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ARTICLE

Rosmanol Potently Inhibits Lipopolysaccharide-Induced iNOS and COX-2 Expression through Downregulating MAPK, NF-κB, STAT3 and C/EBP Signaling Pathways

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Rosmanol is a natural polyphenol from the herb rosemary (*Rosmarinus officinalis L.*) with high antioxidant activity. In this study, we investigated the inhibitory effects of rosmanol on the induction of NO synthase (NOS) and COX-2 in RAW 264.7 cells induced by lipopolysaccharide (LPS). Rosmanol markedly inhibited LPS-stimulated iNOS and COX-2 protein and gene expression, as well as the downstream products, NO and PGE_2. Treatment with rosmanol also reduced translocation of the nuclear factor-κB (NF-κB) subunits by prevention of the degradation and phosphorylation of inhibitor κB (IκB). Western blot analysis showed that rosmanol significantly inhibited translocation and phosphorylation of NF-κB, signal transducer and activator of transcription-3 (STAT3), and the protein expression of C/EBPβ and C/EBPδ. We also found that rosmanol suppressed LPS-induced phosphorylation of ERK1/2, p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling. Our results demonstrate that rosmanol downregulates inflammatory *iNOS and COX-2* gene expression by inhibiting the activation of NF-κB and STAT3 through interfering with the activation of PI3K/Akt and MAPK signaling. Taken together, rosmanol might contribute to the potent anti-inflammatory effect of rosemary and may have potential to be developed into an effective anti-inflammatory agent.

KEYWORDS: Rosmanol; inducible NO synthase (iNOS); NF-κB; RAW 264.7 monocyte/macrophages; lipopolysaccharide (LPS); cyclooxygenase-2 (COX-2)

INTRODUCTION

Much research has linked inflammation to several human diseases including cancers (*1*). Macrophages play an important role in various inflammatory responses by upregulating the expression of pro-inflammatory cytokines and enzymes such as tumor necrosis factor-α (TNF-α), interleukin, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (*2*). LPS is a component of the cell walls of Gram-negative bacteria, and triggers the activation of monocytes and macrophages involved in infection response (*3*, *4*).

Low levels of NO have many biological functions, including neurotransmitters, vascular homeostasis, and wound repair, and have antimicrobial activity against bacterial pathogens. However, excessive NO produced by inducible nitric oxide synthase (iNOS) and its derivatives, such as nitrogen dioxide and peroxynitrite, is implicated in the pathogenesis of septic shock, inflammation and carcinogenesis (*5*). iNOS is widely expressed in various cells, including vascular smooth muscle cells, hepatocytes and kupffer cells, and is highly expresses in LPS-activated macrophages (*6*). Cyclooxygenase-2 (COX-2) is another inducible enzyme that catalyzes biosynthesis of prostaglandins (PGEs), particularly PGE_2 which contributes to pathogenesis of various inflammatory diseases, edema, angiogenesis, invasion and growth of tumor (*7*). Overexpression of COX-2 has been reported to occur in certain epithelial cancer tissue (*8*). COX-2 is also markedly expressed in inflammatory cells stimulated by LPS, pro-inflammatory cytokines and tumor promoters (*8*).

Nuclear factor-κB (NF-κB) is a pivotal transcription factor regulating gene expression involved in inflammation, immunity, and cancer development and progression (*9*). Among the transcription regulators in the promoter regions of iNOS and COX-2, NF-κB seems to be essential for induction of these inflammatory enzymes by LPS (*10*). Stimulating inflammatory cells with LPS

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results in activation of Toll-like receptor 4 and downstream inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB, degrades and leads to NF-κB translocation to the nucleus (*11*). In addition, it has been demonstrated that cytokine is also involved in the induction of transcription activity of NF-κB through mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK), and PI3K/AKT pathways (*11*). Another important transcription factor involved in pro-inflammatory cytokines-induced inflammatory gene expression is signal transducer and activator of transcription (STAT) 3. Previous studies showed that a STAT-binding gamma activated site (GAS) is necessary for expression of iNOS in IFNγ- and LPS-induced RAW 264.7 macrophages (*12*).

