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Induction of Nitric Oxide Production by the Cytostatic Macrolide Apicularen A [2,4-Heptadienamide, *N*-[(1*E*)-3-[(3*S*,5*R*,7*R*,9*S*)-3,4,5,6,7,8,9,10-octahydro-7,14 Dihydroxy-1-oxo-5,9-epoxy-1*H*-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9Cl)] and Possible Role of Nitric Oxide in Apicularen A-Induced Apoptosis in RAW 264.7 Cells

JangJa Hong, Aya Yokomakura, Yasuhiro Nakano, Hyun Seung Ban, Kenji Ishihara, Jong-Woong Ahn, OkPyo Zee, and Kazuo Ohuchi

Laboratory of Pathophysiological Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Aoba-ku, Sendai, Miyagi, Japan (J.J.H., A.Y., Y.N., H.S.B., K.I., K.O.); Division of Ocean Science, Korea Maritime University, Dongsam-dong, Youngdo-ku, Busan, Korea, (J.-W.A.); and Laboratory of Pharmacognosy, Graduate School of Pharmacy, Sungkyunkwan University, Chunchun-dong, Suwon, Kyungi-do, Korea (J.J.H., O.P.Z.)

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ABSTRACT

We previously reported that apicularen A [2,4-heptadienamide, *N*-[(1*E*)-3-[(3*S*,5*R*,7*R*,9*S*)-3,4,5,6,7,8,9,10-octahydro-7,14 dihydroxy-1-oxo-5,9-epoxy-1*H*-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9Cl)], a highly cytostatic macrolide isolated from the myxobacterial genus *Chondromyces*, induces apoptosis in the mouse leukemic monocyte cell line RAW 264.7. To analyze the action mechanism of apicularen A for the induction of apoptosis, effects of apicularen A on nitric oxide (NO) production in RAW 264.7 cells were examined. It was demonstrated that apicularen A at 10 and 100 nM induced nitrite production, whereas apicularen B [2,4-heptadienamide, *N*-[(1*E*)-3-[(3*S*,5*R*,7*R*,9*S*)-7-[[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-3,4,5,6,7,8,9,10-octahydro-14-hydroxy-1-oxo-5,9-epoxy-1*H*-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9Cl)], an *N*-acetyl-glucosamine glycoside of apicularen A, had no effect at 100 nM. The apicularen A-induced nitrite production was accompanied by an increase in the level of inducible nitric-oxide synthase (iNOS) and its mRNA and was suppressed by the NOS inhibitor N^G-monomethyl-L-arginine acetate (L-NMMA). In addition, apicularen A activated nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) and decreased the level of IκB-α and increased that of phosphorylated c-Jun N-terminal kinase (JNK). Furthermore, the apicularen A-induced nitrite production was suppressed by the NF-κB inhibitor Bay 11-7082 [(*E*)-3-(4-methylphenylsulfonyl)-2-propenenitrile] and the JNK inhibitor SP600125 [anthra[1,9-*cd*]pyrazol-6(2*H*)-one]. These findings suggested that apicularen A activates NF-κB and AP-1, thus triggering the expression of iNOS mRNA and iNOS protein and induces NO production. Finally, apicularen A decreased cell growth and survival and cell viability and disrupted the mitochondrial membrane potential. The addition of L-NMMA partially recovered the apicularen A-induced decrease in cell growth and survival and cell viability and the disruption of mitochondrial membrane potential. These findings suggested that NO produced by apicularen A treatment participate partially in the apicularen A-induced apoptosis in RAW 264.7 cells.

The cytostatic macrolides apicularens A and B have been isolated from a variety of strains of the myxobacterial genus *Chondromyces* (i.e., *C. apiculatus*, *C. lanuginosus*, *C. pediculatus*, and *C. robustus)* (Kunze et al., 1998). Structurally, apicularen A features a *trans*-hydroxypyran with a salicylic acid residue within a 10-membered lactone, which bears a highly unsaturated enamide side chain (Fig. 1). This natural product is usually found with varying amounts of its glycoconjugate with *N*-acetyl glucose, known as apicularen B (Fig. 1).

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; apicularen A, 2,4-heptadienamide, *N*-[(1*E*)-3-[(3S,5*R*,7*R*,9*S*)-3,4,5,6,7,8,9,10-octahydro-7,14 dihydroxy-1-oxo-5,9-epoxy-1*H*-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9Cl); apicularen B, 2,4-heptadienamide, *N*-[(1*E*)-3-[(3S,5*R*,7*R*,9*S*)-7-[[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-3,4,5,6,7,8,9,10-octahydro-14-hydroxy-1-oxo-5, 9-epoxy-1*H*-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9Cl); PD98059, 2′-amino-3′-methoxyflavone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; NO, nitric oxide; CHOP, C/EBP homologous protein; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine acetate; NOS, nitric-oxide synthase; MEK, mitogen-activated protein kinase kinase; Bay 11-7082, (*E*)-3-(4-methylphenylsulfonyl)-2-propenenitrile; JNK, c-Jun N-terminal kinase; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; 7-ADD, 7-amino-actinomycin D; iNOS, inducible NOS; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; AP-1, activator protein-1; V-ATPase, vacuolar-type (H^+)-ATPase.

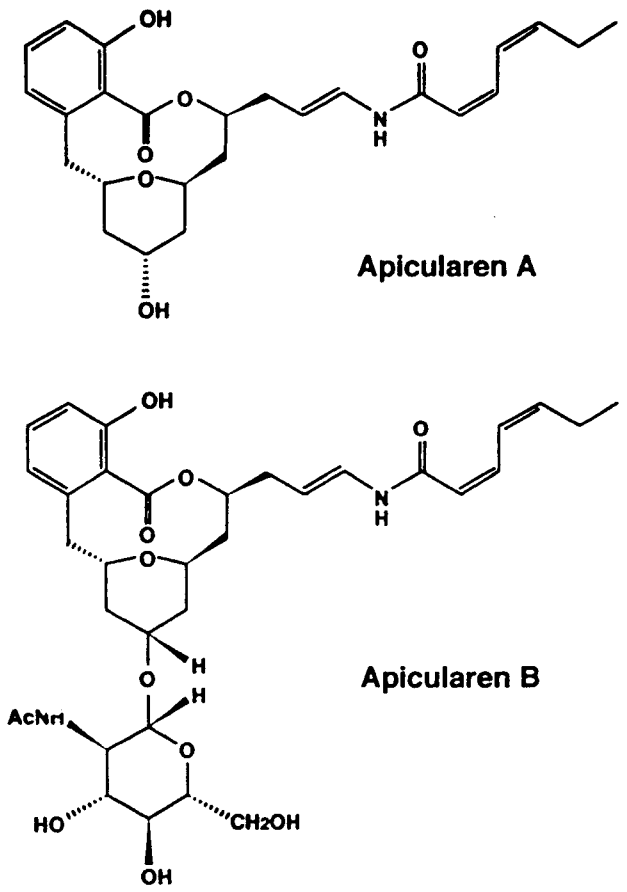

Fig. 1. Chemical structures of apicularens A and B

Apicularen A is reported to be highly cytostatic against a range of human cancer cells, including ovarian, prostate, lung, kidney, leukemia, cervix, and histocytic cell lines and the multidrug-resistant cell line KB-VI with IC_{50} values ranging between 0.227 and 22.7 nM, whereas apicularen B is distinctly less cytostatic than apicularen A with IC_{50} values ranging between 0.317 and 1.8 μM (Kunze et al., 1998; Jansen et al., 2000). In addition, it was found that apicularen A exhibited a potent inhibitory effect on the growth of bovine aortic endothelial cells without any evidence of cytotoxicity even when concentrations were increased to 22.7 nM (Kwon et al., 2002). This compound also showed inhibition of basic fibroblast growth factor-induced invasion and capillary tube formation of bovine aortic endothelial cells at low concentrations (Kwon et al., 2002). Accordingly, apicularen A was suggested to be a novel antiangiogenic compound with potent antitumor activity (Kwon et al., 2002).

