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PC-SPES: A Potent Inhibitor of Nuclear Factor-κB Rescues Mice from Lipopolysaccharide-Induced Septic Shock

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ABSTRACT

Septic shock is the most common cause of death in intensive care units, and no effective treatment is available at present. Lipopolysaccharide (LPS) is the primary mediator of Gram-negative sepsis by inducing the production of macrophage-derived proinflammatory cytokines, in which activation of nuclear factor-κB (NF-κB) plays an important role. PC-SPES is an eight-herb mixture active against a variety of malignancies, including prostate cancer and leukemia. In this study, we demonstrated that PC-SPES inhibited the LPS-induced NF-κB reporter activity in RAW264.7 macrophages. Electrophoretic mobility shift assay showed that PC-SPES inhibited the binding of NF-κB to specific DNA sequences; however, it did not affect either degradation of inhibitory κBα or nuclear translocation of NF-κB. Also, we explored the effect of PC-SPES on LPS-induced mitogen-activated protein (MAP) kinase signaling; PC-SPES did not affect LPS-induced phosphorylation of MAP kinases, including c-Jun NH_2-terminal kinase, p38, and extracellular signal-regulated kinase 1/2. Moreover, PC-SPES decreased the production of proinflammatory cytokines and inducible enzymes, such as tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, cyclooxygenase-2, as well as inducible nitric-oxide synthase in RAW264.7 macrophages and peritoneal macrophages from C57BL/6 mice after the cells were stimulated by either LPS or LPS and interferon-γ. Furthermore, PC-SPES rescued C57BL/6 mice from death caused by LPS-induced septic shock in conjunction with decreased serum levels of TNFα and IL-1β. Together, PC-SPES is a potent inhibitor of NF-κB and might be useful for the treatment of sepsis and inflammatory diseases.

Sepsis is a syndrome referring to an exaggerated inflammatory and immune response to infections, which may ultimately lead to death from septic shock. Gram-negative bacteria are a common cause of septic shock, which is estimated to result in 20,000 deaths annually in the United States (Pinner et al., 1996). The mechanisms by which Gram-negative bacteria cause septic shock have been well studied over the past 20 years. Many treatment strategies have been developed; however, the mortality rate has not substantially improved (Fisher et al., 1994; Abraham et al., 1995; Bone et al., 1995; Giroir et al., 1997).

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacterial outer membrane, can trigger a variety of inflammatory reactions, including the release of proinflammatory cytokines within the blood. The lipid A-moiety of LPS attaches to the LPS-binding protein, and the resulting complex binds with high affinity to the CD14 toll-like receptor-4 complex on macrophages. The resulting activation of these cells induces release of proinflammatory cytokines, including tumor necrosis factor (TNF) α, interleukin (IL)-1β, and IL-6 via activation of the nuclear factor-κB (NF-κB) (Tobias et al., 1989; Gallay et al., 1993; Bohrer et al., 1997; Poltorak et al., 1998; Lawrence et al., 2001; Beutler, 2002).

NF-κB is a generic term for a dimeric transcription factor formed by the hetero- or homodimerization of a number of the rel family members (Karin and Ben-Neriah, 2000). To date, five rel proteins have been identified: RelA (p65), RelB, and cRel, each having transactivation domains, and p50 and p52, which are expressed as the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. These precursors require post-translational processing and do not contain transactivation domains. The most abundant and active forms of NF-κB are dimeric complexes of p50/RelA (p50/p65). NF-κB is considered to play a pivotal role in immune and inflammatory

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ABBREVIATIONS: LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; NF-κB, nuclear factor-κB; COX, cyclooxygenase; iNOS, inducible nitric-oxide synthase; JNK, c-Jun NH_2-terminal kinase; INF, interferon; IκBα, inhibitory κB; DTT, dithiothreitol; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; RA, rheumatoid arthritis.

responses through the regulation of genes encoding proinflammatory cytokines and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS). These proinflammatory cytokines and enzymes are supposed to be critical mediators of septic shock. Therefore, a rationale target for either prevention or treatment of sepsis and septic shock is NF-κB.

Herbal therapies have been used for many centuries in China as treatment for individuals with cancer as well as inflammatory disease (Risberg et al., 1998). The use of herbs in the treatment or prevention of diseases has been dramatically rising in recent years in the United States; however, their medicinal actions have not been fully elucidated (Eisenberg et al., 1998). PC-SPES contains a partially extracted mixture of eight different herbs: *Dendrantherma morifolium* Tzvel, *Ganoderma lucidium* Karst; *Glycyrrhiza glabra* L., *Isatis indigotica* Fort, *Panax pseudoginseng* Wall, *Rabdosia rubescens* Hart, *Scutellaria baicalensis* Georgi, and *Serenoa repens* Small (DiPaola et al., 1998; Hsieh et al., 1998; Darzynkiewicz et al., 2000). In previous studies, we and others have shown that PC-SPES mediated an antiproliferative effect on prostate cancer cells in vivo and in vitro (DiPaola et al., 1998; Eisenberg et al., 1998; Hsieh et al., 1998; Darzynkiewicz et al., 2000; Kubota et al., 2000). In addition, clinical studies showed that PC-SPES reduced prostate specific antigen levels in more than 80% of individuals with prostate cancer (de la Taille et al., 2000; Small et al., 2000). Recently, we showed that PC-SPES activated the c-Jun NH_2-terminal kinase (JNK)/c-Jun/activator protein-1 signal pathway in LNCaP human prostate cancer cells; and our results suggested that activation of this signal pathway might contribute to PC-SPES-induced apoptosis of these cells (Ikezoe et al., unpublished data). Furthermore, we have shown that PC-SPES inhibited growth and induced differentiation of human myelocytic leukemia cells in conjunction with up-regulation of expression of C/EBP-ϵ, a myeloid specific transcription factor (Ikezoe et al., 2003). Thus, PC-SPES might possess a variety of biological properties.

In this study, we found that PC-SPES inhibited LPS-induced NF-κB transcriptional activity in murine macrophages, leading to their decreased production of proinflammatory cytokines, including TNFα, IL-1β, and IL-6, and the inducible enzymes COX-2 and iNOS. Furthermore, PC-SPES rescued C57BL/6 mice from death caused by LPS-induced septic shock in conjunction with decreased serum levels of TNFα and IL-1β.

Materials and Methods

Cell Line. Murine macrophage RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (Invitrogen) containing no detectable LPS (<0.006 ng/ml as determined by the manufacturer).

Chemicals. LPS from *Escherichia coli* serotype *0111:B4*, interferon-γ (IFNγ), warfarin, and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO). PC-SPES (lot 5431219) was obtained from BotanicLab, Inc. (Brea, CA). One capsule contains 320 mg of powdered herbal extracts. Stock solutions of PC-SPES were prepared by exposing these herbal extracts to ethanol (one capsule/1 ml of 70% ethanol) for 1 h at 40°C in a rocking water bath. Baicalin and oridonin were purified from PC-SPES as described previously (Ikezoe et al., 2001, 2002).