Rosemary (*Rosmarinus officinals L.*) leaves are widely used for spice and food flavoring (*13*). Previous studies reported that rosemary possesses pharmacological and physiological properties including antioxidant, anti-inflammatory, anticancer and antiproliferate activities (*14*, *15*). Extract of rosemary contains volatile oil, phenolic acids, flavonoids and diterpenes (*16*). Among the antioxidant substances in rosemary, several phenolic diterpenes such as carnosol and carnosic acid have been shown to exhibit strong antioxidant activity (*16*). Carnosic acid has been demonstrated to be unstable during processing and storage. In the presence of oxygen, carnosic acid is easily transformed into other diterpenes such as carnosol and rosmanol (*17*). Research demonstrates rosmanol to be a potent antioxidant with superoxide anion production inhibition as well as lipid peroxidation and free radical scavenging activities (*16*, *18*). However, there has not been a study to investigate the anti-inflammatory activity of rosmanol. In the present study, we examine the anti-inflammatory effects of rosmanol in LPS-stimulated murine macrophage. The results show that rosmanol suppressed LPS-induced NO and PGE_2 production by inhibiting activation of NF-κB, STAT3 and C/EBP, as well as the upstream, p38, ERK1/2, and PI3K/Ak signaling pathways.

MATERIALS AND METHODS

Reagents. LPS (*Escherichia coli* 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Rosmanol was semisynthesized from carnosol according to the method of Marrero et al. (*19*). Briefly, carnosol in acetone was treated with aqueous sodium bicarbonate (5%) and the mixture was stirred at room temperature for 6.5 h, after which the acetone was evaporated under reduced pressure. The reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate, washed with salt solution and dried over anhydrous sodium sulfate. The residue was purified by silica gel column chromatography eluted with *n*-hexane/acetone (4:1) to yield rosmanol with 99% purity, as determined by high-performance liquid chromatography−mass spectrometry (HPLC−MS).

Liquid Chromatography−Electron Spray Ionization Mass Spectrometry (LC−MS). An HPLC−MS system was composed of an autosampler injector (Leap Technologies, Switzerland), an HP1090 system controller, with a variable UV wavelength 190−500 nm) detector, an ELSD (evaporizing laser scattered deposition) detector and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass, Beverly, MA). ESI-MS conditions were as following: acquisition mode, ESI-positive; mass scan range, 100−800 amu; scan rate, 0.4 s; cone voltage, 25 V; source temperature, 150 °C; probe temperature, 550 °C. Analytical HPLC conditions on HPLC−MS: column, Chromeabond WR C_{18} (Macherey-Nagel, Bethlehem, PA; 3 μm, 120 Å; length and OD, 30 × 3.2 mm); injection volume, 15 μL; flow rate, 2 mL/min; run time, 3 min. The mobile phase consisted of acetonitrile and H_2O with 0.05% TFA and a typical gradient of 10−90% acetonitrile; the gradient varied.

Cell Culture. RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin. When the cells reached a density of $2-3 \times 10^6$ cells/mL, they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Rosmanol dissolved in dimethylsulfoxide (DMSO, as final concentration of 0.05%) and LPS were added. Cells were treated with 0.05% DMSO as vehicle control.

Nitrite Assay. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction(*20*). After centrifugation at 1000*g* for 20 min, 100 μL of each supernatant medium was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an enzyme-linked immunosorbent assay plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA).

Determination of PGE_2. The culture medium of control and treated cells was collected, centrifuged and stored at −80 °C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI).

Western Blot Analysis. The stimulated murine macrophage cell line RAW 264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) on ice for 1 h, followed by centrifugation at 17500*g* for 30 min at 4 °C. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).

