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Decursin inhibits induction of inflammatory mediators by blocking NF-κB activation in macrophages

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Decursin inhibits macrophages activation

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Abbreviations: MMP, matrix metalloproteinase; TNF, tumor necrosis factor; TIMP, tissue inhibitor of matrix metalloproteinase; NOS, nitric oxide synthase.

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Abstract

In the course of screening inhibitors of MMP-9 induction in macrophages, we isolated decursin, a coumarin compound, from the roots of *Angelicae gigas*. As a marker for the screening and isolation, we tested expression of MMP-9 in RAW264.7 cells and THP-1 cells after treatment with bacterial lipopolysaccharide (LPS), the TLR-4 ligand. Decursin suppressed MMP-9 expression in cells stimulated LPS in a dose dependent manner at concentrations below 60 μM without any sign of cytotoxicity. The suppressive effect of decursin was also observed not only in cells stimulated with ligands for TLR4, TLR2, TLR3, and TLR9 but also in cells stimulated with interleukin (IL)-1β, and tumor necrosis factor (TNF)-α indicating that the molecular target of decursin is common signaling molecules induced by these stimulants. In addition to the suppression of MMP-9 expression, decursin blocked nitric oxide production and cytokine (IL-8, MCP-1, IL-1β, and TNF-α) secretion induced by LPS. In order to find out the molecular mechanism responsible for the suppressive effect of decursin, we analyzed signaling molecules involved in the TLR-mediated activation of MMP-9 and cytokines. Decursin blocked phosphorylation of IκB and nuclear translocation of NF-κB in THP-1 cells activated with LPS. Furthermore, expression of a luciferase reporter gene under the promoter containing NF-κB binding sites was blocked by decursin.

These data indicate that decursin is a novel inhibitor of NF-κB activation in signaling induced by TLR ligands and cytokines.

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Macrophages are involved in the pathogenesis of inflammatory diseases such as atherosclerosis and rheumatoid arthritis. Atherosclerosis involves multiple processes including endothelial dysfunction, inflammation, vascular proliferation and extracellular matrix (ECM) degradation (Falk, 1995; Shah, 1998). Especially, macrophages play important roles in inflammation via the production of matrix degrading enzymes, pro-inflammatory cytokines/chemokines, cell adhesion molecules, nitric oxide (NO), cyclooxygenase-2 (COX-2) (Ross, 1999). Dissolution of ECM in the fibrous cap through overexpression of active matrix metalloproteinases (MMPs), renders this structure weak, friable and susceptible to rupture when exposed to hemodynamic stress (Libby, 2002). Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation that leads to the destruction of cartilage and bone. Synovial inflammation involves lining layer thickening and infiltration of inflammatory cells into the sublining area (Cunnane et al., 1998; Vervoordeldonk and Tak, 2002). In normal joints, macrophages are resident cells and cover the synovial layer. The number of macrophages in the joint greatly increases in RA synovium(Kinne et al., 2000) and the degree of increase is strongly correlated with the development of severe cartilage destruction (Bresnihan, 1999; Mulherin et al., 1996; Yanni et al., 1994). Furthermore, selective depletion of macrophages from the synovial lining before the induction of

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experimental arthritis resulted in prevention of both joint inflammation and cartilage destruction (van Lent et al., 1996; Van Lent et al., 1998; Van Lent et al., 1993). Joint destruction is mediated by enzymes degrading ECM such as serine proteases, MMPs and the cathepsins (Cunnane et al., 1998). MMP-9 levels are substantially elevated in the sera and synovial fluid from RA patients (Giannelli et al., 2004; Gruber et al., 1996). Immunohistochemistry studies have demonstrated that MMPs are expressed by cells present within atheromas, but not in normal arteries (Galis, 1994; Nikkari, 1995).

Matrix metalloproteinases are a family of Zn^{2+}-dependent endopeptidases that are responsible for the degradation of most extracellular matrix proteins as well as a number of other proteins during organogenesis, growth and normal tissue turnover. MMPs also mediate tissue remodeling in various pathologic conditions including several inflammatory diseases (Chakraborti et al., 2003). MMP expression level is increased in atherosclerotic lesions and is linked to weakening of the vascular wall by degrading the extracellular matrix (Chen et al., 2005; Renko et al., 2004; Watanabe and Ikeda, 2004; Zeng et al., 2005). The quantity of MMP-9 (Gelatinase B, 92-kD type IV collagenase) is low in healthy lungs, but much higher in several lung diseases where airway remodeling takes place, including asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (Atkinson and Senior, 2003; Holgate et al., 1999; Kelly and Jarjour,

2003; Suzuki et al., 2004; Whelan, 2004). In inflammatory heart diseases, MMPs have been found to play a significant role in the development of myocardial remodeling leading to dilated cardiomyopathy and ventricular dysfunction (Pauschinger et al., 2004). In the context of neuroinflammatory diseases including multiple sclerosis and bacterial meningitis, MMPs have been implicated in processes such as blood-brain barrier and blood-nerve barrier opening, invasion of neural tissue by blood-derived immune cells, shedding of cytokines and cytokine receptors, and direct cellular damage in diseases of the peripheral and central nervous system (Leppert et al., 2001). Infiltrating inflammatory cells are major producers of MMPs and the paracrine/autocrine effect of a repertoire of cytokines on inflammatory cells is likely to cause an imbalance in MMP/TIMP ratio resulting, eventually, in altered extracellular matrix architecture.

To find out agents that could modulate pro-inflammatory activities of macrophages, we screened extracts from 14 medicinal herbs. A coumarin compound, decursin, has been identified to be responsible for the inhibition of LPS-induced MMP-9 expression in the extract of *A. gigantis* Radix. We further investigated the suppressive effect of decursin in the expression of MMP-9 and cytokines in cells treated with other related stimulants.

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Materials and Methods

Plant extracts and purification. For the initial screening of 14 medicinal herb extracts, EtOH extracts in the form of dried pellets were purchased from Plant Diversity Research Center (PDRC), Daejeon, Korea. These pellets were resuspended in EtOH in 20 mg/ml concentration and used for the initial screening. For the purification of the active compound (Data supplement 1), the dried roots of *Angelicae gigas*, harvested in regional area, were purchased from Market of Medicinal Herbs, Daegu, Korea and the identity was confirmed by Dr. J. H. Yang (Daegu Haany University). The dried roots of *A. gigas* (1 kg) were extracted twice with MeOH under reflux using the methods described by Lee *et al*. (Lee, 2002). The extracts were then combined and concentrated under low pressure to afford 21.4 g of the residue. The MeOH extract was suspended in water and then fractionated with equal volumes of CH_2Cl_2 (10.5 g dry weight). The CH_2Cl_2 fraction was further chromatographed on two successive silica gel columns (8×80 cm, Art. 7734 and Art. 9385, Merck, Germany) and eluted with a gradient of *n*-hexane-EtOAc (20:1~8:1) to afford the active compound (1.3 g, 10:1). The structure of the compound was analyzed using ^{1}H-, ^{13}C-NMR and confirmed to be decursin by comparison with literature values (Hata, 1966; Ryu, 1990) (Data supplement 2). The

HPLC was performed using Jasco system consisting of a binary pump PU-2080, an autosampler AS-2055, and photodiode array detector MD-2010. The ZOBAX eclipse XDB C-18 (250×4.6 mm) was used as column and HPLC was operated at room temperature. The eluent consisted of water with 1% acetic acid and acetonitrile with 1% acetic acid. Gradient profile was: 0~40 minutes from 50 to 100%. The flow rate was 1 ml/min. The wavelength of detection was 280 nm.

Reagents and antibodies. Bacterial lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, U.S.A); Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH (PAM) from Bachem AG (Budendorf, Switzerland); poly(I)-poly(C) double strand RNA from Amersham Biosciences (NJ, USA); CpG 1826 (TLR9 ligand) and CpG 2138 (control oligonucleotide) from Invivogen (CA, USA), recombinant IL-1β and TNF-α from R&D Systems; anti-IκB polyclonal antibody, anti-phospho-IκBα monoclonal antibody (clone 5A5), and polyclonal antibodies to Erk1/2 and their phosphorylated forms (Thr202/Tyr204) from Cell Signaling; rabbit polyclonal antibodies specific for iNOS from Transduction Laboratories (San Diego, CA, USA); monoclonal anti-α-tubulin clone B-5-1-2 mouse ascites fluid from Sigma.