Western Blot Analysis. Degradation of IκBα and nuclear translocation of the p65 subunit of NF-κB were studied by Western blot analysis of cytoplasmic and nuclear extracts of LPS-treated RAW264.7 cells. Cells were suspended in ice-cold extraction buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 10 mM NaCl, 0.2 M EDTA, pH 8.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 100 μg/ml phenylmethysulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin. After 10 min of incubation on ice, nuclei were collected by a short spin in a microcentrifuge. The supernatant was saved as a cytoplasmic fraction, and the nuclei were resuspended in ice-cold extraction buffer containing 300 mM NaCl. After 30 min of incubation, supernatant was collected by centrifugation at 15,000*g* for 20 min at 4°C. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad, Hercules, CA). Proteins were resolved by 4 to 15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Biosciences Inc., Piscataway, NJ), and probed sequentially with antibodies. Anti-IκBα (Imgenex, San Diego, CA) and anti-p65 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used.

The effect of PC-SPES on LPS-stimulated MAP kinases was studied using whole cell lysates from RAW264.7 cells. Whole cell lysates were extracted as described previously (Ikezoe et al., 2001) and transferred to an immobilon polyvinylidene difluoride membrane. Anti-*p*-JNK, anti-*p*-p38, and anti-*p*-ERK (all from Cell Signaling Technology Inc., Beverly, MA) antibodies were used. Protein bands were detected by chemiluminescence (Amersham Biosciences Inc.).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated as described previously using TRIzol (Invitrogen) (Chen et al., 1998). One microgram of DNase I-treated RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 50 ng of the resulting complementary DNAs (cDNAs) were used as templates for polymerase chain reaction (PCR). Real-time PCR was carried out by using *Taq* DNA polymerase (QIAGEN, Valencia, CA), 50 ng cDNA for *COX-2* (500–5 ng in serial dilutions for standard curves), or 1 pg for *18S* (10–0.1 pg for standard curve), and SYBR Green I nucleic acid gel staining solution in a 1:60,000 dilution. Primers used for *COX-2* were 5′-GCTGTACAAGCAGTGGCAAA-3′ and 5′-GTGTACGGCTTCAGGGAGAA-3′, which yielded a 101-base pair product. PCR conditions were as follows: a 95°C initial activation for 15 min followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s, and fluorescence determination at the melting temperature of the product for 20 s on an ICycler detection system (Bio-Rad).

Transfections and Reporter Assay. The NF-κB reporter construct (pGL3-NF-κB) containing four copies of NF-κB site cloned into pGL3-basic plasmid (Promega, Madison, WI) was a generous gift from Dr. Moshe Arditi (Cedars-Sinai Medical Center, UCLA School of Medicine). RAW 264.7 cells (2×10^5/ml) were plated on 24-well plates and incubated until 60–80% confluence. Cells were transfected with pGL3-NF-κB by using the GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). After 24 h, cells were preincubated with either PC-SPES or control diluent for 1 h and exposed to LPS (100 ng/ml, 6 h). Luciferase activity in cell lysates was measured by the dual luciferase assay system (Promega), which was normalized by *Renilla reniformis* activities. The results were presented as fold induction, which is the relative luciferase activity of the LPS-treated cells over that of the untreated control cells. All transfection experiments were carried out in triplicate wells and repeated separately at least three times.

Electrophoretic Mobility Shift Assay (EMSA). RAW264.7 cells (2×10^5/ml) treated with either PC-SPES (1 or 2 μl/ml, 1 h) or control diluent were exposed to LPS (100 ng/ml for 30 min) at 37°C. Four micrograms of nuclear extracts were incubated with 16 fmol of ^{32}P-end-labeled NF-κB binding probe. The DNA-protein complex was separated from the free oligonucleotide on a 5% polyacrylamide gel. The specificity of NF-κB DNA binding was examined by competition with a double-stranded mutated oligonucleotide, unlabeled oligonucleotide, and by supershift of the band by anti-p65 antibodies. Gels

were dried and transferred to the membrane and exposed to Kodak XAR film (Eastman Kodak, New Haven, CT). The band intensity was measured by a densitometer.

Measurement of the Cytokines, TNFα, IL-1β, and -6 in Culture Medium and Plasma from C57BL/6 Mice. RAW 267.4 cells or peritoneal macrophages from C57BL/6 mice were plated in 24 wells (2×10^5/ml). Cells were cultured either with PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. These cells were then exposed to LPS (100 ng/ml). Culture medium was collected at the indicated time points and concentrations of TNFα, IL-1β, and IL-6 were measured by murine specific ELISA kits (BD PharMingen, San Diego, CA). Blood samples from mice were obtained retro-orbitally using heparinized tubes, collected in plasma separation tubes, and the centrifuged (3,000*g*, 10 min) supernatants were subjected to ELISA.

Induction and Measurement of iNOS and COX-2. RAW 264.7 cells (2×10^5/ml) were plated in 100-mm dishes and treated with PC-SPES (2 μl/ml) for 1 h. Then, cells were exposed to either 100 ng/ml LPS alone for 6 h or the combination of LPS (100 ng/ml) and IFNγ (100 IU/ml) for 24 h for measurement of COX-2 and iNOS, respectively. Whole cell lysates were extracted as described previously (Ikezoe et al., 2001) and resolved on a 5 to 15% SDS-polyacrylamide gel. Levels of iNOS and COX-2 were analyzed by Western blot analysis by using rabbit anti-iNOS (Santa Cruz Biotechnology Inc.) and anti-COX-2 (Santa Cruz Biotechnology Inc.) antibodies, respectively.

Effect of PC-SPES on Mortality of C57BL/6 Mice Challenged with LPS. Female mice, at 8 to 10 weeks of age, 18 to 20 g (Harlan, Indianapolis, IN) were injected intraperitoneally with either PC-SPES (160 μl) or control diluent (70% ethanol, 160 μl). In our previous studies, this amount of PC-SPES was administered orally to nude mice without any side effects (Kubota et al., 2000). Two hours later, mice received intraperitoneal injection of either *E. coli 0111:B4* LPS (500 mg/mouse in 200 μl of PBS) or PBS (200 μl). The survival of the mice was monitored for 72 h after injection of LPS, after which no further loss of mice occurred.

Statistical Analysis. Statistical differences in cytokine production and COX-2 expression were determined by two-tailed Student's *t* test. Statistical differences in survival curves among the groups of mice were analyzed by log rank test. For both analyses, Prism and Instat software (GraphPad Software Inc., San Diego, CA) were used.