An equal amount of total cellular protein (50 μg) was resolved by SDS−polyacrylamide minigels and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) as described previously (*20*). The membrane was then blocked at room temperature for 1 h with blocking solution (20 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide) followed by incubation with the primary antibody, overnight, at 4 °C. The membrane was then washed with 0.2% TPBS (0.2% Tween-20/PBS) and subsequently probed with antimouse, antirabbit, or antigoat IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Buckinghamshire, U.K.). Primary antibodies of specific protein were purchased from various locations as listed: for anti-COX-2 and anti-iNOS, Transduction Laboratories (Lexington, KY); for anti-C/EBPβ and C/EBPδ, Santa Cruz Biotechnology (Santa Cruz, CA); for anti-IκBα, anti-p65, antiphospho (Ser 32)-specific IκBα, New England Biolabs (Ipswich, MA); for anti-β-actin monoclonal antibody from Oncogene Science (Oncogene Science Inc., Uniondale, NJ); for antiphospho-STAT3 (Tyr705 and Ser727), antiphospho-Akt (Ser473), antiphospho-p65 (Ser536), antiphospho-p38 (Thr180/Tyr182), antiphospho-ERK1/2 (Thr202/Tyr204), STAT3, ERK, p38, and Akt antibodies, Cell Signaling Technology (Beverly, MA) were used to determine the level of phosphorylated proteins.

Semiquantitative RT-PCR. Total RNA was isolated from mouse macrophage RAW264.7 cell using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Changes in the steady-state concentration of mRNA in iNOS, COX-2 and β-actin were assessed by reverse-transcription polymerase chain reaction (RT-PCR). A total of 2 μg of RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, U.K.) to a final volume of 20 μL. RT reactions were performed at 50 °C for 50 min and 70 °C for 15 min in Gene Cycler thermal cycler (Bio-Rad). The thermal cycle conditions were initiated at 95 °C for 1 min, and 30 cycles of amplification (94 °C for 30 s, 58 °C for 25 s, and 72 °C for 1 min), followed by extension at 72 °C for 3 min. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. Amplification of β-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944−2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ (3416−3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094−1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931−1954); G3PDH,

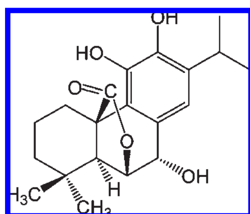

Figure 1. Chemical structure of rosmanol.

forward primer 5′-TGAAGGTAGGTGTGAACGGATTTGGC-3′, reverse primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

Transient Transfection and Luciferase Assay. The luciferase assay was performed as described by George et al. (*21*) with some modifications. RAW 264.7 cells were seeded in a 60 mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco BRL, Life Technologies, Inc.). The cells were then transfected with a pNFκB-Luc plasmid reporter gene (Stratagene, Jalla, CA), murine iNOS promoter plasmid (piNOS/GL3) (*22*) and COX-2 promoter plasmid (pCOX2/GL3) (*22*) using LipofectAMINE reagent (Gibco BRL, Life Technologies, Inc.). After 24 h of incubation, the medium was replaced with complete medium. After another 24 h, the cells were trypsinized and equal numbers of cells were plated in 24-well tissue culture plates for 5 h. The cells were then incubated with 100 ng/mL LPS and either rosmanol or pyrrolidine dithiocarbamate (PDTC) for different periods of time. Luciferase activity was assayed by means of the britelite plus luciferase reporter gene kit (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.1 min/well.

Extraction of Nucleus and Cytosolic Protein. Nuclear and cytoplasmic extracts were prepared as previous reported with slight modification (*36*). The cells were suspended in hypotonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5.0 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1.0% NP-40, and 5.0 mM $MgCl_2$) for 10 min, on ice, followed by centrifugation at 4000*g* for 15 min. The supernatants containing cytosolic proteins were collected. The pellet containing nuclei was resuspended in hypertonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5.0 mM EDTA, 1.0 PMSF, 1.0% NP-40, 5.0 mM $MgCl_2$, and 2% NaCl) for 3 h, on ice, followed by centrifugation at 17500*g* for 30 min. The supernatants containing nucleus proteins were collected for further Western blot analysis.

Statistical Analysis. Data are presented as means ± SE for the indicated number of independently performed experiments. One way Student's *t*-test was used to assess the statistical significance between the LPS- and rosmanol plus LPS-treated cells. A *P*-value <0.05 was considered statistically significant.