Recently, we have reported that apicularen A but not apicularen B at 10 to 100 nM induced apoptosis in RAW 264.7 cells, a mouse leukemia monocytic cell line (Hong et al., 2003b). Apicularen A but not apicularen B also induced apoptosis in HL-60 cells, a human leukemia cell line, at 10 and 100 nM (Hong et al., submitted). Consequently, we speculated that apicularen A might be a candidate for an antileukemic drug. The p44/42 mitogen-activated protein kinase (MAPK) inhibitor PD98059 rescued RAW 264.7 cells from the apicularen A-induced decrease in cell growth and survival as determined by 3-(4,5-dimethyl(thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, whereas the p38 MAPK inhibitor SB203580 augmented the effect of apicularen A (Hong et al., 2003b). Therefore, we suggested that the activation of p44/42 MAPK is proapoptotic and the activation of p38 MAPK is antiapoptotic in apicularen A-treated RAW 264.7 cells. However, the precise mechanism for the induction of apoptosis by apicularen A has remained to be clarified. To further elucidate the biological activities of apicularens A and B, we examined effects of these compounds on nitric oxide (NO) production in RAW 264.7 cells because NO is a potent inducer of apoptosis in various types of cells in vitro (Shimaoka et al., 1995; Lincoln et al., 1996; Leist et al., 1997) including RAW 264.7 cells (Messmer et al., 1995; Jun et al., 1999; Gotoh et al., 2002). It is reported that NO-induced apoptosis in RAW 264.7 cells is mediated by the endoplasmic reticulum stress pathway involving p50ATF6 and C/EBP homologous protein (CHOP) (Gotoh et al., 2002). ATF6 exists constitutively as a transmembrane protein p90ATF6 in the endoplasmic reticulum under nonstressed conditions. The endoplasmic reticulum stress induces proteolysis of p90ATF6 and releases a soluble transcription factor p50ATF6, which is transported into the nucleus, binds to the endoplasmic reticulum stress responsive element of the *CHOP* gene, and activates its transcription (Yoshida et al., 2000). CHOP functions as a transcription factor, and it is reported that CHOP expression results in down-regulation of Bcl-2 expression, depletion of cellular glutathione, and exaggerates production of reactive oxygen species (McCullough et al., 2001). The aim of the present study is to clarify whether apicularen A induces NO production and to clarify roles of NO in apicularen A-induced apoptosis in RAW 264.7 cells.

Materials and Methods

Drugs. Apicularen A and apicularen B were purified from culture medium of *C. apiculatus* JW 184 according to the method described by Jansen et al. (2000). The compounds isolated were identified by a comparison of spectral data (NMR, infrared, and UV) and $[\alpha]_D$ using authentic compounds, and the purity of each isolated compound was confirmed to be more than 99%. Chemical structures of apicularens A and B are shown in Fig. 1.

Lipopolysaccharide (LPS) and N^G-monomethyl-L-arginine acetate (L-NMMA), a nonspecific inhibitor of nitric-oxide synthase (NOS), were purchased from Wako Pure Chemicals (Osaka, Japan). The mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 was purchased from New England Biolabs (Beverly, MA). The inhibitor of IκB-α phosphorylation Bay 11-7082 and the inhibitor of c-Jun N-terminal kinase (JNK) SP600125 were purchased from Calbiochem (Darmstadt, Germany). All the drugs except for LPS were dissolved in dimethyl sulfoxide (DMSO). LPS was dissolved in the medium. An aliquot of each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to 0.1%. The control medium contained the same amount of the vehicle.

Cell Culture. RAW 264.7 cells were obtained from Riken Gene Bank (Tsukuba, Japan) and cultured at 37°C under 5% CO_2/95% air in Eagle's minimal essential medium (Nissui, Tokyo, Japan) containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1% nonessential amino acid solution (Sigma-Aldrich), penicillin G potassium (18 μg/ml), and streptomycin sulfate (50 μg/ml) (Meiji Seika Kaisha, Ltd., Tokyo, Japan). The cells at passage number 10 or lower were used for experiments.

Measurement of Nitrite. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for the

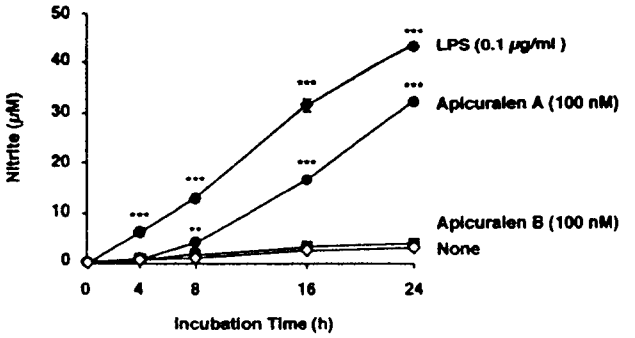

Fig. 2. Time changes in nitrite production. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for the period indicated at 37°C in 0.5 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: **, $P < 0.01$; ***, $P < 0.001$ versus the corresponding control.

specified period at 37°C in 0.5 ml of medium in the presence or absence of drugs. After incubation, nitrite levels in the conditioned medium were determined using Griess reagent (Green et al., 1982).

Measurement of Cell Growth and Survival. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 32 h at 37°C in 0.5 ml of medium containing various drugs, then 10 μl of phosphate-buffered saline containing MTT (Sigma-Aldrich) (5 mg/ml) was added, and the cells were further incubated for 4 h at 37°C. After the removal of the medium, 100 μl of DMSO was added, and the absorbance at 595 nm was determined (Mosmann, 1983).

Measurement of Cell Viability by Flow Cytometry. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 36 h at 37°C in 0.5 ml of medium containing apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml) in the presence of various concentrations of L-NMMA. After incubation, the cells were scraped off the plate, washed three times with medium, and incubated for 30 min at room temperature in the dark in 0.5 ml of phosphate-buffered saline (PBS) containing 10 μg of 7-amino-actinomycin D (7-ADD; Sigma-Aldrich). The fluorescence of the cells stained with 7-ADD was analyzed by flow cytometry using FACScan (BD Biosciences, San Jose, CA), and the percentage of the nonviable cells was calculated (Hong et al., 2003a).

Detection of Mitochondrial Membrane Potential. To analyze mitochondrial depolarization, a marker of apoptosis, RAW 264.7 cells were stained with a mitochondrial membrane potential-dependent dye DePsipher (Trevigen, Inc., Gaithersburg, MD). RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 2 ml of medium in the presence or absence of apicularen A (100 nM), apicularen B (100 nM), and L-NMMA (100 μM). For the microscopic observation, the cells were washed three times with PBS and stained using a DePsipher Kit (Trevigen Inc.) and observed under a florescence microscope (IX70; Olympus, Tokyo, Japan). For the flow cytometric analysis, the cells were washed three times with PBS, scraped off the plate, and stained using a DePsipher kit (Trevigen Inc.). Subsequently, the intensities for green fluorescence (FL1; a maximal emission at 530 nm) and red fluorescence (FL2; a maximal emission at 590 nm) were analyzed by flow cytometry using FACScan (BD Biosciences), and the percentage of the cells with decreased mitochondrial membrane potential ($\Delta\psi_m$) was calculated.