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Cell culture and activation. A murine macrophage cell lines, RAW264.7 and a human macrophage cell line, THP-1 were cultured as described by American Type Culture Collection. To test the suppressive effects of herbal extracts and purified compounds on the RAW264.7 and THP-1 cells, cells were washed three times with medium (DMEM for RAW264.7 and RPMI 1640 for THP-1 cells) with 0.1% fetal calf serum and then seeded on sterile 96-well tissue culture plate (5×10^4 and 1 x 10^5 cells/well for RAW264.7 and THP-1 cells, respectively). We then pretreated the cells with 10~30 μg/ml of herbal extracts for overnight or 20~60 μM of decursin for 2~3 hours. After the pretreatment, cells were stimulated with 0.1~1 μg/ml LPS (Sigma, St. Louis, U.S.A.) in the continued presence of decursin. Cell lysates were obtained at various time points after the activation and used for Western blotting. For the detection of MMP-9 or cytokine expression, the supernatants were collected 24 hours after the activation and used for gelatin zymogram or ELISA, respectively. BV-2 mouse microglial cell line was generously provided by Dr. E. Choi at Korea University (Seoul, Korea). The BV-2 cells were maintained in DMEM supplemented with 5% FBS, 2 mM glutamine, and penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD), and were treated with decursin and/or stimulants in a manner similar to RAW264.7 and THP-1 cells. In order to obtain the peritoneal macrophages, Balb/c mice were injected

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intraperitonelly (3ml/mouse) with 3% Brewer thioglycollate medium containing 0.3mM thioglycollate (Difco, Detroit, MI, USA). Four days later, cell were harvested by lavage with cold PBS and plated into appropriate wells. Cells were allowed to adhere for 2 hr and then washed free of nonadherent cells.

Nitrite quantification. After BV-2 microglia cells were treated with activating agents in 96-well plates, NO_2^- concentration in culture supernatants was measured to assess NO production in microglial cells. Fifty μl of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

Western blotting, ELISA and gelatin zymogram. Cell lysates were prepared using triple-detergent lysis buffer and used for Western blot analysis as described previously (Lee et al., 2001). Cytokine levels in the culture supernatant were measured using sandwich ELISA (R&D Systems). The detection limits were < 10 pg/ml for all the cytokines. The MMP-9 activity in the culture supernatant was determined by

performing substrate gel electrophoresis as described before (Lee et al., 2001).

Cell viability test (MTT assay). To determine the RAW264.7 cell viability, cells (5×10^4 cells/well) were washed three times with 0.1% serum DMEM medium and then seeded on sterile 96-well tissue culture plate. Decursin was then added at concentration of 20~60 μM for 3 hours. After the pretreatment, the cells were stimulated with 100 ng/ml LPS (Sigma, St. Louis, U.S.A) for 24 hours in the continued presence of decursin. Cell viability was evaluated in each well by the addition of 50 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 2.5 mg/ml in PBS). After 4 hours of incubation, the cell-free supernatants were removed completely from each well and 150 μl of dimethylsulfoxide (DMSO) was added. The optical densities of the wells were measured using a spectrophotometric multiwell microplate reader (Multiskan MS, Lab-system, Finland) at wavelength of 540 nm.

Immunofluorescence assay. For the detection of intracellular location of NF-κB p65 subunit, RAW264.7 cells ($2x10^5$/well in 24 well plates) were cultured in sterile cover slips and treated with Decursin and LPS as described above. At various times after the LPS treatment, the cells were fixed with 4% formaldehyde in PBS for 30 min

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and washed with PBS for 5 min. The fixed cells were then stained with 0.5μg/ml Hoechst staining solution (Sigma) for 20 min at 37 °C and washed. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at RT, washed with 0.02% Tween-20 in PBS for 20 min, and 0.02% Tween-20/1% BSA in PBS for 5 min. The permeabilized cells were then treated with 2 μg/ml of anti-p65 monoclonal antibody (SC-8008, Santa Cruz, USA) for 45 min at 37°C, washed with 0.02% Tween-20/1% BSA in PBS for 5 min. Cell were then incubated in a 1:50 dilution of Alexa Fluor 488-labeled goat anti-mouse antibody (A-21121, Molecular Probes) for 45 min at 37°C, and washed with 0.02% Tween-20 in PBS for 5 min and PBS for 5 min. Finally, the cover slips with cells were dried in 37 °C oven for 45 min and mounted in a 1:1 mixture of Xylene and Malinol.

Real time RT-PCR. Five microgram of total RNAs isolated from cells were treated with RNase free DNase (BD-Pharmingen), and then used to generate first-strand cDNAs using RevertAid^{TM} first strand cDNA synthesis kit with 500 ng oligo $(dT)_{12-18}$ primers. PCR primers were designed with ABI PRISM Primer Express 2.0 (Applied Biosystems) and made by Geno Tech Corp (Korea). Primers were designed for 123 and 143 bp for human and mouse MMP-9 and 52 and 452 bp for human and mouse GAPDH

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PCR products, respectively. Primer sequences are 5'ttctacggccactactgtgcct3'(forward) and 5'aatcgccagtacttcccatcct3' (reverse) for human MMP-9, 5'aaaccagaccccagactcctctct3' (forward) and 5'gaggacacagtctgacctgaacca3' (reverse) for mouse MMP-9, and 5'tgggctacactgagcaccag3' (forward) and 5'gggtgtcgctgttgaagtca3'(reverse) for human GAPDH and 5'accacagtccatgccatcac3' and 5'tccaccaccctgttgctgta3' for mouse GAPDH. Real time PCR reaction was performed in ABI PRISM 7300 sequence detector (Applied Biosystems) using SYBR green PCR mix (Applied Biosystems) with cDNA corresponding to 125 ng of original total RNA and 400 nM primers in a 20 μl volume. The threshold cycle (Ct) values for MMP-9 reactions were normalized with Ct value from corresponding GAPDH reactions. The specificity of the PCR reaction was confirmed by control reaction such as PCR reaction with templates processed without reverse transcriptase and PCR reaction without template. After the PCR reaction, the PCR products were run on 2% agarose gel to confirm the size and purity of the PCR products.

Luciferase reporter assay. RAW264.7 cells were plated and overnight cultured in 24-well plates (2×10^5 cells/well). These cells were transiently transfected with 1 μg of plasmid DNA containing 0.1 μg of NF-κB-firefly-luciferase reporter construct

(containing 4 tandem repeats of NF-κB binding sites), 0.1 μg of pRL-TK construct (containing Renillar luciferase gene under the thymidine kinase promoter, Promega), and inert filler plasmid using SuperFect transfect reagent (Qiagen) according to the manufacturer's instruction. The luciferase activities were determined using the Dual-Luciferase reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's recommended protocol. Relative firefly luciferase activity was determined by normalization with Renillar luciferase activity.

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Results

In order to find out agents which could suppress pro-inflammatory activities of macrophages, we screened extracts from 14 medicinal herbs (Table 1). As a marker for the screening, we tested suppression of MMP-9 induction in RAW264.7 cells stimulated with LPS. MMP-9 activity in the culture supernatant was measured using gelatin zymogram. As shown in table 1 and figure 1, MMP-9 induction was suppressed by extracts of *A. gigantis* Radix and *Cinnamomi Ramulus.* In case of extract from *C. Ramulus*, cytotoxicity was observed in the concentrations range used in this experiment.

Purification of the causative compounds from the roots of *A. gigas* (data supplement 1) led to the identification of decursin (Fig 2A). In order to confirm the suppressive effect of the purified compound, RAW264.7 cells were activated with LPS in the presence of decursin which had been added 2 hours before the activation. Decursin suppressed MMP-9 induction in a dose dependent manner (Fig. 2B). Cytotoxicity was not detected in these treatment conditions (Fig 2C). These data indicate that decursin, which has been purified form the root of *A. gigas,* blocks MMP-9 induction in LPS stimulated macrophages without affecting cytotoxicity. Decursin also blocked MMP-9 induction in LPS-treated human macrophage cell line, THP-1 (Fig 3A).