Results

Effect of PC-SPES on LPS-Induced NF-κB Reporter Activity in RAW264.7 Cells. We previously demonstrated that PC-SPES had an antiproliferative activity against a variety of types of cancer (Kubota et al., 2000; Ikezoe et al., 2001; Huerta et al., 2002). This prompted us to begin to determine its mechanism of action; and in preliminary studies, we have found that PC-SPES had NF-κB inhibitory activity (data not shown). This encouraged us to explore the anti-inflammatory activity of this agent. LPS (100 ng/ml, 6 h) induced NF-κB reporter activity in RAW 264.7 cells by 30-fold; and pretreatment of cells with PC-SPES inhibited this LPS-induced NF-κB reporter activity in a dose-dependent manner. One or 2 μl/ml PC-SPES inhibited this reporter activity by 43 and 75%, respectively (Fig. 1A). PC-SPES was not toxic to RAW 264.7 cells under the culture conditions used for these experiments, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (data not shown).

Further studies explored the ability of major components of PC-SPES to inhibit LPS-induced NF-κB reporter activity in RAW 264.7 cells (Fig. 1B). Oridonin, which was purified from one of the component of PC-SPES, *Rabdosia rubescens* (Ikezoe et al., 2002), inhibited reporter activity by 46% ($p = 0.001$); however, baicalin (Ikezoe et al., 2001) inhibited this reporter activity by only 15%. These data suggested that oridonin may represent one of the major components of PC-SPES producing the anti-NF-κB activity. Recently, warfarin (0.15 μg/1cap) and indomethacin (0.28 μg/capsule) were identified in this lot of PC-SPES (Sovak et al., 2002); however, our control studies showed that both warfarin (10^{-5} M) and indomethacin (2×10^{-6} M, 1 h) failed to inhibit NF-κB reporter activity in RAW 264.7 cells under identical culture conditions (Fig. 1B). To certify that the inhibition of reporter activity mediated by PC-SPES was specific to LPS-induced NF-κB, RAW 264.7 cells were transfected with pG5E4Tlux (luciferase reporter gene with GAL4 DNA binding sites in the promoter) and pSVGAL4-VP-16 (GAL4-VP-16 expression vector). GAL4-VP-16 induced pG5E4Tlux reporter activity (Fig. 1C). PC-SPES (2 μl/ml) did not inhibit this reporter activity, suggesting that the inhibition of the transcription factor NF-κB by PC-SPES was specific, and it did not inhibit all transcription factors.

The ability of PC-SPES to inhibit LPS-induced NF-κB transcriptional activity was further confirmed by EMSA (Fig. 2A). RAW264.7 cells were cultured with LPS (100 ng/ml, 30 min); nuclear lysate was isolated and was found to bind approximately 50-fold greater to the NF-κB DNA binding site (lane 2) compared with nuclear lysate from the same cells not cultured with LPS (lane 1). Exposure of the RAW264.7 cells to both PC-SPES (2 μl/ml) and LPS decreased the LPS-induced binding formation by 50% (lane 4). The LPS-induced NF-κB/DNA complex was competed with 50-times molar excess of unlabeled oligonucleotides (lane 6), but not with same molar excess of mutated oligonucleotides (lane 7), and the retarded band was supershifted by anti-p65 antibody (lane 8) confirming the specificity of NF-κB band.

Activation of NF-κB involves two important steps: 1) phosphorylation and subsequent degradation of IκBα caused by IκB kinase resulting in release of NF-κB, and 2) the nuclear translocation of the activated NF-κB. To elucidate the effect of PC-SPES on these steps, control and PC-SPES-treated RAW 264.7 cells were exposed to LPS (100 ng/ml) for various durations. The kinetics of IκBα phosphorylation and degradation were studied by Western blot analysis by using cytoplasmic extracts. As shown in Fig. 3, A and B, no significant difference in the pattern of IκBα phosphorylation and degradation after treatment with PC-SPES was observed (Fig. 3, A and B). Next, to study the accumulation of the activated NF-κB into the nucleus, the appearance of the p65 subunit of NF-κB in the nuclear extracts of control and PC-SPES-treated RAW 264.7 cells were examined. Exposure of RAW 264.7 cells to LPS (100 ng/ml) for 15 min induced accumulation of NF-κB into the nucleus (Fig. 3C). These results were nearly identical when the experiments were repeated with PC-SPES-treated RAW264.7 cells. These results suggested that probably PC-SPES inhibited NF-κB transcriptional activity via inhibition of the ability of NF-κB to bind to DNA of the target genes.

Effect of PC-SPES on LPS-Induced Cytokine Production by Macrophages. To determine the effect of PC-SPES on production of LPS-induced proinflammatory cytokines, RAW 264.7 macrophages were cultured with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h and then exposed to LPS (100 ng/ml). Three hours of exposure of cells to LPS stimulated RAW 264.7 cells to secrete 4,400 $\pm$ 3,400 pg/ml TNFα. However, pretreatment of the cells with PC-SPES for 1 h decreased the mean level of