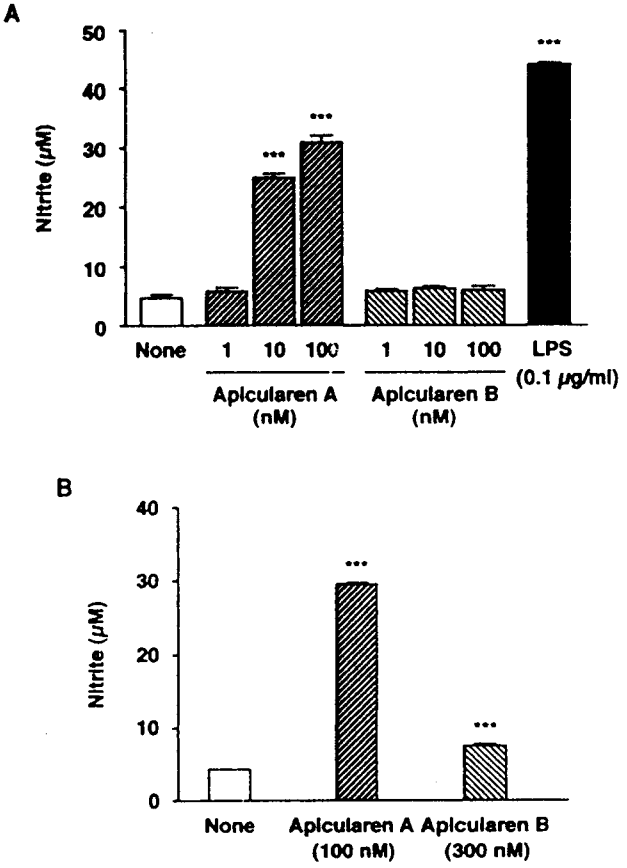

Fig. 3. Effects of various concentrations of apicularen A and apicularen B on nitrite production. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 0.5 ml of medium in the presence of 1, 10, and 100 nM apicularen A or apicularen B, 0.1 μg/ml LPS (A), or in the presence of 100 nM apicularen A or 300 nM apicularen B (B). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples for (A) and three samples for (B) with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the control.

Western Blotting Analysis. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated at 37°C for 1 h for the detection of IκB-α and JNK and for 24 h for the detection of iNOS in 2 ml of medium in the presence or absence of drugs. After incubation, Western blotting for iNOS, IκB-α, actin, and JNK was carried out as described previously (Ban et al., 2004). The levels of each protein were quantified by scanning densitometry, and the individual band density value for each point was expressed as the relative density signal.

Semiquantitation of the RNA Level of iNOS by Reverse Transcription-Polymerase Chain Reaction (PCR). RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 6 h at 37°C in 2 ml of medium in the presence or absence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). The cells were washed with ice-cold PBS, and the total RNA was extracted using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The yield of RNA extracted was determined by spectrophotometry. One microgram of each sample was reverse-transcribed for 1 h at 37°C in 20 μl of buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$) containing 5 μM random hexamer oligonucleotides (Invitrogen, Carlsbad, CA), 500 μM 2′-deoxynucleotide 5′-triphosphate (dNTP; Takara Bio Inc., Shiga, Ja-

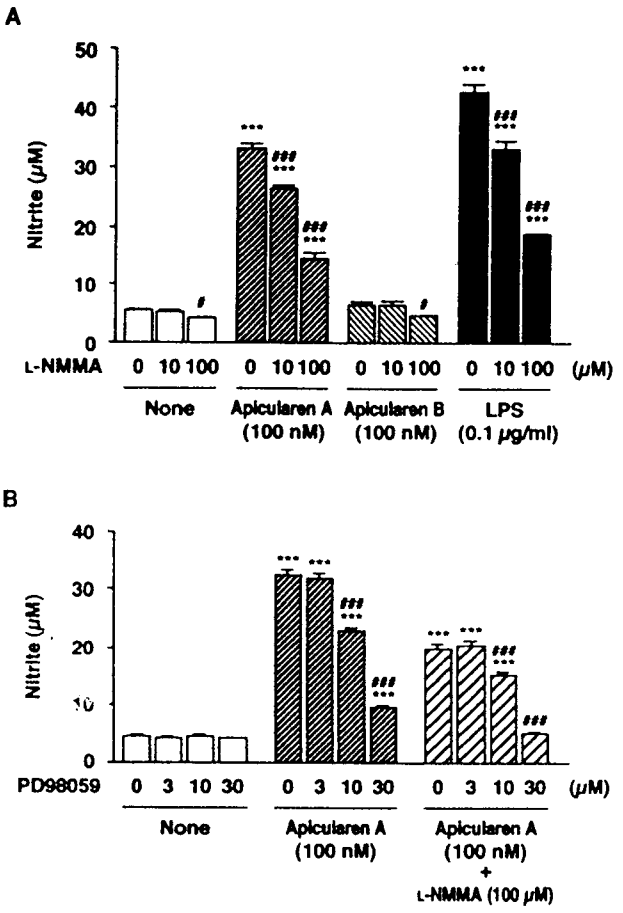

Fig. 4. Effects of L-NMMA and PD98059 on apicularen A-induced nitrite production. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 0.5 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml), and the indicated concentrations of L-NMMA (A) or in the presence of apicularen A (100 nM), L-NMMA (100 μM), and the indicated concentrations of PD98059 (B). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control and #, $P < 0.05$; ###, $P < 0.001$ versus the corresponding control.

pan), and 10 mM dithiothreitol (Takara Bio Inc.). The sequences of primers for iNOS used were (forward) 5′-GTGTTCCACCAGGAGATGTTG-3′ and (reverse) 5′-CTCCTGCCCACTGAGTTCGTC-3′, which amplify a 576-base pair fragment of iNOS. PCR mixtures consisted of 10 μl of the reverse-transcribed RNA solution and 40 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$) containing 0.2 μM each primer, 200 μM 2′-deoxynucleotide 5′-triphosphate, and 1.25 U of *Taq* polymerase (Takara Bio Inc.). PCR was performed for 27 cycles: 30 s denaturation at 94°C, 1 min annealing at 54°C, and 1 min extension at 72°C using a thermal cycler (PCR Thermal Cycler SP; Takara Bio Inc.). The level of mRNA for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined as an internal control. The PCR primers for rat GAPDH were (forward) 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and (reverse) 5′-TCCTTGGAGGCCATGTAGGCCAT-3′, which amplify a 249-base pair GAPDH fragment (Robbins and McKinney, 1992). PCR was performed for 24 cycles: 30 s denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C. After PCR, 10 μl of the reaction mixture was loaded onto a 1.5% agarose minigel, and the PCR products were visualized by ethidium bromide staining after electrophoresis. The levels of mRNA for iNOS and GAPDH were quantified by scanning densitometry, and the ratio of mRNA for iNOS to GAPDH was calculated.

Electrophoretic Mobility Shift Assay (EMSA). RAW 264.7 cells (4×10^6 cells) were incubated for 4 h at 37°C in 4 ml of Eagle's minimal essential medium containing 10% fetal bovine serum in the presence or absence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). After incubation, the nuclear extract was prepared according to the method described previously (Ban et al., 2004). EMSA was carried out according to the protocol accompanying the Gel Shift Assay System (Promega, Madison, WI). Briefly, the double-stranded oligonucleotide probes containing NF-κB- and AP-1-binding sequences were end-labeled with 1.85 MBq of [γ-^{32}P]ATP (111 TBq/nmol; PerkinElmer Life and Analytical Sciences, Boston, MA) using T4 polynucleotide kinase. The nuclear extract (4 μg) was incubated at room temperature for 20 min with 4 μl of ^{32}P labeled probe in a binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 mg/ml poly(dI-dC), and 20% glycerol). DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 4% acrylamide gel, and the gel was vacuum-dried and visualized with a GS-250 Molecular Imager (Bio-Rad, Hercules, CA) (Ban et al., 2004).