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We then tested whether decursin blocks MMP-9 induction in cells activated with stimulants other than LPS. MMP-9 expression induced by PAM (a synthetic lipoprotein for TLR2 stimulation) was blocked by decursin in both THP-1 (Fig 3A) and RAW264.7 cells (Fig 3B) in a dose dependent manner. MMP-9 expression induced by CpG DNA, the TLR9 ligand, was also blocked by decursin in RAW264.7 cells. Since stimulation of TLR4, TLR2, and TLR9 induce MyD88 mediated signaling, we stimulated the cells with IL-1β which also induces MyD88 mediated signaling events. As expected, IL-1β induced MMP-9 expression was blocked by decursin in both RAW264.7 cells and THP-1 cells. We then tested MyD88 independent stimulants such as double strand RNA (poly I:C, the TLR3 ligand) and TNF-α. Pre-treatment of RAW264.7 and THP-1 cells with decursin blocked MMP-9 expression induced by these stimulants. These data indicate that the suppressive effect of decursin is not restricted to LPS stimulation, but general.

We then questioned whether the suppressive effect of decursin can occur in primary macrophages. Peritoneal macrophages, isolated from thioglycollate injected mice, were stimulated with LPS in the presence of decursin. As shown in figure 3C, decursin blocked MMP-9 induction in these cells, indicating that the suppressive effect of decursin is not restricted to immortalized macrophage-like cells.

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Next, we analyzed whether the suppressive effect of decursin is durable. For that purpose, we transiently exposed both RAW264.7 and THP-1 cells to decursin and then the decursin is removed from the culture medium by extensive washing. The suppressive effect of decursin was not detected in this condition (data supplement 3).

We then tested whether decursin blocks induction of other inflammatory mediators such as pro-inflammatory cytokines. Decursin blocked LPS induced expression of MCP-1, IL-8, TNF-α and IL-1β in a dose dependent manner in THP-1 cells (Fig 4A). Decursin also blocked TNF-α expression in RAW264.7 cells activated with LPS (Fig 4B). Furthermore, decursin blocked TNF-α production from peritoneal macrophages (Fig 4C). These data demonstrate that decursin blocks LPS induced cytokine expression in both immortalized and primary macrophages.

Next, we sought to determine whether decursin blocks activation of microglia, a form of macrophages resident in the central nervous system. Stimulation of BV-2 mouse microglia cells with LPS induced strong NO production. Decursin blocked the NO production in a dose dependent manner (Fig 5) without affecting cellular viability (data not shown). Inhibitory effects of decursin on the LPS-induced NO production were accompanied by the attenuation of inducible nitric oxide synthase (iNOS) induction (Fig. 5 inset). Collectively, our current data prove that decursin blocks

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inflammatory activation of macrophages with respect to the pro-inflammatory cytokine expression, NO production, and MMP-9 induction.

In order to find out whether decursin suppresses MMP-9 gene expression at transcriptional level, THP-1 cells were stimulated with LPS in the presence or absence of decursin and the relative amounts of MMP-9 mRNA were measured by real-time RT-PCR. As shown in figure 6A, treatment with decursin but not with the vehicle control blocked LPS induced upregulation of MMP-9 mRNA levels. MMP-9 mRNA levels were also tested in RAW264.7 cells in different time points after LPS stimulation. Decursin completely blocked MMP-9 mRNA induction at 2 hours after the LPS stimulation and about 70% at 4 hours after the activation (Fig 6B).

Since decursin blocked transcriptional activation of MMP-9 gene, it is likely that it blocks signaling events involved in transcriptional activation of MMP-9 gene. Expression of MMP-9 and cytokine genes requires NF-κB activation and its subsequent translocation into the nucleus. In resting cells, NF-κB/IκB complexes are present in the cytoplasm. Activation of cells under appropriate condition leads to phosphorylation and subsequent degradation of IκB. The free NF-κB then translocates into the nucleus to activate genes with NF-κB binding sites. So we tested whether decursin blocks NF-κB nuclear translocation. As shown in figure 7, LPS stimulation caused translocation of

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part, but not all, of the NF-kB from the cytoplasm into the nucleus within 60 min after the activation while the presence of 80 μM of decursin blocked it (compare Fig 7A and 7B). Nuclear translocation of NF-κB was partially blocked in the presence of 20 and 40 μM of decursin and 80 μM decursin blocked it completely (Fig 7C). Decursin also blocked nuclear translocation of NF-κB in THP-1 cells activated with LPS (data supplement 4). Decursin completely blocked phosphorylation and subsequent degradation of IκB in both RAW264.7 cells (Fig 7D, E and F) and in THP-1 cells (Data supplement 5). These data demonstrate that the suppression of IκB degradation and subsequent NF-κB nuclear translocation is responsible for the anti-inflammatory activities of decursin.

We then questioned whether the suppressive effect of decursin is restricted to NF-κB activation pathway or affects mitogen-activated protein kinase (MAPK) pathways as well. In order to test the activation of Erk, one of the three MAPKs, we used both peritoneal macrophages and RAW264.7 cells. As shown in figure 7G for the peritoneal macrophages, phosphorylation of Erk occurred 60 min after LPS treatment and the presence of 80 μM of decursin failed to achieve a significant reduction in Erk phosphorylation. The same results were obtained in RAW264.7 cells further confirming that decursin do not affect activation of Erk (data supplement 6).

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Since our data point out that decursin suppresses MMP-9 transcription through inhibition of NF-κB activation, we confirmed the suppressive effect of decursin on the NF-κB signaling pathway using NF-κB reporter assay. RAW264.7 cells were transiently transfected with plasmids including luciferase gene under the promoter containing 4 tandem repeats of NF-kB binding sites. As expected, decursin blocked the expression of the reporter gene after stimulation with either LPS or double strand RNA (poly I:C) (Fig 8). These data further confirm that the decursin blocks signaling events leading to the activation of NF-κB.

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Discussion

Our present data provide the first evidence that decursin is an anti-inflammatory agent in macrophage functions. Decursin blocked various responses of macrophages to stimulants including TLR ligands and inflammatory cytokines. Although current data do not identify the molecular target, presence of decursin causes suppression of IκB degradation and subsequent NF-κB nuclear translocation. Since NF-κB is the key regulatory molecule in the transcriptional activation of the genes encoding MMP-9, proinflammatory cytokines, and iNOS (the enzyme responsible for the production of NO), treatment with decursin will lead to the transcriptional suppression of these genes. In case MMP-9, we confirmed that decursin blocked induction of MMP-9 mRNA levels in macrophages activated with LPS (Fig. 6).

Based on the present findings, decursin must be added to what is now a very long list of compounds, some natural and others synthetic, that inhibit signaling via the NF-κB pathway. Like decursin, a large number of these compounds are electrophiles that are characterized by the presence of an olefinic linkage conjugated to the C=O group of a ketone, ester or lactone. Two of these conjugated systems are present in the molecular structure of decursin. It remains to be determined why compounds in this general category tend to inhibit NF-κB-dependent signaling.

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Decursin needs to be present continuously for the inhibition of LPS induced expression of MMP-9 (data supplement 3). This indicates that decursin's effect is not through a covalent modification of its molecular targets. It is possible that decursin is an inhibitor of an enzyme, such as kinase, involved in LPS induced NF-κB activation. It is also possible that decursin may interfere with the interaction between signaling molecules in NF-κB activation pathway. The inhibitory effect of decursin appears to be specific to NF-κB signaling pathway, since addition of 80 μM of decursin, which was sufficient to suppress NF-κB activation, failed to block phosphorylation of Erk.

The suppressive effect of decursin is not restricted to cultured cell lines but can occur in primary macrophages. Thus, it is expected that decursin will have similar effect *in vivo*, unless decursin is rapidly converted into other inactive compounds via metabolic enzymes or masked by some serum components, etc.