RESULTS

Effect of Rosmanol on the Production of Nitrite and PGE_2 in RAW 264.7 cells. The effects of rosmanol (**Figure 1**) on the level of nitrite and prostaglandin in the culture media of RAW 264.7 cells were determined at 24 h after cotreatment with 100 ng/mL LPS and various concentrations of rosmanol. As shown in **Figure 2A**, rosmanol inhibited LPS-induced nitrite production in a dose-dependent manner, but did not interfere with the reaction between nitrite and Griess reagents (data not shown). Treatment with rosmanol also significantly decreased LPS-induced PGE_2 production (**Figure 2B**). We also performed an indirect nitrite assay to examine the possibility of rosmanol that inhibits the intrinsic activity of NOS enzyme and as a result of NO production. Cells were stimulated with LPS for 12 h and washed with PBS to remove LPS. The cells were then treated with different doses of rosmanol, and the nitrite in the media was determined after another 12 h. The result (**Figure 3A**) showed that rosmanol was unable to inhibit nitrite production in these cells indicating

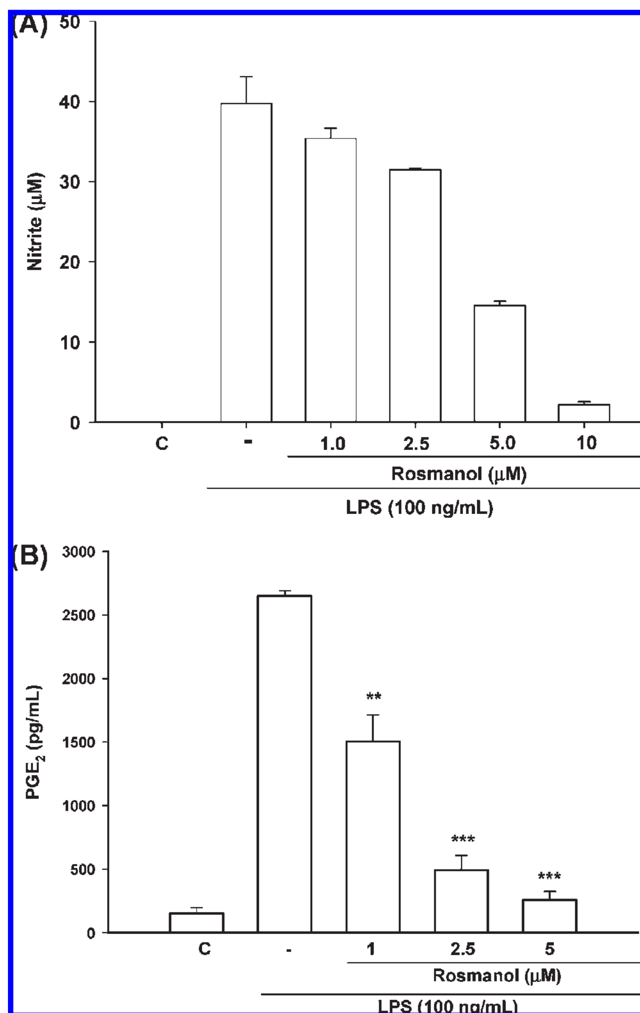

Figure 2. Effects of rosmanol on LPS-induced NO and PGE_2 production in RAW 264.7 macrophages. The cells were treated with 100 ng/mL LPS only or with different concentrations of rosmanol. After incubation for 24 h, 100 μL samples of culture media were collected for (**A**) nitrite and (**B**) PGE_2 assay. The values are expressed as means ± SE of triplicate tests. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 indicate statistically significant differences from the LPS-treated group.

that the inhibitory effect is not attributed to influencing the activity of NOS enzyme. The Western blotting analysis also demonstrated that treatment with rosmanol did not affect the iNOS and COX-2 protein levels in LPS-stimulated cells (**Figure 3B**).

Rosmanol Inhibited LPS-Induced iNOS and COX-2 Gene and Protein Expression. It has been reported that iNOS and COX-2 are the key enzymes for the production of nitrite and PGE_2, respectively (*23*, *24*). Therefore, we next investigated the effects of rosmanol on LPS-induced iNOS and COX-2 protein by Western blotting analysis. As shown in **Figure 4A**, the protein levels of iNOS and COX-2 were undetectable in RAW264.7 cells without LPS-stimulation. Treatment with LPS alone markedly increased iNOS and COX-2 protein levels, whereas cotreatment with rosmanol significantly and concentration-dependently suppressed the expression of iNOS and COX-2 proteins. The reduced expression of iNOS and COX-2 protein was consistent with reductions in total nitrite and PGE_2 in culture media (**Figure 2**). In addition, RT-PCR was done to investigate whether rosmanol suppressed LPS-mediated induction of iNOS and COX-2 through pretranslational mechanism. The results from RT-PCR