Statistical Analysis. The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons and Student's *t* test for unpaired observations.

Results

Induction of Nitrite Production by Apicularen A and Apicularen B. A significant increase in nitrite production by apicularen A at 100 nM was observed at 8 h and thereafter (Fig. 2). The increase in nitrite production by apicularen A at 8 h was low, while the effect of LPS (0.1 μg/ml) on nitrite production appeared earlier than that of apicularen A (100 nM) (Fig. 2). Apicularen A at 1000 nM did not induce nitrite production at 4 h but increased it at 8 h to almost the same level as that of 100 nM apicularen A (data not shown). Apicularen B at 100 nM had no significant effect on nitrite production at 8 to 24 h (Fig. 2). At 24 h of incubation, levels of nitrite in the conditioned medium were increased by apicularen A at 10 and 100 nM but not at 1 nM, whereas apicularen B at 1 to 100 nM had no effect (Fig. 3A), but at 300 nM, apicularen B increased nitrite production slightly but significantly (Fig. 3B). These findings indicate that apicularen A more potently increases NO production than apicularen B.

Effects of L-NMMA and PD98059 on the Apicularen A-Induced Nitrite Production. As well as LPS (0.1 μg/ml)-induced nitrite production, apicularen A (100 nM)-induced nitrite production at 24 h was inhibited by L-NMMA at 10 and 100 μM in a concentration-dependent manner (Fig. 4A). Spontaneous nitrite production in the control group and the apicularen B (100 nM)-treated group at 24 h was also inhibited by L-NMMA at 100 μM (Fig. 4A). The MEK inhibitor PD98059 at 10 and 30 nM also inhibited the apicularen A (100 nM)-induced nitrite production at 24 h (Fig. 4B). Suppression of the apicularen A (100 nM)-induced nitrite production by L-NMMA (100 μM) was further augmented by PD98059 at 10 and 30 nM (Fig. 4B).

Effects of Apicularen A on the Levels of iNOS Protein and mRNA. On treatment with apicularen A at 10 and 100 nM, protein levels of iNOS were increased at 24 h, but apicularen B (10 and 100 nM) had no effect (Fig. 5A). The effect of apicularen A was time-dependent. At 8 h, apicularen A at 10 and 100 nM slightly increased iNOS protein levels

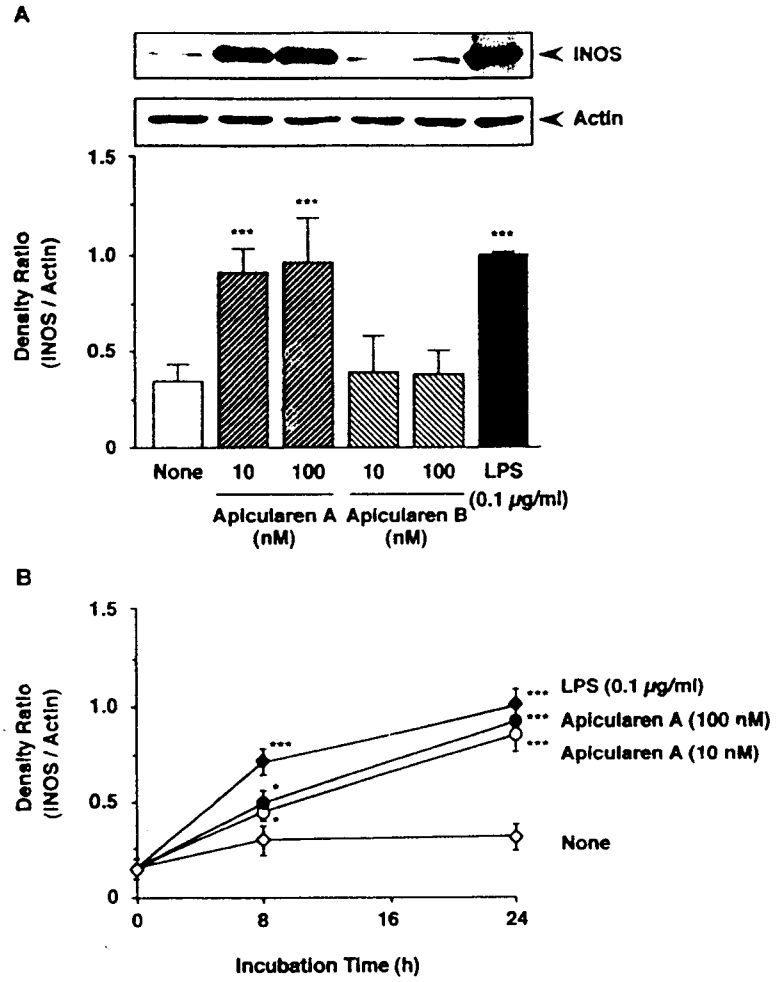

Fig. 5. Effects of apicularen A on the levels of iNOS protein. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 2 ml of medium in the presence of apicularen A (10, 100 nM), apicularen B (10, 100 nM), or LPS (0.1 μg/ml) (A) or 8 and 24 h at 37°C in 2 ml of medium in the presence of apicularen A (10, 100 nM) or LPS (0.1 μg/ml) (B). The protein levels of iNOS and actin were determined by Western blotting analysis. The density ratios of iNOS to actin were calculated, and the mean density ratio in the LPS group at 24 h is set to 1.0. Values are the means from three samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control (A). *, $P < 0.05$; ***, $P < 0.001$ versus the corresponding nonstimulated control (B).

(Fig. 5B). LPS (0.1 μg/ml) also increased the protein level of iNOS at 8 and 24 h (Fig. 5, A and B). In accordance with the increase in protein levels of iNOS, iNOS mRNA levels at 6 h were increased by apicularen A (100 nM) and LPS (0.1 μg/ml) but not by apicularen B (100 nM) (Fig. 6).

Activation of NF-κB and AP-1 by Apicularen A. To clarify the mechanism of action of apicularen A for the expression of iNOS protein, effects of apicularen A on the activation of NF-κB and AP-1 were examined by EMSA. Treatment with apicularen A (100 nM) for 4 h increased the activation of both NF-κB and AP-1, but treatment with apicularen B (100 nM) had no effect (Fig. 7, A and B). LPS (0.1 μg/ml) treatment also activated NF-κB and AP-1 (Fig. 7, A and B). These findings suggested that the increase in the levels of iNOS mRNA by apicularen A is induced through the activation of NF-κB and AP-1.