Decursin has been studied as a chemical with various biological activities including anti-cancer, anti-oxidant, anti-platelet aggregation, and anti-bacterial activities. The anti-tumor activity of decursin was reported by Lee et al. (Lee et al., 2003) who reported that decursin not only increased the survival time of mice injected with the Sarcoma-180 ascitic tumor but also reduced the tumor volumes and weights. The underling mechanism of decursin's anti-tumor activity has been investigated by Yim et al. (Yim et

al., 2005) who reported that decursin treatment induced G1 or G2-M arrest in human prostate carcinoma cells. Furthermore, decursin caused apoptosis in the cancer cells via both caspase dependent and independent mechanisms.

Our data might provide additional mechanism responsible for the anti-tumor activity of decursin. It is well noted that inflammation is linked to cancer development (Clevers, 2004; Crowe, 2005; Philip et al., 2004; Schwartsburd, 2004) and activation of MMPs is involved in tumor metastasis (Klein et al., 2004; Mannello et al., 2005; Vihinen et al., 2005). The suppression of cytokine/MMP-9 expression and subsequent inflammation by decursin may contribute to its anti-tumor effect. But it remains to be investigated to see whether decursin can block inflammatory activities associated with oncogenesis and metastasis.

In addition to the activities described above, decursin exhibited significant protective activity against glutamate-induced neurotoxicity when added to primary cultures of rat cortical cells at concentrations ranging from 0.1 to 10 μM (Kang et al., 2005). Decursin may exert neuroprotective effects because of its inhibitory effects on neurotoxic microglial activation as determined in Fig. 5.

Our data added one more effect of decursin, anti-inflammatory effects on macrophages, to the growing number of its biological effects. Further investigation is

required to find out the molecular target(s) of this compound in NF-κB pathway and to find out whether this compound can suppress other inflammatory reactions mediated by macrophages.

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Footnotes

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Figure legends

Figure 1. The extracts from *A. gigais* and *C. cassia* block LPS induced expression of MMP-9. RAW264.7 cells were incubated for 15 hr in the presence of 10 to 30 μg/ml of ethanol extracts from Angelicae gigantis Radix (**A**), Cinnamomi Ramulus (**B**), or Trichosanthes Semen (**C**) and then stimulated with 1 μg/ml LPS for 24 hrs. The culture supernatants were collected subjected to gelatin zymogram as described in Materials and Methods. As a vehicle control, 0.1% ethanol was used.

Figure 2. Decursin blocks MMP-9 expression in RAW264.7 cells stimulated with LPS without affecting cell viability. A, chemical structure of decursin. **B**, RAW264.7 cells were pretreated with 20, 40, or 60 μM of decursin for 2 hrs before stimulation with 100 ng/ml of LPS. The culture supernatants were collected 24 hrs after activation and subjected to gelatin zymogram. **C**, RAW264.7 cells were treated as described in **B** and cell viability was tested with MTT assay as described in Materials and Methods.

Figure 3. Decursin blocks induction of MMP-9 in monocytic cells lines stimulated with various agents and in primary macrophages stimulated with LPS. THP-1 cells (**A**), RAW264.7 cells (**B**), and peritoneal macrophages (**C**) were pretreated with 20, 40,

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or 60 μM of decursin for 2 hrs and activated with LPS, PAM, dsRNA (poly I:C), TNF-α, IL-1β, or CpG 1826 DNA at indicated concentrations. As a vehicle control, 0.06% ethanol was used. The culture supernatants were then collected 24 hrs after the activation and MMP-9 levels were analyzed using gelatin zymogram.

Figure 4. Decursin blocks cytokine expression induced by LPS in monocytic cell lines. THP-1 cells (**A**), RAW264.7 cells (**B**), and peritoneal macrophages (**C**) were pretreated with 20, 40, or 60 μM of decursin for 2 hours and activated with 1 μg/ml LPS. Culture supernatants were collected in 24 hours and the cytokine concentrations were measured using sandwich ELISA. Measurements were done in triplicate and the error bars represent the standard deviation **, significant difference from LPS treatment ($P < 0.01$); ***, $P < 0.001$..

Figure 5. Decursin blocks LPS-induced NO production in microglia cells. BV-2 microglial cells were treated with LPS (100 ng/ml) in the presence of 1 - 50 μM of decursin for 24 hrs, and then the production of nitric oxide was measured by Griess reaction. Values represent mean ± SD. *, significant difference from LPS treatment ($P < 0.05$); ***, $P < 0.001$. Inset: BV-2 cells were left untreated (lane 1) or treated with LPS

(100 ng/ml) (lane 2) or LPS plus decursin (50 μM) (lane 3) for 8 hours, and then the expression of iNOS protein (*upper*) was assessed by Western blot analysis. Detection of α-tubulin was also carried out to confirm the equal loading of proteins (*lower*).

Figure 6. Decursin suppresses MMP-9 expression at transcription level. A, THP-1 cells, pretreated for 3 hrs with either 60 μM decursin or 0.06% ethanol as a vehicle control, were stimulated with 1μg/ml of LPS. One hour after the stimulation, total cellular RNAs were collected and subjected to real time RT-PCR analysis. **B**, RAW264.7 cells were treated with 80 μM of decursin overnight before stimulation with 1 μg/ml of LPS. Total cellular RNAs were collected at indicated times and used for real time RT-PCR analysis. Ct values from MMP-9 measurements were normalized with the values from corresponding GAPDH reactions. ***, significant difference from LPS treatment ($P < 0.001$).

Figure 7. Decursin blocks phosphorylation and degradation of IκB and activation of NF-κB. RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence (**A**) or presence (**B**) of 80 μM decursin which had been added 3 hours before the activation. At indicated times after the LPS addition, subcellular location of NF-κB p65 subunit was

tested using immunofluorescence assay. Note that nuclear translocation of p65 subunit is not complete but part of the cytoplasmic p65 translocated from the cytoplasm to the nucleus so that the distinction between the nucleus and the cytoplasm blurs 60 min after the activation. **C**, RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence or presence of 20, 40, or 80 μM decursin which had been added 3 hours before the activation. Subcellular location of NF-κB p65 subunit was tested using immunofluorescence assay at 60 min after the activation. **D**, RAW264.7 cells were stimulated as in **C**. Total cell lysates obtained 1 hr after the activation and the levels of IκB and its phosphorylated form in the cellular lysates were analyzed using Western blotting. **E** and **F**, RAW264.7 cells were stimulated with 1 μg/ml LPS in the absence or presence of 80 μM decursin. The levels of IκB in the cellular lysates were analyzed using Western blotting. **G**, peritoneal macrophages were stimulated with 1 μg/ml LPS in the absence or presence of 80 μM decursin. The levels of Erk 1/2 and their phosphorylated forms were analyzed using Western blotting at indicated time points. Band intensities were measured by densitometer and normalized with band intensities of corresponding loading controls. The normalized quantification values are written below each band.

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Figure 8. Decursin blocked NF-κB activation in luciferase reporter assay. RAW264.7 cells were transiently transfected with plasmids containing firefly luciferase reporter gene under a promoter containing 4 tandem repeats of NF-κB binding site and Renilla luciferase expression construct as an internal control. The transfected cells were then pretreated with 10 or 40 μM of decursin for 2 hrs and activated with LPS (A), or poly I:C (B) for 6 hrs at indicated concentrations before measuring the luciferase activities. As a vehicle control, 0.04% ethanol was used. *, significant difference from LPS treatment ($P < 0.05$); **, $P < 0.01$.

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Table 1. List of herbal extracts used for the initial screening and their effects on LPS-induced MMP-9 expression.