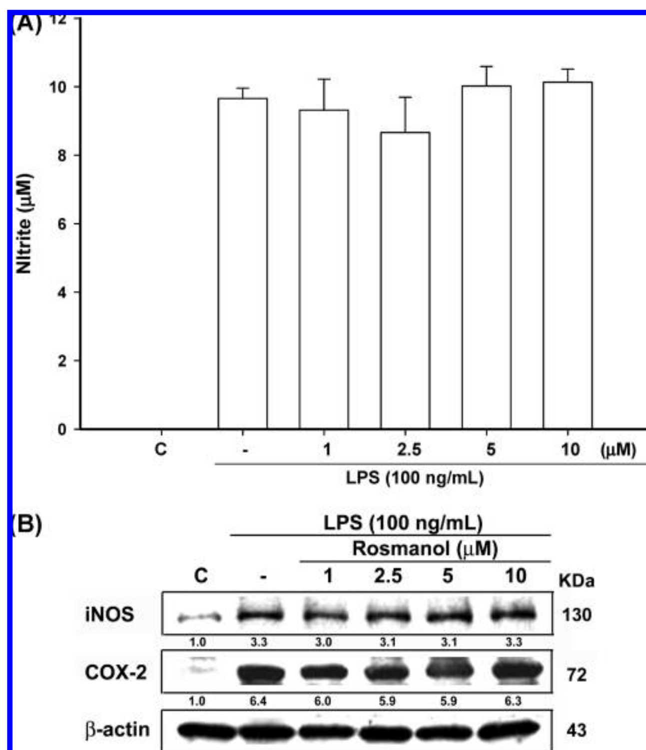

Figure 3. Effect of rosmanol on the activity of iNOS enzyme in RAW 264.7 cells. The cells were stimulated with 100 ng/mL LPS for 12 h, and were washed with PBS to remove LPS before treated with different concentration of rosmanol for another 12 h. The culture media and cell lysates were then collected for (**A**) nitrite assay and (**B**) Western blot analysis. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

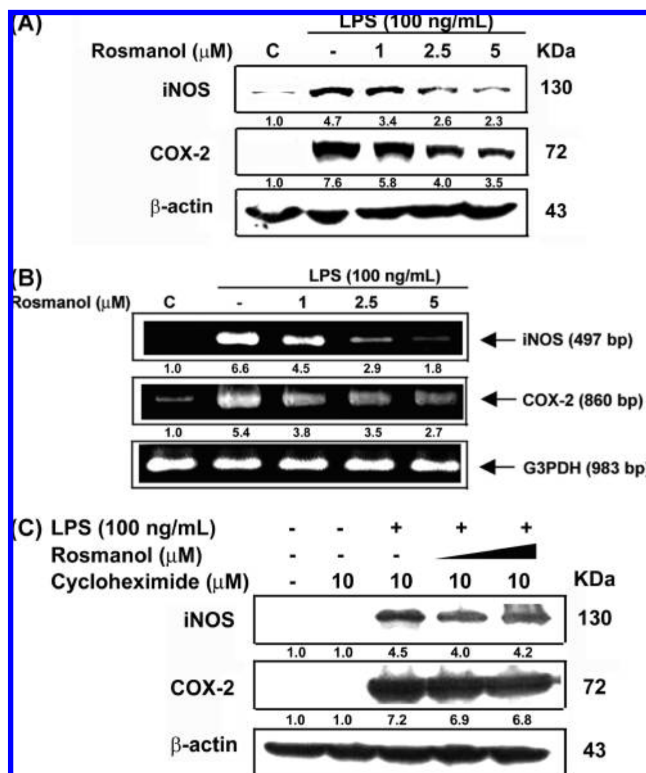

Figure 4. Effects of rosmanol on LPS-induced iNOS and COX-2 gene and protein expression in RAW 264.7 cells. Cells were treated with 100 ng/mL LPS only or with rosmanol for 24 h (**A**) or stimulated with LPS for 12 h and after treatment with CHX (10 μg/mL) for 2 h to stop subsequently protein synthesis; then rosmanol (2.5 and 5 μM) was added into culture media for a further 2 h (**C**). The levels of iNOS or COX-2 in cell lysates were analyzed by Western blotting. β-Actin was used as a loading control. (**B**) Cells were treated with 100 ng/mL LPS only or with rosmanol as described previously; after incubation for 5 h, total RNA was isolated and the mRNA expressions of iNOS and COX-2 were determined by semiquantitative RT-PCR. This experiment was repeated three times with similar results. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