Effects of Apicularen A on the Levels of IκB-α and Phosphorylation of JNK. Incubation of RAW 264.7 cells with apicularen A (100 nM) decreased the level of IκB-α at 1 h, but apicularen B (100 nM) had no effect (Fig. 8). LPS (0.1 μg/ml) also decreased the level of IκB-α at 1 h (Fig. 8A). In addition, incubation of RAW 264.7 cells with apicularen A (100 nM) for 1 h induced phosphorylation of JNK, but apicularen B (100 nM) had no effect (Fig. 8B). LPS (0.1 μg/ml) also phosphorylated JNK at 1 h (Fig. 8B). These findings suggested that apicularen A activated NF-κB by decreasing the

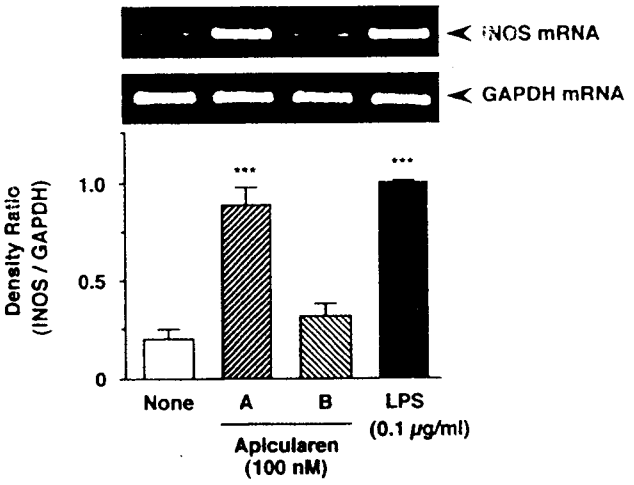

Fig. 6. Effects of apicularen A on the levels of iNOS mRNA. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 6 h at 37°C in 2 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). Total RNA was extracted and reverse transcription-PCR for iNOS mRNA and GAPDH mRNA was performed. The density ratios of iNOS mRNA/GAPDH mRNA were calculated, and the mean density ratio in the LPS group is set to 1.0. Values are the means from three samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control.

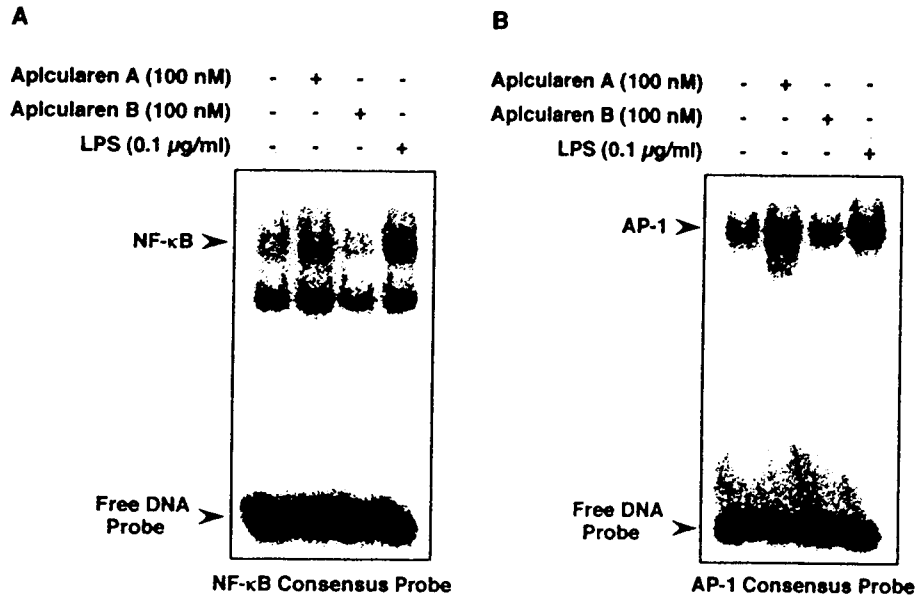

Fig. 7. Activation of NF-κB and AP-1 by apicularen A. RAW 264.7 cells (2×10^6 cells) were incubated for 24 h at 37°C in 4 ml of medium. The cells were then washed three times with medium and further incubated for 4 h at 37°C in 4 ml of medium in the presence (+) or absence (−) of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). After incubation, nuclear proteins were extracted, and the amount of NF-κB (A) and AP-1 (B) bound to each DNA probe was detected by EMSA. Similar results were obtained in three separate sets of experiments.

level of IκB-α and activated AP-1 through phosphorylation of JNK.

Effects of Bay 11-7082 and SP600125 on the Apicularen A-Induced Nitrite Production. The apicularen A-induced nitrite production at 24 h was inhibited by the NF-κB inhibitor Bay 11-7082 and the JNK inhibitor SP600125 in a concentration-dependent manner (Fig. 9A). Combined treatment with Bay 11-7082 at 3 μM and SP600125 at 10 μM further inhibited the apicularen A (100 nM)-induced nitrite production, but the complete inhibition was not induced (Fig. 9B). These findings suggest that the activation of NF-κB and AP-1 participated in the apicularen A-induced nitrite production.

Effects of L-NMMA on the Apicularen A-Induced Decrease in Cell Growth and Survival. Incubation of RAW 264.7 cells with apicularen A at 100 nM for 36 h decreased the cell growth and survival as determined by MTT assay (Fig. 10A). In the presence of L-NMMA, the apicularen A (100 nM)-induced decrease in cell growth and survival at 36 h was partially alleviated in a concentration-dependent manner at

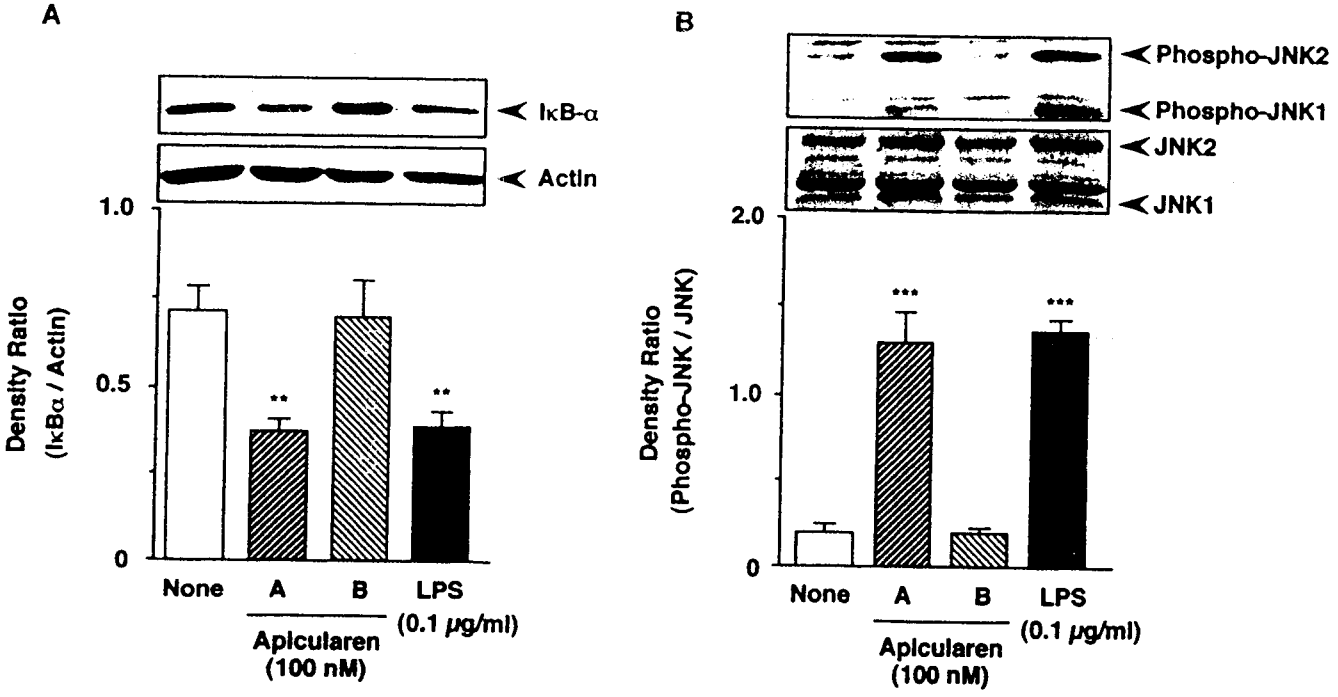

Fig. 8. Effects of apicularen A on the level of IκB-α, and the phosphorylation of JNK. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 1 h at 37°C in 2 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml). The protein levels of IκB-α and actin (A) and JNK and phospho-JNK (B) were determined by Western blotting analysis. The density ratios of IκB-α/actin (A), and phospho-JNK/JNK (B) were calculated. Values are the means from three samples with the S.E.M. shown by vertical bars. Statistical significance: **, $P < 0.01$; ***, $P < 0.001$ versus the nonstimulated control.