Herbal drug	Specific name	Family	Growing district	Product Number*	Suppressive activity
Angelicae gigantis Radix	*Angelica gigas*	Umbellifere	Korea	CA01-031	+++
Cinnamomi Ramulus	*Cinnamomum cassia*	Lauraceae	China	CA01-009	++
Trichosanthes Semen	*Trichosanthes kirilowii*	Cucurbitaceae	China	CA02-018	-
Cnidii Rhizoma	*Cnidium offisinale*	Umbellifere	Korea	CA03-071	+
Pinelliae Tuber	*Pinellia ternata*	Araceae	China	CA04-015	+
Gardiniae Fructus	*Gardenia jasminoides*	Rubiaceae	Korea	CA03-078	-
Aurantii Nobilis Pericarpium	*Citrus unshiu*	Rutaceae	Korea	CA03-068	-
Paeoniae Radix alba	*Paeonia albiflora Pallas*	Paeoniaceae	Korea	CA02-034	-
Cyperi Rhizoma	*Cyperus rotundus*	Cyperaceae	Korea	CA03-088	-
Liriopis Tuber	*Liriope platyphylla*	Liliaceae	Korea	CA03-007	+
Ponciri Fructur	*Poncirus trifoliata*	Rutaceae	China	CA02-094	-
Aurantii Fructur	*Citrus aurantiun*	Rutaceae	China	CA03-059	-
Atractylodis Rhizoma	*Atractylodes japonica*	Compositae	China	CA03-069	-
Corydlais Tuber	*Corydalis ternata*	Papaverceae	China	CA04-082	-

*Product number from Plant Diversity Research Center (PDRC), Daejeon, Korea

Figure 1

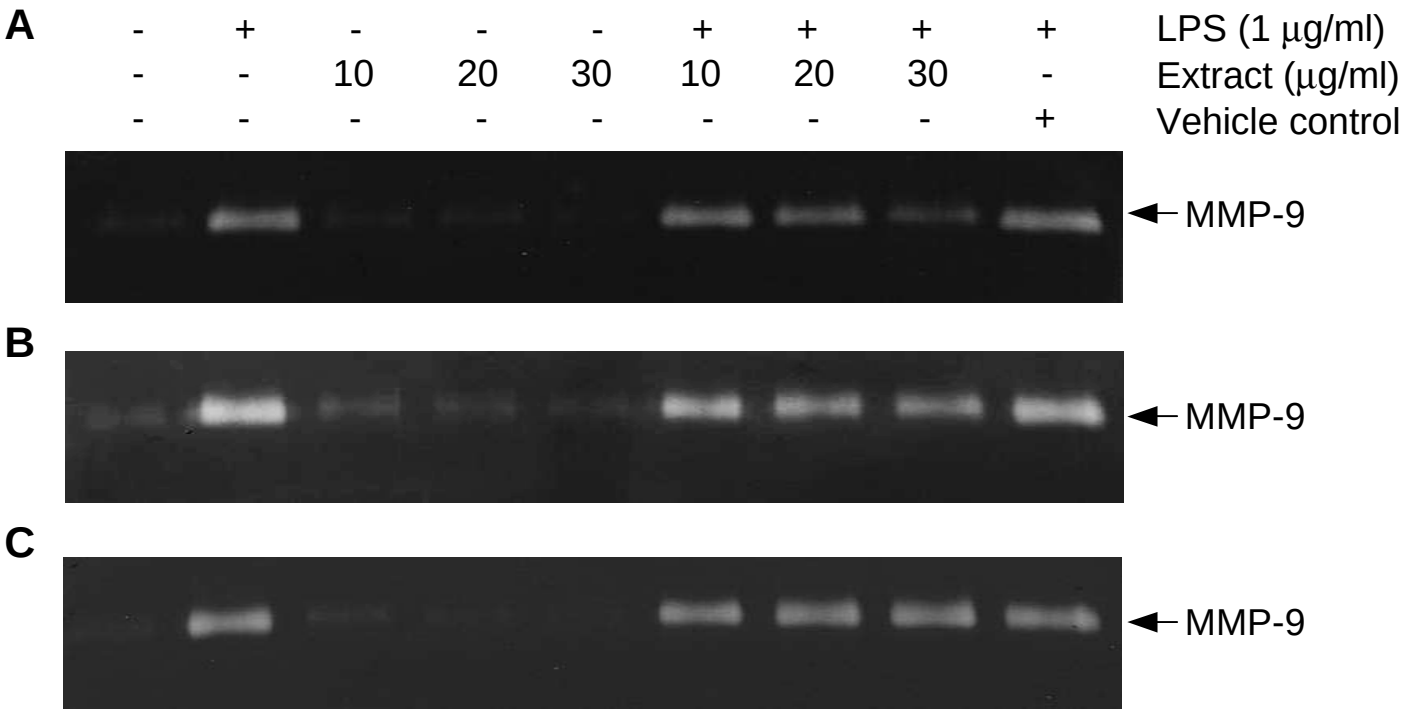


Figure 2

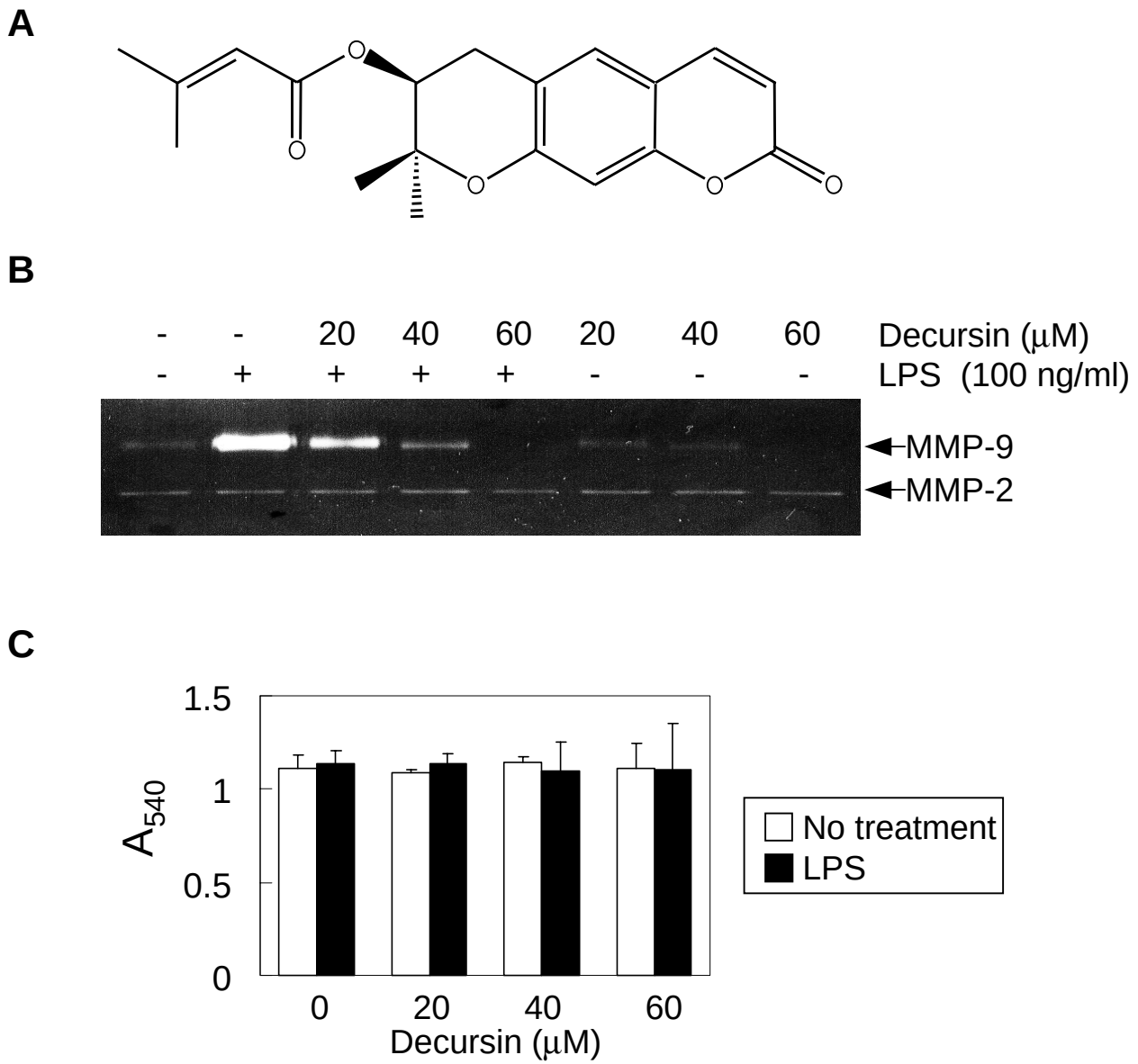
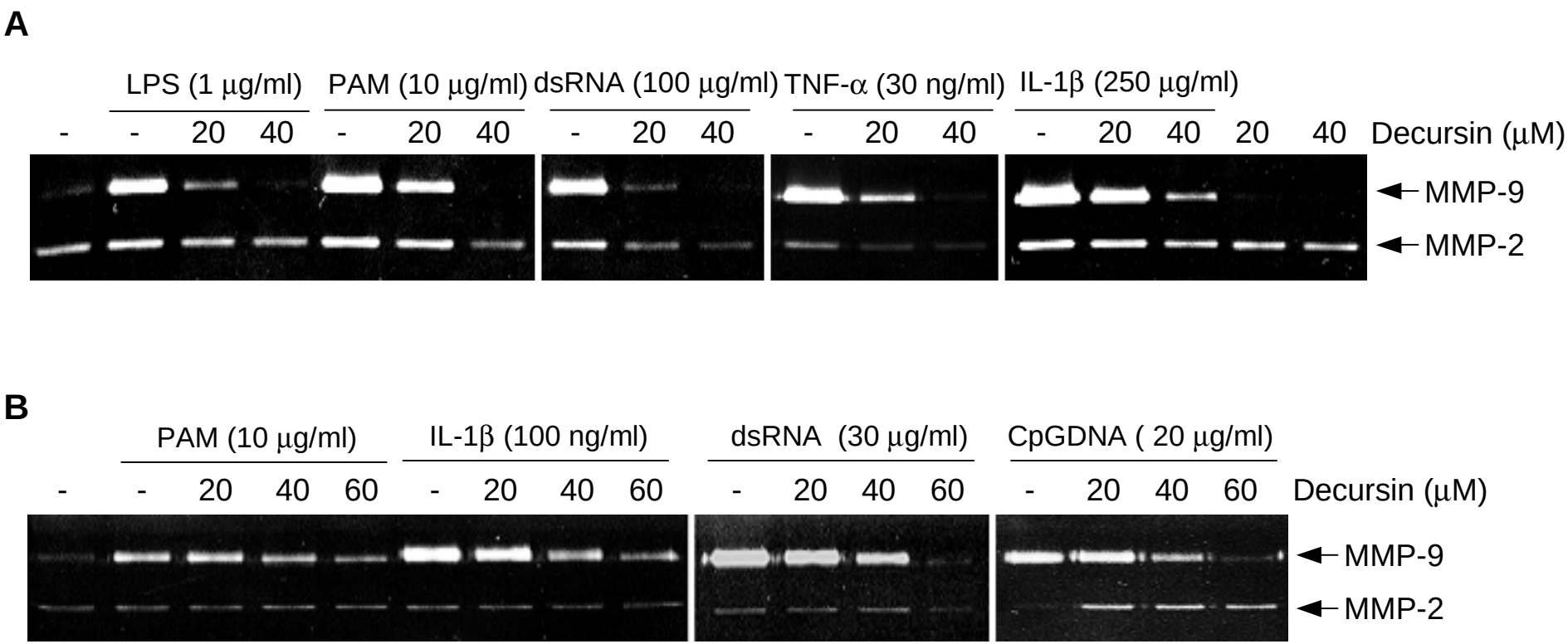