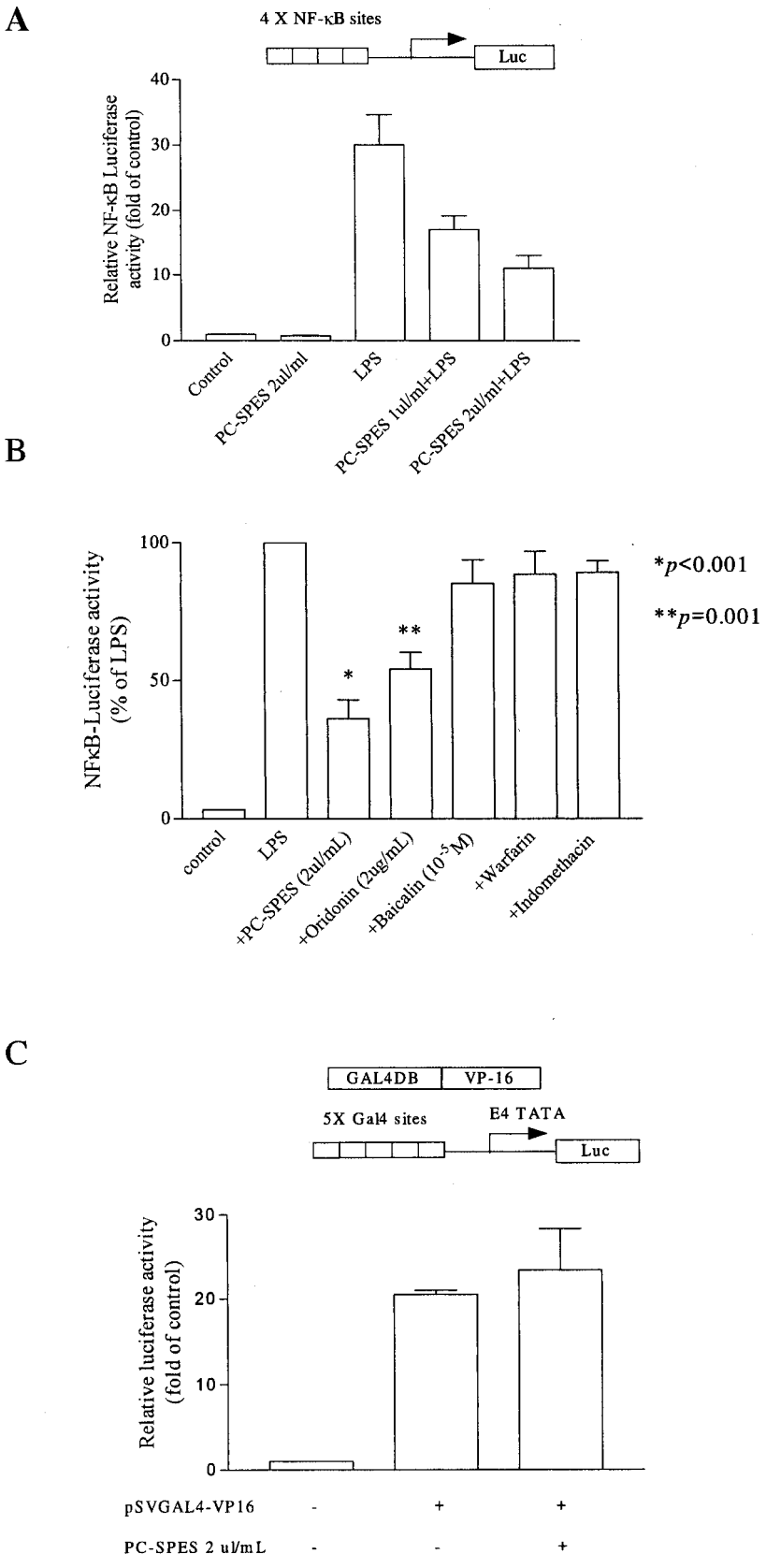

Fig. 1. A, effects of PC-SPES on the NF-κB transcriptional activity in RAW264.7 cells. The construct (NF-κB-Luc) containing the four copies of NF-κB binding sites attached to pGL3 luciferase reporter plasmid is shown at the top. RAW264.7 cells were transfected with NF-κB luciferase plasmid (0.8 μg). These cells were then cultured either with PC-SPES or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without LPS (100 ng/ml) for 6 h, at which time luciferase activity was measured. B, effect of oridonin and baicalin on the NF-κB transcriptional activity in RAW264.7 cells. RAW264.7 cells were transfected with NF-κB luciferase plasmid (0.8 μg). These cells were then cultured either with PC-SPES (2 μl/ml), oridonin (2 μg/ml), baicalin (10^{-5} M), warfarin (10^{-5} M), indomethacin (2×10^{-6} M), or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and cultured either with or without LPS (100 ng/ml) for 6 h at which time luciferase activity was measured. C, effect of PC-SPES on GAL4-VP-16 transcriptional activity. The construct (pG5E4Tlux) containing the five concatermized GAL4 binding sites attached to the luciferase reporter and the expression vector containing the DNA binding domain of GAL4 fused to VP-16 are shown at the top. RAW264.7 cells were cotransfected with pG5E4Tlux (0.8 μg) and pSVGAL4-VP16 (50 ng), and cultured with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 6 h. Lysates from these cells were subjected to luciferase assay. Results represent the mean ± S.D. of three experiments with triplicate dishes per experimental point. pRL-SV40-Luciferase (*R. reniformis* luciferase) vector was cotransfected for normalization. GAL4DB, DNA binding domain of GAL4.

LPS-induced TNFα to 303 $\pm$ 140 pg/ml ($p < 0.04$) (Fig. 4A). In additional experiments, a 6-h exposure of RAW 264.7 cells to LPS resulted in a mean secretion of 350 $\pm$ 300 pg/ml IL-6 and 51 $\pm$ 30 ng/ml IL-1β. Pretreatment of these cells with PC-SPES for 1 h, washing them, and exposing these cells to LPS for 6 h decreased levels of IL-6 to 31 $\pm$ 27 pg/ml ($p = 0.05$) and levels of IL-1β to 3 $\pm$ 4 ng/ml ($p = 0.02$) (Fig. 4, B and C). Also, the ability of PC-SPES to inhibit the production of proinflammatory cytokines in peritoneal macrophages from C57BL/6 mice was studied. Peritoneal macrophages were obtained from mice under anesthesia, cultured with PC-SPES (2 μl/ml) for 1 h, and exposed to LPS (100 ng/ml) for 3 h. LPS induced production of 1,300 $\pm$ 600 pg/ml TNFα in these cells and a 1-h pulse pretreatment with PC-SPES (2 μl/ml) inhibited production of TNFα by about 90% (Fig. 4D).

Effect of PC-SPES on LPS-Stimulated MAP Kinases. Previous studies showed that LPS activated MAP kinases, including JNK, extracellular signal-regulated protein kinase (ERK), and p38 (Guha and Mackman, 2001). LPS-activated MAP kinases mediated cytokine production in macrophages (Scherle et al., 1998). Therefore, we explored whether PC-SPES affects LPS-stimulated MAP kineses in RAW264.7 cells. Control RAW264.7 cells constitutively expressed phosphorylated forms of ERK and p38, and exposures of these cells to LPS (100 ng/ml) increased their levels by 3- and 2-fold at 15 min, respectively. Phosphorylated form of JNK was negligible in RAW264.7 cells and exposure of these cells to LPS (100 ng/ml) induced phosphorylation of JNK at time 15 min, which was sustained at 30 min (Fig. 5); PC-SPES did not down-regulate levels of LPS-induced phosphorylated forms of MAP kinases in RAW264.7 cells compared with control diluent-treated cells (Fig. 5).

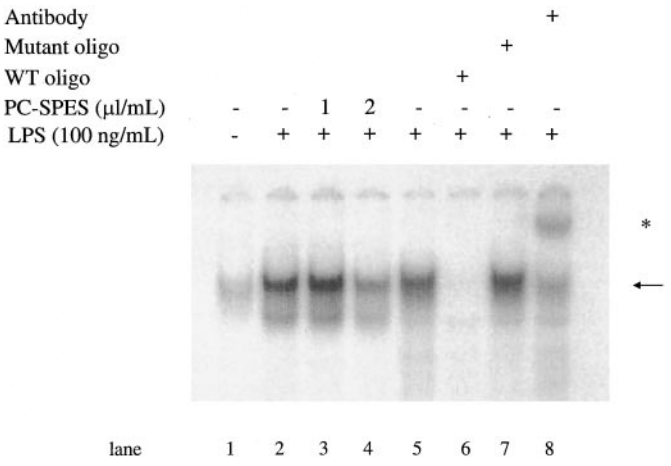

Fig. 2. Effect of PC-SPES on NF-κB DNA binding in RAW264.7 cells. RAW264.7 cells were preincubated either with PC-SPES or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without LPS (100 ng/ml) for 30 min. Nuclear extracts were prepared and EMSA was performed as described under *Materials and Methods*. The arrow indicates the gel location of NF-κB bound to DNA. *, supershifted band.