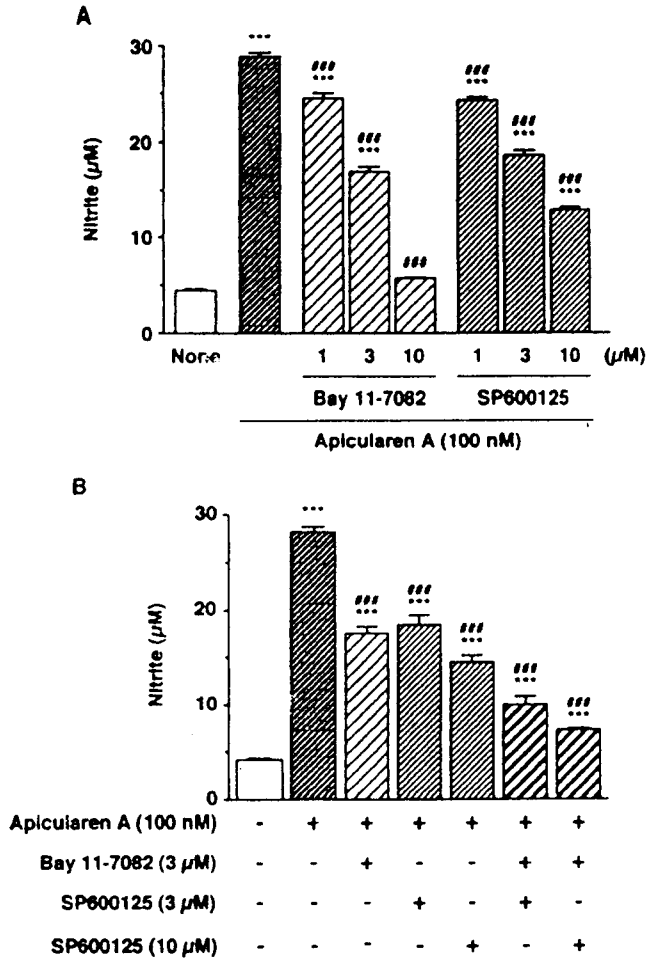

Fig. 9. Effects of Bay 11-7082 and SP600125 on apicularen A-induced nitrite production. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 0.5 ml of medium in the presence of apicularen A (100 nM) and the indicated concentrations of Bay 11-7082 or SP600125 (A) or in the presence (+) or absence (−) of the indicated concentrations of Bay 11-7082 or SP600125 (B). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control; ###, $P < 0.001$ versus the apicularen A control.

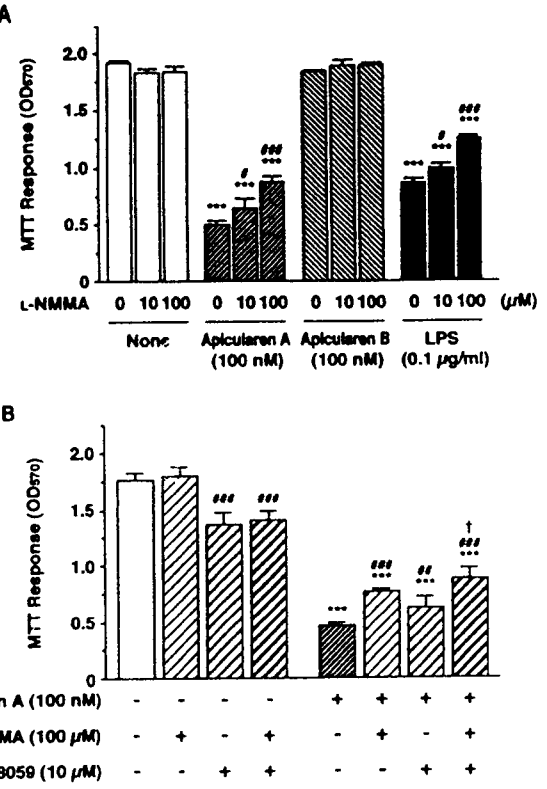

Fig. 10. Effects of L-NMMA and PD98059 on the apicularen A-induced decrease in cell growth and survival. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 36 h at 37°C in 0.5 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml), and the indicated concentrations of L-NMMA (A) or in the presence (+) or absence (−) of apicularen A (100 nM), L-NMMA (100 μM), and PD98059 (10 μM) (B). The cell growth and survival were determined by the MTT assay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control, #, $P < 0.05$; ###, $P < 0.001$ versus the corresponding control (A). ***, $P < 0.001$ versus the nonstimulated control, ##, $P < 0.01$; ###, $P < 0.001$ versus the apicularen A control; †, $P < 0.05$ versus the apicularen A + PD98059 group (B).

10 and 100 μM (Fig. 10A). Apicularen B at 100 nM showed no effect on the cell growth and survival at 36 h (Fig. 10A). Treatment with LPS (0.1 μg/ml) also decreased the cell growth and survival at 36 h, which was partially recovered by L-NMMA at 10 and 100 μM (Fig. 10A). These findings suggest that NO produced by treatment with apicularen A participate, in part, in the apicularen A-induced decrease in cell growth and survival. Treatment with the MEK inhibitor PD98059 (10 μM) partially attenuated the apicularen A (100 nM)-induced decrease in cell growth and survival at 36 h, although PD98059 at 10 μM by itself decreased cell growth and survival in nonstimulated cells (Fig. 10B). In addition, the combined treatment with PD98059 (10 μM) and L-NMMA (100 μM) further augmented the L-NMMA (100 μM)-induced increase in cell growth and survival (Fig. 10B). These findings suggest that the activation of p44/42 MAPK partially participate in the apicularen A-induced apoptosis.

Effects of L-NMMA on the Apicularen A-Induced Decrease in Cell Viability. Incubation of the cells with apicularen A (100 nM) decreased the viability of the cells at 36 h as assessed by staining with 7-ADD, whereas apicularen B at 100 nM did not affect the viability (Fig. 11). In the presence of L-NMMA, the apicularen A (100 nM)-induced decrease in cell viability was partially alleviated in a concentration-dependent manner at 10 and 100 μM (Fig. 11). LPS (0.1 μg/ml) also decreased the cell viability, which was recovered by L-NMMA at 10 and 100 μM (Fig. 11). These findings suggest that NO produced by treatment with apicularen A participate, in part, in the apicularen A-induced decrease in cell viability.