analysis showed that LPS treated alone resulted in gene expression of iNOS and COX-2 (**Figure 4B**). As compared to the LPS alone group, cotreatment with rosmanol markedly decreased the levels of iNOS and COX-2 mRNA in a dose-dependent manner, but did not affect the expression of the housekeeping gene G3PDH. We also investigated the effect of rosmanol on LPS-induced iNOS and COX-2 protein level. RAW264.7 cells were stimulated with LPS for 12 h, then protein synthesis was stopped by treatment with cycloheximide (CHX, 10 μM) for 2 h, and then rosmanol was added into culture media for a further 2 h. The result, as seen in **Figure 4C**, shows that the addition of rosmanol to LPS-stimulated cells after CHX treatment had no significant effect on iNOS and COX-2 protein level, suggesting rosmanol did not affect the protein stability or degradation of iNOS and COX-2. These data suggest that rosmanol may inhibit the expression of iNOS and COX-2 at the transcription level.

Rosmanol Inhibits NF-κB Nuclear Translocation and Activation in LPS-Stimulated Macrophages. Previous research demonstrated that NF-κB activation is critical for induction of both iNOS and COX-2 by LPS or other inflammatory cytokines (*25*). Because levels of iNOS and COX-2 mRNA were inhibited by rosmanol, we then examined the effects of rosmanol on the activation of NF-κB. The translocation of NF-κB was measured by extracts of nucleus and cytosol and subjected to Western blot analysis. As shown in **Figure 5A**, when observed at 60 min stimulation with LPS caused NF-κB subunits, p50 and p65 to translocate to the nucleus. Rosmanol significantly decreased the nuclear levels of p50 and p65 in LPS-stimulated macrophage. PARP, a nuclear protein, and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction. Moreover, it has been reported that phosphorylation of p65 causes the transcriptional activity of NF-κB (*26*). Treatment with rosmanol also suppressed the phosphorylation of p65 at serine 536 in a dose dependent manner. In an additional study, RAW 264.7 cells were transiently transfection with a NF-κB-dependent luciferase reporter plasmid and then treated with LPS alone or with rosmanol. As shown in **Figure 5B**, LPS-induced transcriptional activity of NF-κB was strongly reduced by rosmanol. To further determine the effects of rosmanol on iNOS and COX-2 promoter activities, piNOS/GL3 and pCOX2/GL3 were also transfected into RAW264.7 cells. **Figures 5C** and **5D** show that transfected cells treated with LPS for 15 h markedly increased the iNOS and COX-2 promoter activities by 3.3- and 10.1-fold, respectively, as compared with the control group. Rosmanol strongly inhibited the promoter activities both of iNOS and COX-2, even more than PDTC (known as a NF-κB inhibitor) group for COX-2 promoter activity.

Inhibitory Effects of Rosmanol on LPS-Induced Phosphorylation and Degradation of IκB. LPS-mediated activation of NF-κB is related to phosphorylation and proteolytic degradation of IκB. Therefore, we further investigated the effects of rosmanol on LPS-induced phosphorylation and degradation of IκB. It was found that treatment with LPS caused the serine-phosphorylation

