the effects of melittin

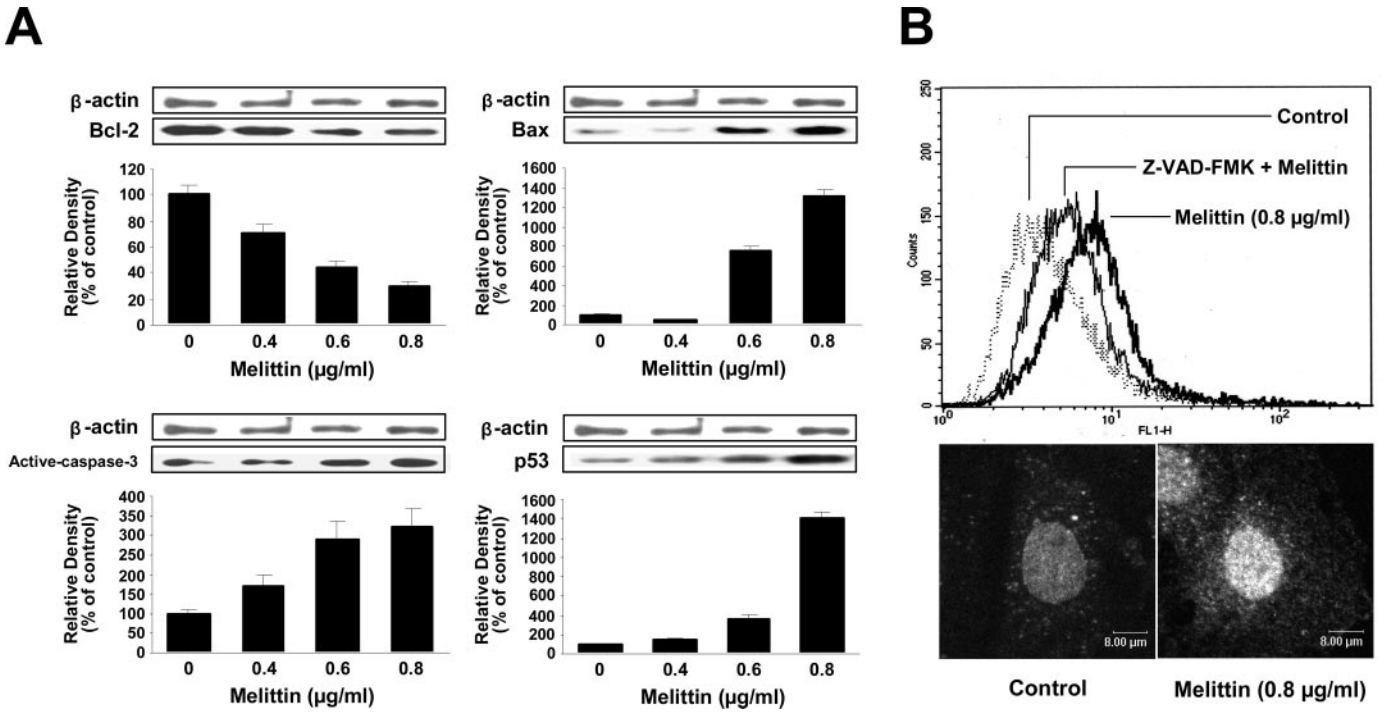

Fig. 5. Effects of melittin on apoptosis regulatory protein expression. Cells were incubated for 24 h with or without ~0.4 to 0.8 μg/ml melittin, and the expression of Bcl-2, Bax, active-caspase-3, and p53 were determined by Western blot analysis (A). Effect of melittin on caspase-3 activation was also examined by flow cytometry and confocal microscopy analysis (B). All experiments were performed under PDGF-BB stimulation. The values are mean ± S.D. (n = 3).

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, because these two events are essential for the nuclear translocation and activation of NF-κB (Romashkova and Makarov, 1999). We found that melittin significantly inhibited the PDGF-BB-induced IκBα phosphorylation and its degradation (Fig. 3A). 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. 4B) and NF-κB p50 subunit nuclear translocation (Fig. 4C) in response to the action of PDGF-BB. We recently demonstrated that BV and melittin strongly reduced NF-κB activation through directly binding to the p50 subunit (Park et al., 2004). Therefore, our results strongly suggest that melittin suppresses NF-κB activation, leading to an inhibition of VSMC proliferation and an increase in VSMC 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 seem to lead to two distinct end effectors and that they are regulated independently by various stimulators and intermediate signal transduction molecules (Rommel et al., 1999; Zimmermann and Moelling, 1999). 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 VSMC. In our study, melittin markedly inhibited the PDGF-BB-induced phosphorylation of Akt (Fig. 4, A and C). However, melittin weakly affected the ERK1/2 phosphorylation induced by PDGF-BB for 5 min (Fig. 4B). It has been recently reported that AKT activation induces cell proliferation and enhances resistance to apoptosis signaling through regulation of NF-κB (Grandage et al., 2005; Jeong et al., 2005). Therefore, our results suggested that the Akt pathway was involved in melittin-induced proapoptotic effect through suppression of NF-κB.

Recently, Jang et al. (2003) and Hong et al. (2005) 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 BclII expression. Therefore, we were interested in investigating whether melittin induces expressions of apoptosis regulatory proteins in VSMC. It was found that, consistent with the increase of the induction of apoptosis, the expression of proapoptotic proteins p53, Bax, and active-caspase-3 was dose-dependently increased but that antiapoptotic protein Bcl-2 was decreased (Fig. 5). 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 VSMC.

In summary, we have demonstrated that BV and melittin inhibit cell proliferation and induce apoptosis in rat aortic VSMC. In particular, melittin potently inhibited PDGF-BB-induced phosphorylation and degradation of IκB and markedly suppressed activation of NF-κB and phosphorylation of Akt and weakly inhibited phosphorylation of ERK1/2. Melittin also increased expression of proapoptotic protein p53, Bax, and caspase-3 and decreased antiapoptotic protein Bcl-2. The antiproliferative effect of BV and melittin on VSMC may be due to induction of apoptosis via suppressions of NF-κB and Akt activation and enhancement of proapoptotic signaling pathway.

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