Figure 3 A and B



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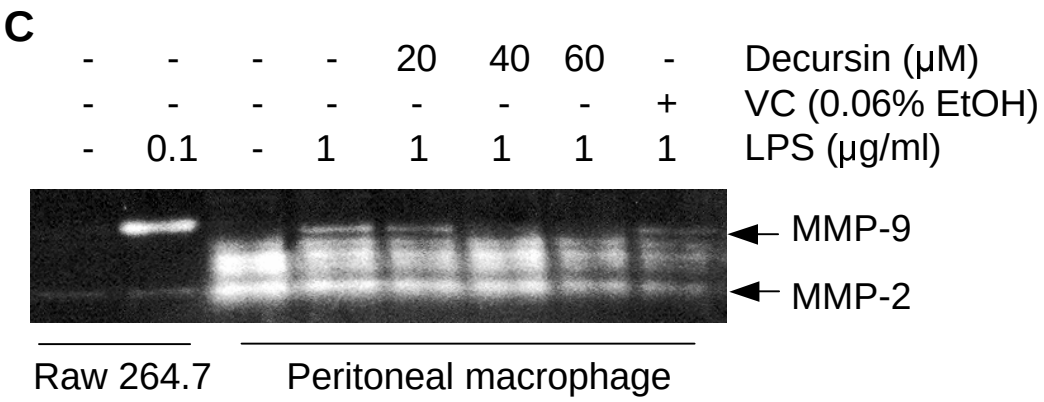

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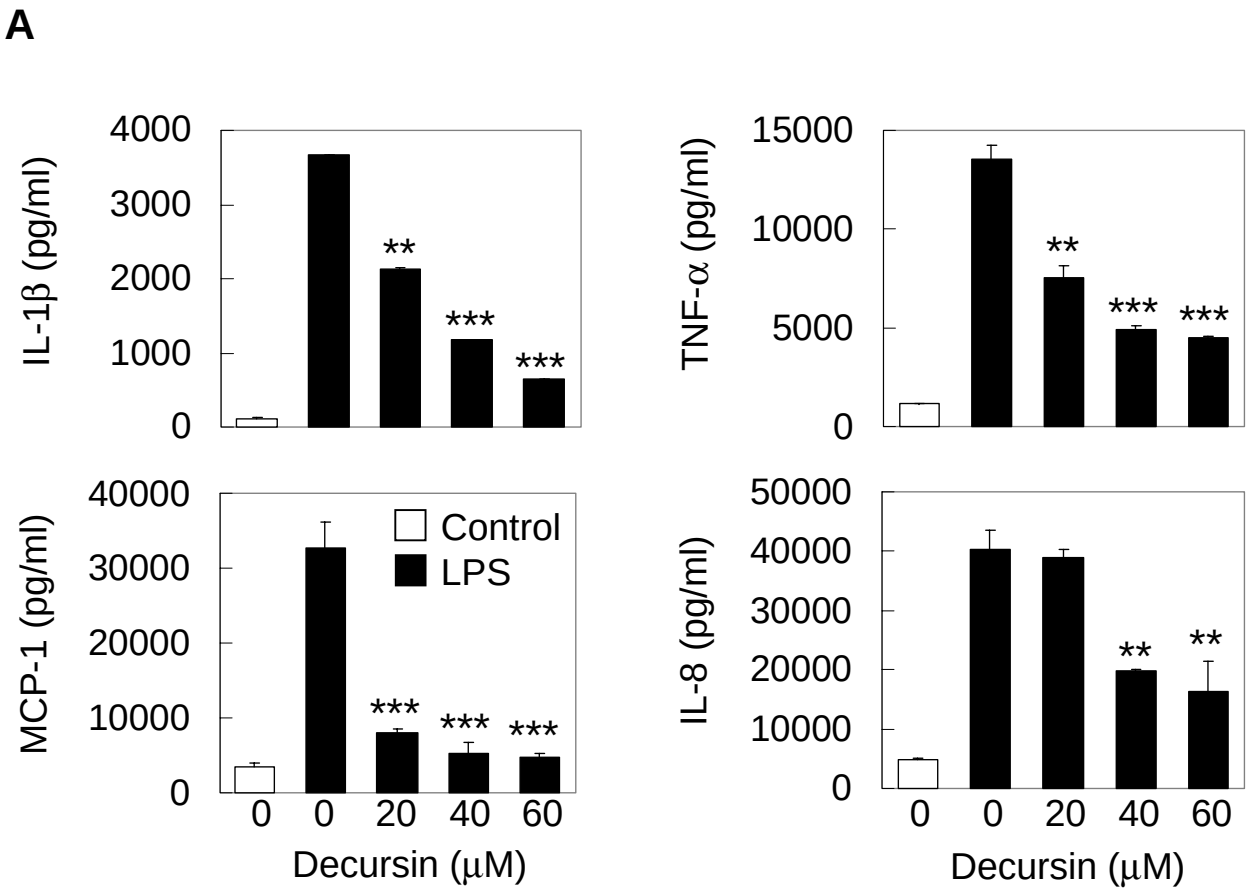
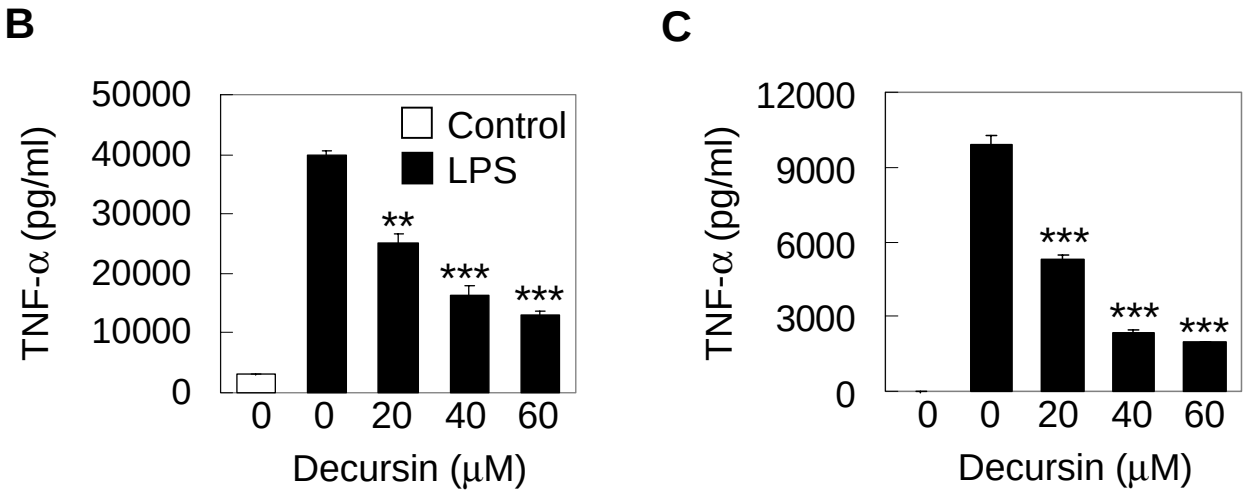