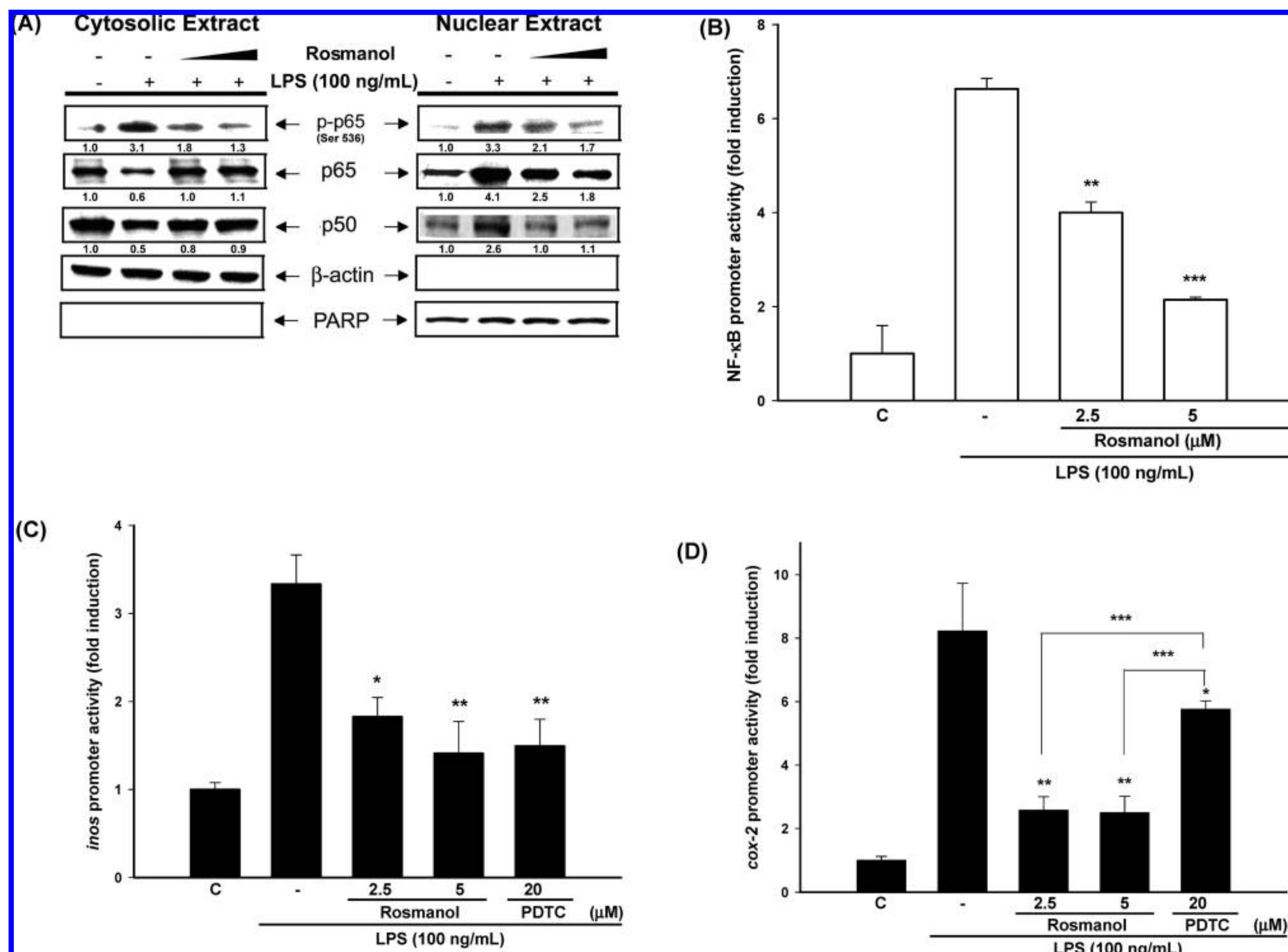

Figure 5. Effect of rosmanol on NF-κB activation and iNOS and COX-2 promoter activities in RAW 264.7 cells. (**A**) The cells were treated with 100 ng/mL LPS alone or with rosmanol (2.5 and 5 μM) for 1 h. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting. (**B**) pNF-κB-Luc reporter plasmid, (**C**) murine iNOS promoter plasmid (piNOS/GL3) and (**D**) COX-2 promoter plasmid (pCOX2/GL3) were transiently transfected into RAW264.7 cells. Cells were then treated with 100 ng/mL LPS in the presence or absence of rosmanol (2.5 and 5 μM) or PDTC (20 μM) for 6 h (for pNF-κB-Luc reporter plasmid), 18 h (piNOS/GL3 plasmid) and 15 h (for pCOX2/GL plasmid). After incubation, cells were harvested and luciferase activities were determined as described in Materials and Methods. The results are expressed as means ± SE of triplicate tests. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ indicate statistical differences from the LPS-treated group.