Effects of L-NMMA on the Apicularen A-Induced Disruption of the Mitochondrial Membrane Potential. Fluorescence microscopic analysis demonstrated that most RAW 264.7 cells lost mitochondrial membrane potential after incubation for 24 h at 37°C in the presence of apicularen A (100 nM) (Fig. 12A). The disruption of mitochondrial membrane potential by apicularen A (100 nM) was partially recovered by L-NMMA (100 μM) (Fig. 12A). The flow cytometry analysis

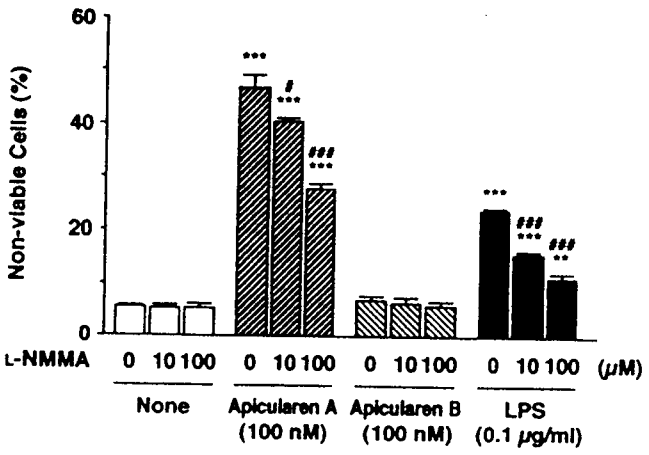

Fig. 11. Effects of L-NMMA on the apicularen A-induced decrease in viability of the cells. RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 0.5 ml of medium in the presence of apicularen A (100 nM), apicularen B (100 nM), or LPS (0.1 μg/ml) and the indicated concentrations of L-NMMA. The viability of the cells was determined by flow cytometry using 7-ADD. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: **, $P < 0.01$, ***, $P < 0.001$ versus the nonstimulated control; #, $P < 0.05$; ###, $P < 0.001$ versus the corresponding control.

also demonstrated that apicularen A (100 nM) induced disruption of the mitochondrial membrane potential at 24 h, which was partially recovered by L-NMMA (100 μM) (Fig. 12B). Apicularen B had no effect on the mitochondrial membrane potential (Fig. 12, A and B). These findings suggest that NO produced by apicularen A partially participate in the apicularen A-induced apoptosis in RAW 264.7 cells.

Discussion

NO is a radical produced from L-arginine by NOS and plays a significant role as a cellular second messenger (Palmer et al., 1988). iNOS produces a high output of NO, whereas constitutively expressed NOS generates a physiologically active low level of NO (Kubes, 2000). It is possible that a high level of NO is associated with anticancer, bactericidal, and antiparasitic effects probably due to the formation of reactive radicals including peroxynitrite (Szabo and Ohshima, 1997).

In this article, we report for the first time that the cytostatic macrolide apicularen A induces nitrite production in RAW 264.7 cells. However, apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, had no effect at 100 nM (Figs. 2 and 3A), but at 300 nM, it increased nitrite production at 24 h slightly but significantly (Fig. 3B). Therefore, it was suggested that much higher concentrations of apicularen B may induce apoptosis of RAW 264.7 cells, and the concomitantly produced NO may also contribute, in part, to the apicularen B-induced apoptosis. Less cytostatic activity of apicularen B than apicularen A is also reported in human cancer cell lines (Kunze et al., 1998). We also reported that apicularen A induced apoptosis in RAW 264.7 cells more potently than apicularen B (Hong et al., 2003b). The weaker biological activity of apicularen B than apicularen A might be explained by the glycosylation of apicularen A. For example, the glycosylation of isoflavones reduced the inhibitory effect of isoflavones on 12-*O*-tetradecanoylphorbol 13-acetate-induced prostaglandin E_2 production in rat peritoneal macrophages (Yamaki et al., 2002). The mechanism by which glycosylation of apicularen A reduces the biological activity remains to be elucidated. Results of time course changes in nitrite production (Fig. 2) indicated that a longer time lag is necessary for the apicularen A-induced nitrite production than for the LPS-induced nitrite production. Therefore, the possibility remained that apicularen A induces some cytokines such as interleukin-1β, which in turn induces nitrite production. LPS binds to LPS-binding protein and then binds to the membrane receptor CD14 (Wright et al., 1990), but the receptor of apicularen A responsible for nitrite production has not yet been identified. In RAW 264.7 cells, apicularen A increased the level of iNOS mRNA (Fig. 6) and induced iNOS protein expression (Fig. 5), and the apicularen A-induced nitrite production was inhibited by the NOS inhibitor L-NMMA (Fig. 4). These findings indicated that the apicularen A-induced nitrite production is due to the expression of the *iNOS* gene. The expression of the *iNOS* gene is regulated by the binding of transcription factors to several consensus sequences of the gene. The promoter region of the *iNOS* gene contains the binding sites for NF-κB, AP-1, and CCAAT/enhancer-binding protein, and the component essential for the expression of the *iNOS* gene is NF-κB (Xie et al., 1994). As shown in Fig. 7, apicularen A-induced activation of NF-κB and AP-1. The NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine at 1.5 mM did not activate NF-κB and AP-1 at 4 h (data not shown). In addition, apicularen A (100 nM)-induced activation of NF-κB and AP-1 at 4 h was not suppressed by L-NMMA (100 μM) (data not shown). These findings suggested that the apicularen A-induced activation of NF-κB and AP-1 is not mediated by NO. The apicularen A-induced production of nitrite was inhibited by Bay 11-7082 (Fig. 9), an inhibitor of IκB-α phosphorylation (Pierce et al., 1997), and by SP600125 (Fig. 9), a specific inhibitor of JNK (Bennett et al., 2001). These findings indicated that the expression of the *iNOS* gene caused by apicularen A in RAW 264.7 cells is induced by the activation of NF-κB and AP-1. Consistent with the suppression of nitrite production by Bay 11-7082 and SP600125, the apicularen A-induced decrease in cell proliferation and survival, as determined by MTT assay, was ameliorated in part (data not shown).

In our previous report (Hong et al., 2003b), we described that SB203580, an inhibitor of p38 MAPK, enhanced the apicularen A-induced decrease in cell growth and survival as determined by MTT assay, suggesting that the activation of p38 MAPK by apicularen A is antiapoptotic. In RAW 264.7 cells, it is reported that p38 MAPK is critical for LPS-induced iNOS expression, NO production, and the activation of NF-κB DNA-binding activity (Chen and Wang, 1999). Therefore, it was suggested that the activation of p38 MAPK induced by apicularen A also participates in the activation of NF-κB (Fig. 7), expression of iNOS mRNA (Fig. 6) and iNOS protein (Fig. 5), and nitrite production (Figs. 2 and 3). In this study, we suggested that concomitantly produced NO by apicularen A treatment partially participate in the apicularen A-induced apoptosis. However, 10 μM SB203580 had no effect on the apicularen A-induced nitrite production (data not shown). Therefore, it was suggested that the antiapoptotic effect of SB203580 (Hong et al., 2003b) is not mediated by inhibition of NO production. Previously, we reported that the MEK inhibitor PD98059 partially attenuated the apicularen A-induced decrease in cell growth and survival as determined by the MTT assay in a certain period after the exposure to apicularen A in RAW 264.7 cells (Hong et al., 2003b). The combined treatment with PD98059 (10 μM) and