Effect of PC-SPES on Induction of Inducible Enzymes in Macrophages. The expression of the inducible enzymes, COX-2, and iNOS are also regulated by NF-κB. Therefore, the effect of PC-SPES on induction of COX-2 and iNOS was studied. RAW 264.7 or peritoneal murine macrophages were cultured with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. Cells were washed twice with PBS and exposed to LPS (100 ng/ml). Three hours later, the cells were harvested, RNA was extracted and subjected to reverse transcriptase. Synthesized cDNAs were used for real-time PCR with SYBR Green to measure the level of *COX-2*. PC-SPES dramatically down-regulated LPS-induced expression of *COX-2* (Fig. 6, A and B). The RAW 264.7 cells treated with LPS had about a 19-fold increase in *COX-2* RNA levels and PC-SPES inhibited this induction by 55% (Fig. 6A). Furthermore, LPS induced expression of *COX-2* RNA about 37-fold in peritoneal macrophages and PC-SPES almost completely inhibited this induction (Fig. 6B).

The ability of PC-SPES to inhibit LPS-induced COX-2 was also confirmed at the protein level. COX-2 protein was not detectable in control RAW 264.7 cells. LPS (100 ng/ml, 6 h) dramatically induced expression of COX-2, and a 1-h pulse pretreatment of the cells with PC-SPES (2 μl/ml) down-regulated protein levels of COX-2 by 70% (Fig. 6C). On the other hand, COX-1 was constitutively expressed in control untreated cells, and levels of this protein were not modulated by PC-SPES. Expression of iNOS was also induced in RAW 264.7 cells cultured in the presence of LPS (100 ng/ml) and IFNγ (100 IU/ml) for 24 h, and pretreatment of these cells with PC-SPES prominently decreased the level of LPS-induced iNOS (Fig. 6C).

Effect of PC-SPES on in Vivo LPS-Induced Cytokine Production and Lethality. To determine the effect of PC-SPES on LPS-induced proinflammatory cytokine levels in vivo, mice were injected with *E. coli 0111:B4* LPS either in the absence or presence of PC-SPES, and cytokine levels were measured in the sera up to 3 h after injection of LPS. Injection of LPS induced strong induction of TNFα and IL-1β in C57BL/6 mice. After 1.5 and 3 h, serum levels of TNFα and

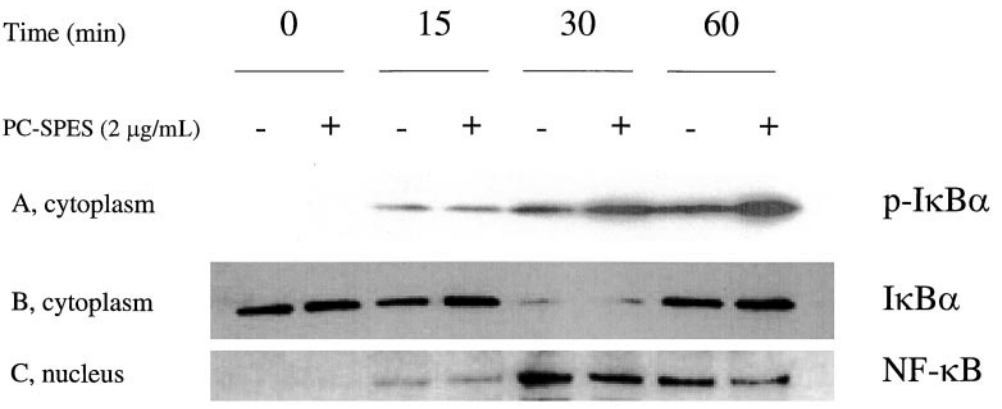

Fig. 3. Effect of PC-SPES on LPS-induced phosphorylation (A), degradation of IκBα (B), and nuclear translocation of NF-κB (p65) (C) in RAW264.7 cells. RAW264.7 cells were pretreated either with PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and either treated with (+) or without (−) LPS (100 ng/ml) for the indicated time periods. The cytoplasmic (A and B) and nuclear extracts (C) of these cells were prepared and subjected to Western blot analysis to measure the level of IκBα and p65 of NF-κB, respectively.