Figure 4 B and C



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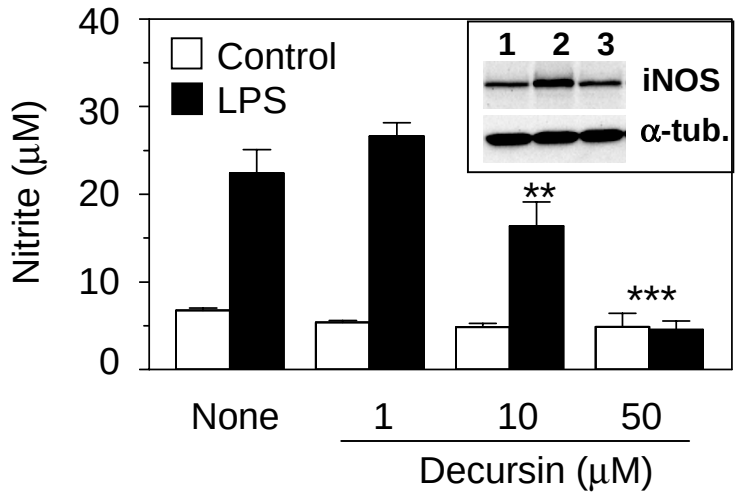

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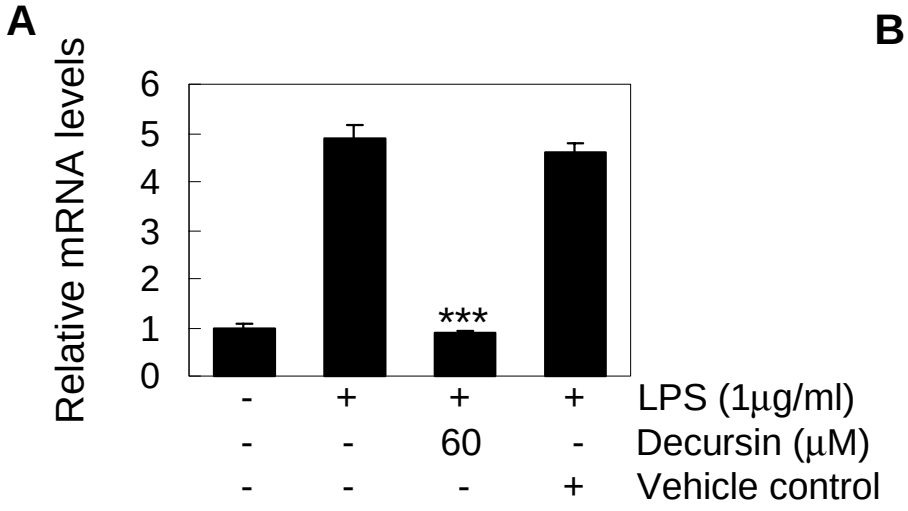