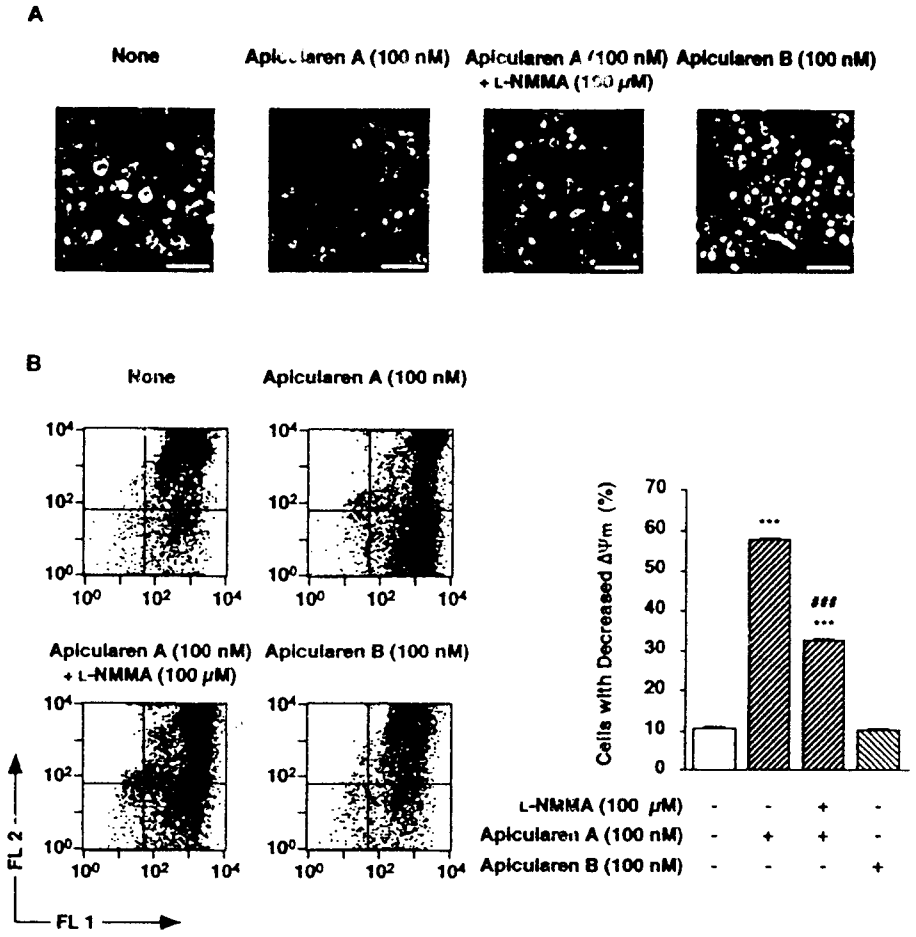

Fig. 12. Effects of L-NMMA on the apicularen A-induced disruption of the mitochondrial membrane potential. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37°C in 2 ml of medium. The cells were then washed three times with medium and further incubated for 24 h at 37°C in 2 ml of medium in the presence (+) or absence (−) of apicularen A (100 nM), apicularen B (100 nM), and L-NMMA (100 μM). After incubation, the cells were stained with DePsipher and observed under fluorescence microscopy (A). The bar represents 50 μm. The intensities for green fluorescence and red fluorescence were analyzed by flow cytometry, and the percentage of the cells with decreased mitochondrial membrane potential ($\Delta\psi_m$) was calculated (B). Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: ***, $P < 0.001$ versus the nonstimulated control; ###, $P < 0.001$ versus apicularen A group.

L-NMMA (100 μM) in the presence of apicularen A (100 nM) further increased the L-NMMA (100 μM)-induced increase in cell growth and survival at 36 h (Fig. 10B). Furthermore, apicularen A (100 nM)-induced nitrite production at 24 h was inhibited by PD98059 (Fig. 4B). However, PD98059 (10 μM) did not completely prevent the apicularen A-induced decrease in cell growth and survival in the presence of L-NMMA (100 μM) and apicularen A (100 nM) at 36 h (Fig. 10B). Therefore, the activation of p44/42 MAPK partially participates in the apicularen A-induced apoptosis as described previously (Hong et al., 2003b). It is reported that p44/42 MAPK activation is not involved in LPS-induced nitrite production in RAW 264.7 cells (Chen and Wang, 1999). However, PD98059 partially inhibited the apicularen A-induced nitrite production (Fig. 4B), suggesting that p44/42 MAPK activation is partially involved in the apicularen A-induced nitrite production. Participation of p44/42 MAPK in interleukin-1β-induced nitrite production is also reported in rat pancreatic islets (Larsen et al., 1998). Further studies are necessary to address the significance of the activation of p38 MAPK and p44/42 MAPK in the apicularen A-induced apoptosis.

NO seems to be a bifunctional regulator of apoptosis. It exerts an antiapoptotic action in vascular endothelial cells (Dimmeler et al., 1997), hepatocytes (Kim et al., 1997), eosinophils (Hebestreit et al., 1998), and splenocytes (Genaro et al., 1995). On the other hand, it induces apoptosis in vascular endothelial cells (Lincoln et al., 1996), macrophages (Albina et al., 1993; Shimaoka et al., 1995), pancreatic islet cells (Heller et al., 1995), thymocytes (Fehsel et al., 1995), and neurons (Leist et al., 1997). In RAW 264.7 cells, it is also reported that NO induces apoptosis (Messmer et al., 1995; Jun et al., 1999; Gotoh et al., 2002). As we reported previously, apicularen A at 10 and 100 nM induced apoptosis in RAW 264.7 cells, it induced a DNA ladder, increased the percentages of subG1 cells, and annexin V-positive but propidium iodide-negative cells (Hong et al., 2003b). In the present study, we found that apicularen A induces nitrite production in RAW 264.7 cells (Figs. 2 and 3) at concentrations at which apoptosis was induced, and treatment with L-NMMA partially recovered the apicularen A-induced decrease in cell growth and survival as determined by MTT assay (Fig. 10), cell viability as determined by 7-ADD staining (Fig. 11), and disruption of the mitochondrial membrane potential (Fig. 12). In addition, in RAW 264.7 cells, the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine at 0.15, 0.45, and 1.5 mM decreased the cell growth and survival in the presence or absence of apicularen A (100 nM) at 24 h in a concentration-dependent manner (data not shown). Therefore, it was suggested that the NO produced in response to apicularen A treatment participates, in part, in the apicularen A-induced apoptosis in RAW 264.7 cells.

It is reported that compounds having a benzolactone enamide core including salicylihalamide A, lobatamides A–F, and oximidines I and II inhibit mammalian vacuolar-type (H^+)-ATPases (V-ATPases) (Boyd et al., 2001), and the V-ATPase inhibitors bafilomycin A1 (Bowman et al., 1988) and

concanamycin A (Dröse et al., 1993) induce apoptosis in several kinds of cancer cells (Ohta et al., 1998; Ishisaki et al., 1999). V-ATPases play an important role in the regulation of the activity in organelles of the central vacuolar system, and the internal acidification of intracellular compartments such as lysosomes, endosomes, Golgi complexes, and secretary granules has been suggested to play a critical role in the mechanism of cell survival (Wieczorek et al., 1999). Because apicularen A has a benzolactone enamide core (Fig. 1), it is possible that apicularen A inhibits V-ATPases, thus inducing apoptosis in RAW 264.7 cells (Hong et al., 2003b).

As to the possible utility of apicularen A for clinical treatments of cancer, it is prerequisite to clarify whether apicularen A does not induce apoptosis in nontumoral cells of the patients.

In conclusion, we demonstrated here for the first time that the cytostatic macrolide apicularen A induces nitrite production in RAW 264.7 cells at concentrations which induce apoptosis in this murine macrophage cell line. The results of this study suggested that apicularen A-induced apoptosis is partially mediated by NO produced by apicularen A because L-NMMA partially ameliorated the apicularen A-induced decrease in the cell proliferation, viability, and the mitochondrial membrane potential. Apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, had no effect on nitrite production.

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Address correspondence to: Dr. Kazuo Ohuchi, Laboratory of Pathophysiological Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980-8578, Japan. E-mail: ohuchi-k@mail.pharm.tohoku.ac.jp

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