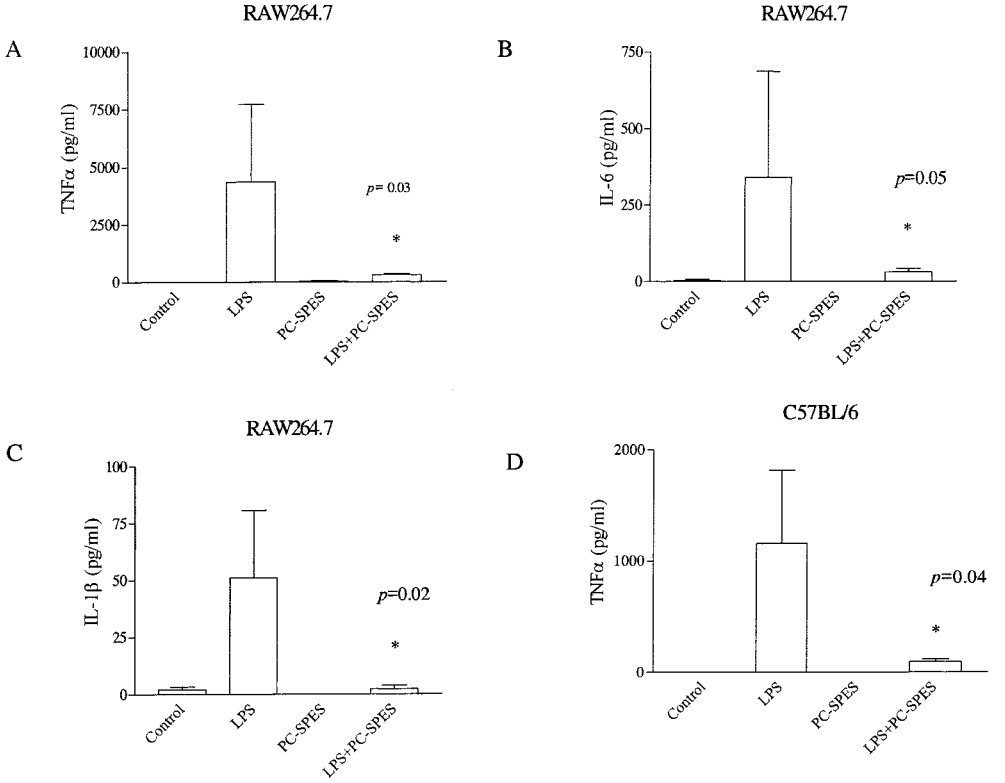

Fig. 4. Effect of PC-SPES on LPS-induced production of proinflammatory cytokines: TNFα (A and D), IL-6 (B), IL-1β (C) in RAW264.7 cells (A–C), and macrophages from C57BL/6 mice (D). RAW264.7 cells were pretreated with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without LPS (100 ng/ml) for 3 h for measurement of TNFα (A) or for 6 h for measurement of IL-1β (B) and IL-6 (C). Cell culture supernatant was analyzed for cytokine levels by ELISA. D, peritoneal macrophages were obtained from C57BL/6 mice and pretreated either with PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and cultured either with or without LPS (100 ng/ml) for 3 h for measurement of TNFα in the conditioned culture medium. Results represent the mean $\pm$ S.D. of three experiments with triplicate dishes per experimental point.

IL-1β reached a mean 3.6 $\pm$ 1.0 ng/ml and 480 $\pm$ 106 pg/ml, respectively (Fig. 7, A and B). Preadministration of PC-SPES (2 h before LPS challenge) to these mice decreased their LPS-induced serum levels of TNFα and IL-1β to 1.6 $\pm$ 2.4 ng/ml and 142 $\pm$ 92 pg/ml, respectively ($p < 0.05$). On the other hand, neither control diluent nor PC-SPES alone affected serum levels of these cytokines (Fig. 7, A and B).

The proinflammatory cytokines TNFα and IL-1β may play an important role in the fatal outcome of Gram-negative sepsis. We hypothesized that inhibition of these LPS-induced proinflammatory cytokines by administration of PC-SPES would make mice less susceptible to a lethal dose of LPS. Mice were injected with either PC-SPES or control diluent; and 2 h later, they were challenged intraperitoneally with 500 mg of *E. coli 0111:B4* LPS. Intraperitoneal injection of LPS into control mice resulted in death of 50% (5/10) of the mice within 72 h (Fig. 8). In contrast, all mice were rescued by administration of PC-SPES.

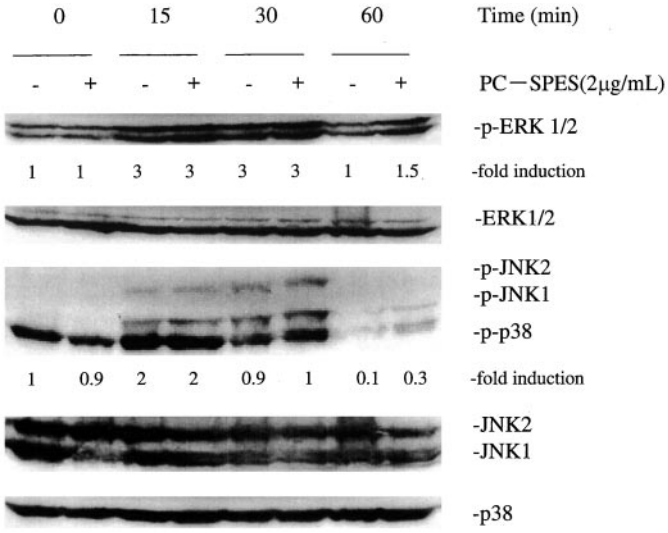

Fig. 5. Effect of PC-SPES on LPS-induced MAP kinases in RAW264.7 cells. RAW264.7 cells were pretreated either with PC-SPES (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and either cultured with (+) or without (−) LPS (100 ng/ml) for the indicated time periods. Whole cell lysates of these cells were prepared and subjected to Western blot analysis to measure the level of either phosphorylated or total level of ERK, p38, and JNK. The band intensities were measured by densitometry.

Discussion

This is the first observation demonstrating that PC-SPES acted as an immune modulator via inhibition of NF-κB transcriptional activity in murine macrophages. PC-SPES inhibited the binding of NF-κB to its consensus sequences on target genes, without affecting either degradation of IκBα or translocation of NF-κB to the nucleus.

The X-ray structure of RelA showed that it possesses cysteine residues in its DNA binding site, which was found to be critical for optimal protein/DNA interaction (Kumar et al., 1992; Chen et al., 1998). PC-SPES could affect cysteine residues that contain sulfhydryl groups, resulting in disruption of protein/DNA interaction. In fact, our studies demonstrated that treatment of nuclear extracts with DTT, which was able to prevent alkylation of free sulfhydryls, reversed the PC-SPES-induced inhibition of binding of NF-κB to its DNA binding site in the *COX-2* promoter (T. Ikezoe, Y. Yang, T. Saitoh, D. Heber, R. McKenna, S. Chen, H. Taguchi, and H. P. Koeffler, manuscript in preparation), suggesting that cysteine residues necessary for protein/DNA binding were affected by PC-SPES. These results are reminiscent of the