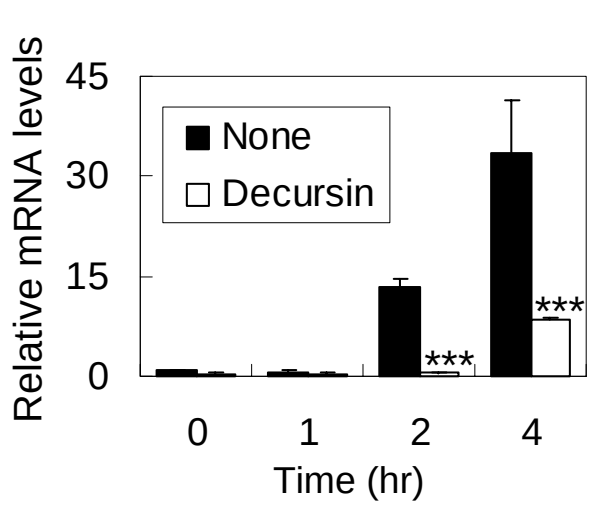

Figure 7 A, B and C

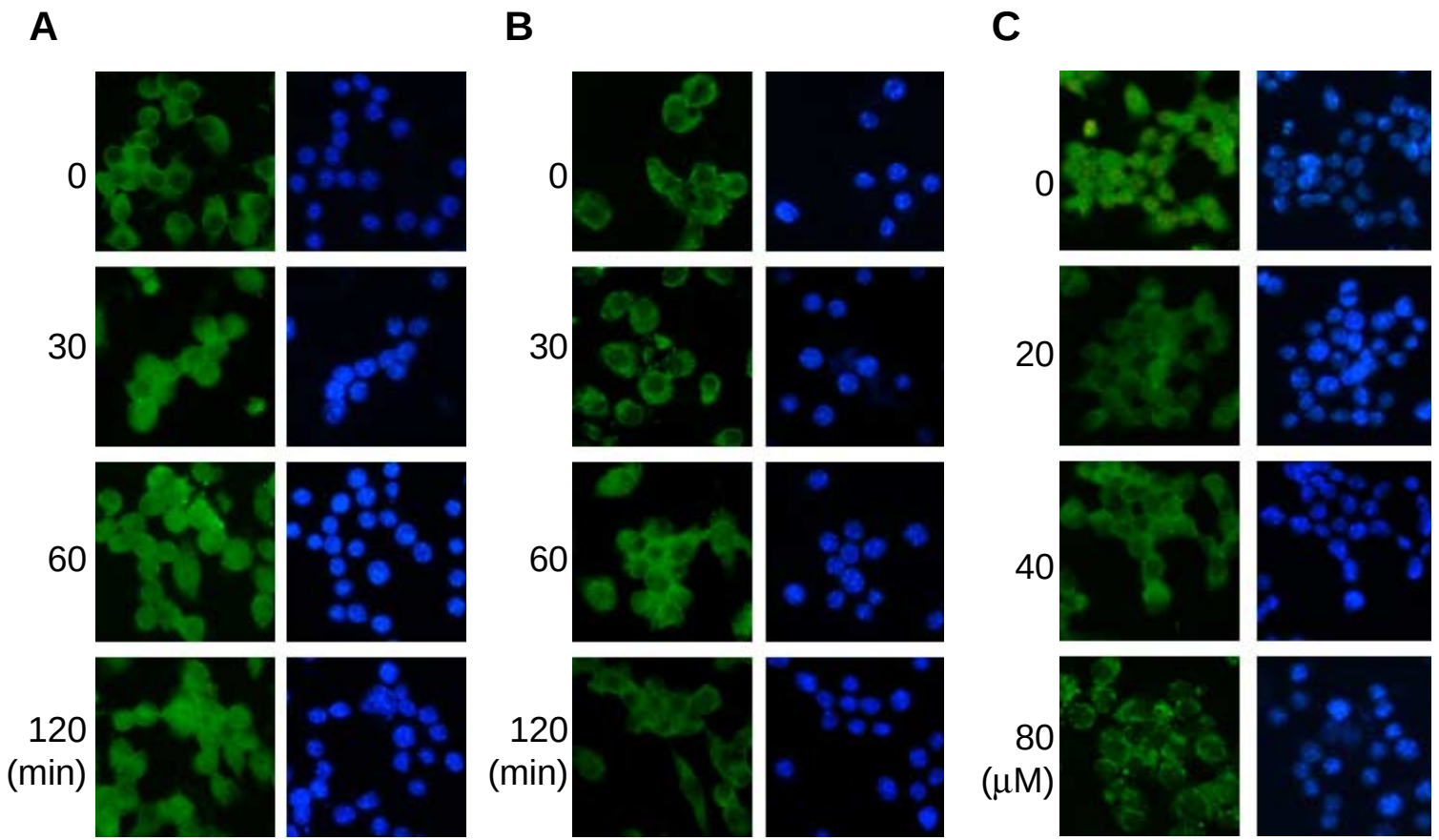

Figure 7 D and E

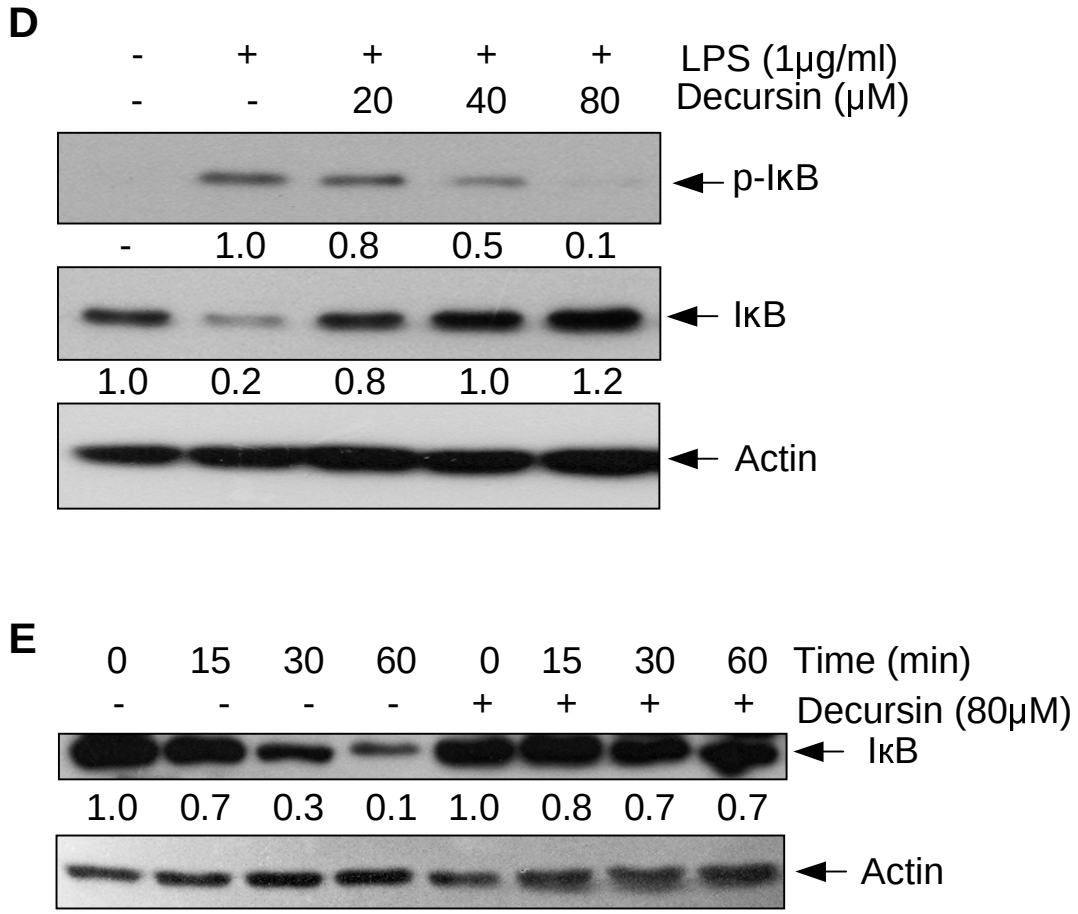

Figure 7F and G

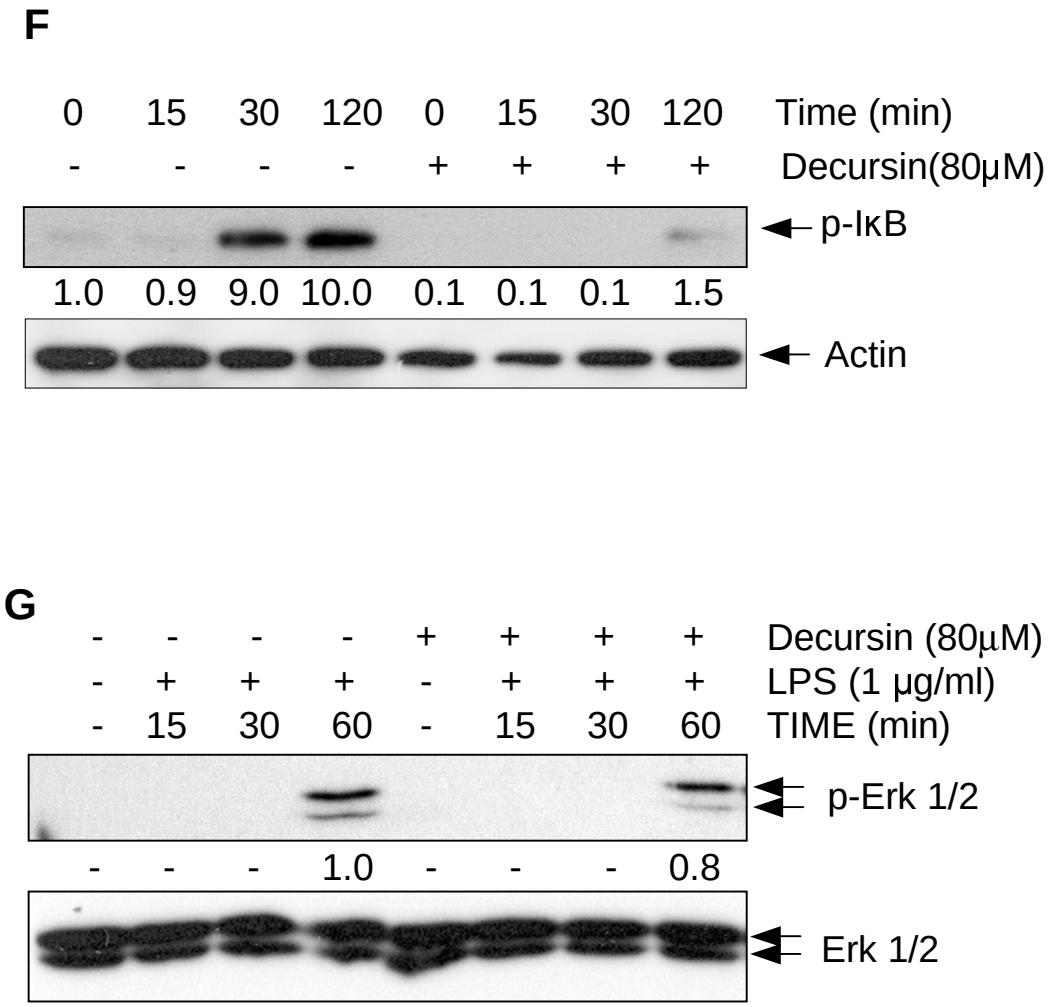

Figure 8 A and B