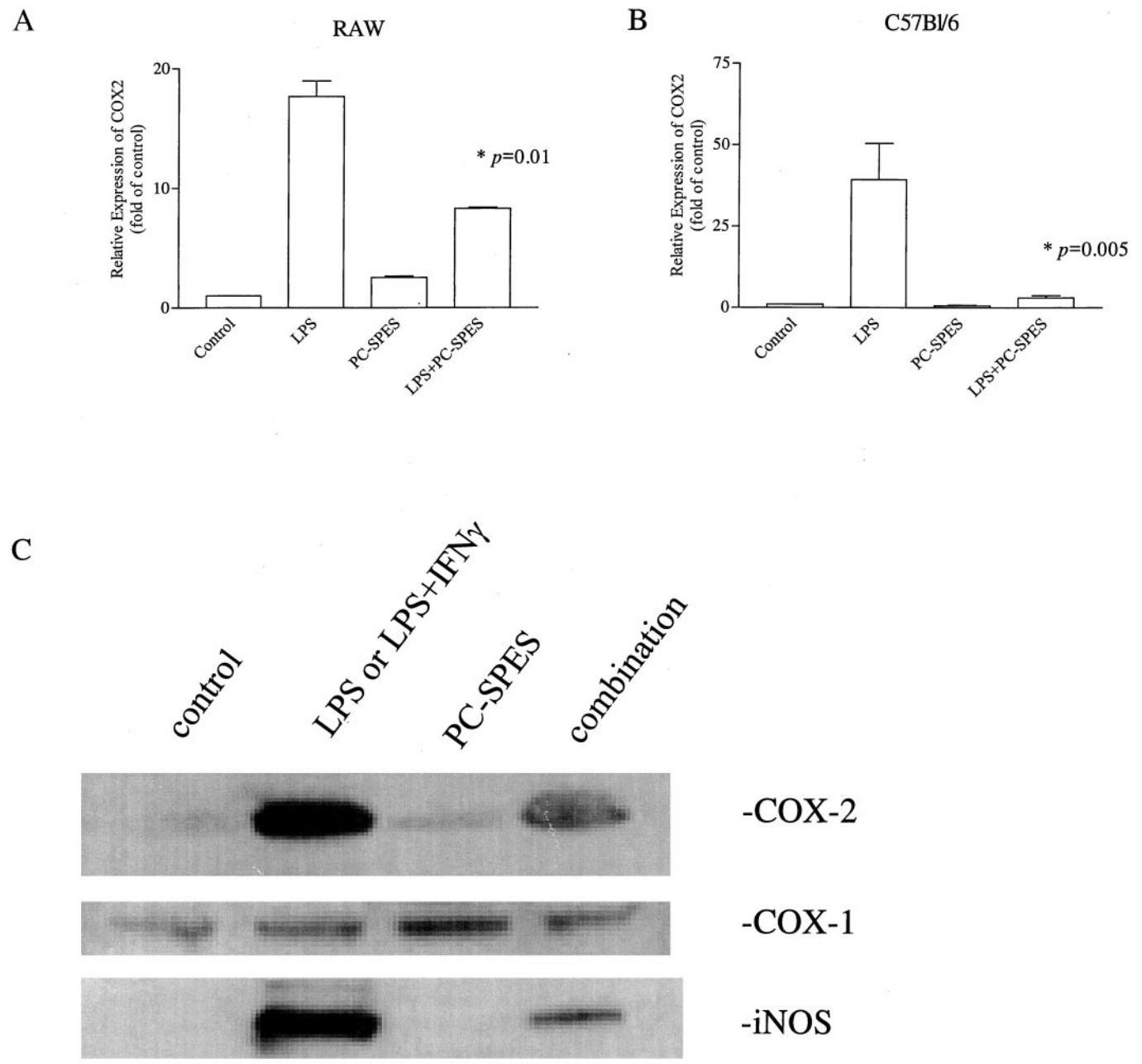

Fig. 6. Effect of PC-SPES on level of inducible enzymes COX-2 and iNOS in RAW 264.7 cells and macrophages from C57BL/6. RAW 264.7 cells (A) and peritoneal macrophages from C57BL/6 mice (B) were pretreated with either PC-SPEC (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. At the end of the treatment, cells were washed twice with PBS and cultured either with or without LPS (100 ng/ml) for 3 h. Cells were harvested and RNA was extracted. cDNAs were synthesized and subjected to real-time PCR by using SYBR Green I nucleic acid gel staining solution as described under *Materials and Methods*. Results represent the mean ± S.D. of three experiments with triplicate dishes per experimental point. C, Western blot analysis. RAW 264.7 cells were pretreated with either PC-SPEC (2 μl/ml) or control diluent (ethanol, 0.28%) for 1 h. Cells were washed twice with PBS and cultured with either LPS (100 ng/ml, 6 h) for the COX-1 and -2 studies or the combination of LPS (100 ng/ml, 24 h) and INFγ (100 IU/ml, 24 h) for the iNOS studies. Whole cell lysates were extracted and Western blot analysis was performed as described under *Materials and Methods* to measure levels of either COX-2 or iNOS.

mode of action of several other compounds. For example, avicin (Haridas et al., 2001), a family of triterpenoid saponins from *Acacia victoricae* Bentham, and kamebakaurin (Lee et al., 2002) from *Isodon japonicus* Hara inhibited NF-κB transcriptional activity by inhibition of binding of NF-κB to specific DNA sequences, and DTT treatment reversed this inhibition. Moreover, kamebakaurin failed to inhibit the binding of NF-κB when Cys-62 in the DNA-binding site of NF-κB was mutated, suggesting that kamebakaurin inhibited NF-κB transcriptional activity by modifying Cys-62 of NF-κB. Of interest, oridonin (Ikezoe et al., 2002), purified from *Rabdosia rubescens* Hara, which is one of the eight herbs in PC-SPES, possesses the same basic terpene structure as avicins (Haridas et al., 2001) and kamebakaurin (Lee et al., 2002). Furthermore, we recently have found that oridonin could inhibit TNFα-induced NF-κB activity in human T lymphocytes as measured by reporter assay, and EMSA showed that oridonin inhibited the binding of NF-κB to its DNA binding

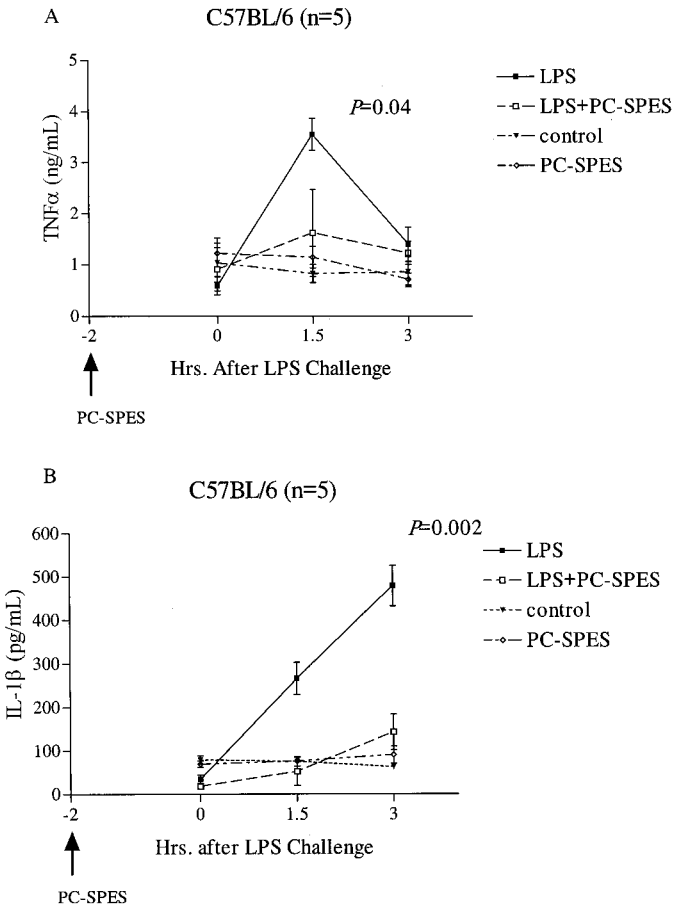

Fig. 7. Effect of PC-SPES on the LPS-induced serum levels of proinflammatory cytokines in C57BL/6 mice. C57BL/6 mice were given either PC-SPES (160 μl) or control diluent (70% ethanol, 160 μl) intraperitoneally. Two hours later, mice were challenged with either LPS (500 mg/mouse in 200 μl of PBS) or PBS (200 μl). Blood samples were obtained at the indicated times retro-orbitally, and serum was screened for levels of TNFα (A) and IL-1β (B). Results represent the mean ± S.D. of five experiments.

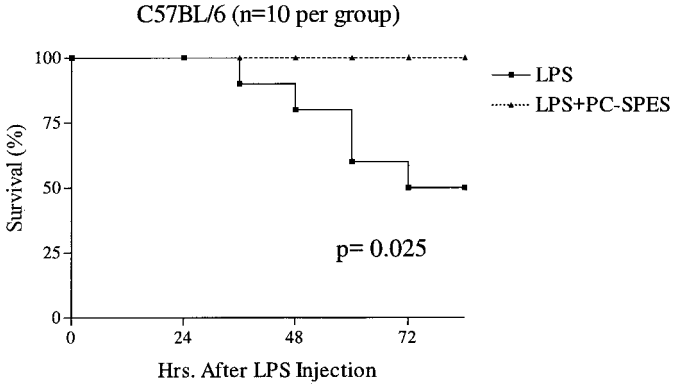

Fig. 8. Effect of PC-SPES on LPS-induced mortality. C57BL/6 mice were injected with either PC-SPES (160 μl) or control diluent (70% ethanol, 160 μl) intraperitoneally; and 2 h later, they were challenged with either LPS (500 mg/mouse, in 200 μl of PBS) or PBS (200 μl). Survival was determined during the 72-h period after LPS injection; a duration after which no further loss of animals occurred (n = 10/group). Kaplan-Meier survival curves are shown.

sequences (T. Ikezoe, Y. Yang, T. Saitoh, D. Heber, R. Mc Kenna, S. Chen, H. Taguchi, and H. P. Koeffler, manuscript in preparation). These findings are congruent with our hypothesis that the anti-inflammatory effects of PC-SPES emanate in part from oridonin affecting NF-κB activity.

Importantly, PC-SPES inhibited expression of LPS-inducible enzymes COX-2 and iNOS in murine macrophages. Both enzymes are critical for the response of tissues to injury or infectious agents and are essential components of the inflammatory response, as well as repair of injury and carcinogenesis (Suh et al., 1998). Physiological activity of these enzymes may provide a benefit to the organism; however, the aberrant or excessive expression of either COX-2 or iNOS has been implicated in a variety of pathological processes, including arthritis, carcinoma, as well as septic shock. For example, elevated expression of COX-2 and iNOS was found in synovial tissue and cartilage from individuals with rheumatoid arthritis (RA), and their metabolic products nitric oxide and prostaglandin E_2, which are pleiotropic inflammatory mediators, were overproduced in the affected joints (Amin et al., 1999). Thus, selective inhibitors of COX-2, iNOS, or both may have a therapeutic role in RA. Perhaps, PC-SPES has anti-rheumatoid arthritis activity via inhibition of NF-κB leading to down-regulation of proinflammatory cytokines as well as COX-2 and iNOS in the affected joints.

Aberrant expression of COX-2 and iNOS has been observed in many types of cancer, including those from colon and lung (Rao et al., 2002). A murine colon cancer model, the Apc^{min} mouse, seems to validate the importance of *COX-2* and *iNOS* gene in carcinogenesis. These mice have a germ line, nonsense mutation at codon 716 of the adenomatous polyposis coli (*APC*) gene, and they spontaneously develop multiple polyps in their small and large intestines at the age of 10 to 12 weeks (Oshima et al., 1996). Therefore, Apc^{min} mice are considered to be useful for analysis or prevention of human familial adenomatous polyposis and sporadic colorectal cancers. The polyp formation was dramatically prevented in Apc^{min} mice when they were either treated with known COX-2 and iNOS inhibitors (Oshima et al., 1996, 2001; Rao et al., 2002), or the mice were engineered to have a deletion of either the *COX-2* or the *iNOS* gene (Oshima et al., 1996; Ahn et al., 2001). Similarly, we have discovered that administration of PC-SPES to these mice can prevent tumor development in a large proportion of the mice (Huerta et al., 2002). Future studies will further explore the ability of PC-SPES to inhibit the proliferation of colon and lung cancer cells in conjunction with down-regulation of COX-2 and iNOS in these cells.

We found that PC-SPES inhibited LPS-induced production of IL-6 in macrophages. IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and carcinogenesis (Willenberg et al., 2002). Its signal is mediated by two pathways. One is through the IL-6 receptor and gp-130, which signals through the Janus family tyrosine kinase-signal transducer and activator of transcription proteins. The second is through the Ras mitogen-activated protein kinase pathway. Multiple myeloma cells often have enhanced expression of IL-6, resulting in activation of both their Janus family tyrosine kinase-signal transducer and activator of transcription and their Ras signal pathways (Frassanito et al., 2002). Further studies found increased levels of IL-6 in the serum from individuals with multiple myeloma as well as Castleman's disease (Hawley and Berger, 1998; Kakiuchi et al., 2002). Thus, dysregulation of IL-6 may play an important role in carcinogenesis and/or progression of these malignancies. Further

studies will explore whether PC-SPES inhibits the proliferation of these cells via inhibition of their secretion of IL-6.

Together, PC-SPES decreased the production of proinflammatory cytokines and inducible enzymes in murine macrophages in part via inhibition of NF-κB transcriptional activity. Furthermore, it rescued C57BL/6 mice from LPS-induced septic shock in conjunction with decreased serum levels of the proinflammatory cytokines TNFα and IL-1β. Therefore, PC-SPES might be useful for the treatment of septic shock as well as many kinds of inflammatory diseases, including RA. Recently, warfarin or a warfarin-like compound and possibly indomethacin have been found in the lot of PC-SPES that we used (Sovak et al., 2002). The final concentration of PC-SPES (2 μl/ml) used in this study would theoretically contain approximately 2×10^{-6} M of these reagents; in control plates at these concentrations, they did not inhibit NF-κB transcriptional activity (Fig. 2B). Recently, a new batch of PC-SPES was produced which was shown not to contain any warfarin or indomethacin as determined by gas chromatography and mass spectrometry, performed by AEGIS Analytical Laboratories (Nashville, TN), but we found that it had nearly the same anti-NF-κB activity as reported here (data not shown). Our data showed that one component of PC-SPES, oridonin, clearly has anti-inflammatory activity. Further studies are needed to clarify the major functional components of PC-SPES.

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