of IκB protein, as evidenced by the presence of anti-Ser32-phospho-specific IκB antibody after 15−120 min and the degradation of IκB after 30 min. Levels of IκB gradually recovered after 45−120 min (**Figure 6A**). Treatment with rosmanol effectively attenuated the increased phosphorylation of IκBα and sustained the IκBα protein content (**Figure 6B**). The pattern of inhibition on IκB phosphorylation by rosmanol is parallel to the pattern of inhibition on its degradation. These results suggest that rosmanol blocked the phorphorylation and the degradation of IκBα protein and can prevent the activation and translocation of NF-κB to the nucleus and further inhibit the downstream transcriptional activity.

Rosmanol Inhibits Phosphorylation and Nuclear Translocation of STAT3 and C/EBP Expression in LPS-Stimulated Macrophages. STAT3 is another key signaling molecule involved in cytokine-induced inflammatory response and also important for LPS-induced iNOS expression in murine macrophage (*27*). We next determined the effects of rosmanol on the activation of STAT3 by measuring the levels of pSTAT3 (Ser727 and Tyr705) in nuclear extracts. As shown in **Figure 7A**, LPS treatment caused nuclear translocation and phosphorylation of STAT3 (Ser727 and Tyr705) in RAW264.7 cells. As compared with LPS treatment, rosmanol markedly reduced the nuclear translocation and phosphorylation of STAT3 (Ser727 and Tyr705) induced by LPS but did not affect the total levels of STAT3 protein. It is well-known that transcription factor CCAAT/enhancer-binding protein (C/EBP) is also involved in regulating iNOS and COX-2 gene expression (*28*). Therefore, we also determined whether C/EBP is an important target for rosmanol in LPS-treated macrophage. The results, seen in **Figure 7B** show that cells treated with LPS increased the expression of both C/EBPβ and C/EBPδ within 2 h, whereas both greatly decreased with rosmanol treatment. These results indicate that rosmanol inhibited LPS-induced inflammatory gene expression not only through blockage of the activation of NF-κB but also through the STAT3 and C/EBP signaling pathways.

Rosmanol Inhibits Phosphorylation of PI3K/Akt, p38, and ERK1/2 MAPK Kinase in LPS-Stimulated Macrophages. MAPKs pathways are the major signaling molecules involved in various cellular responses. Previous research showed that p38 and p44/42 MAPK play an important role in LPS-mediated induction of iNOS and COX-2 in murine macrophages (*11*). In addition, cytokine-medicated activation of the PI3K/Akt pathway also involves the activation of NF-κB and downstream inflammatory gene expression (*29*). To determine whether rosmanol can modulate these upstream signaling pathways, we investigated the

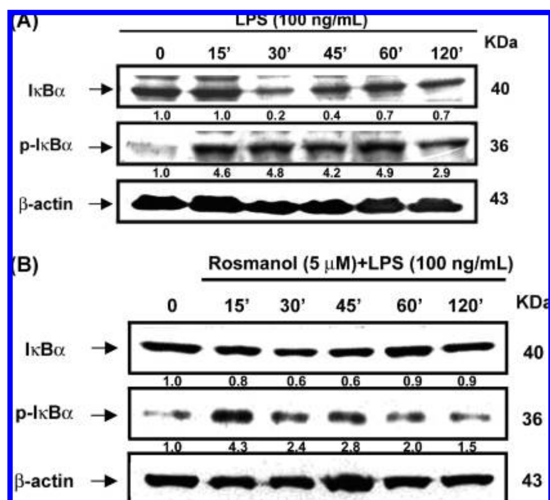

Figure 6. Effects of rosmanol on LPS-stimulated phosphorylation and degradation of IκBα in RAW 264.7. The cells were treated with (**A**) 100 ng/mL LPS alone or (**B**) cotreatment with LPS and rosmanol for different times. Total cell lysates were prepared for Western blot analysis. These experiments were repeated three times with similar results. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

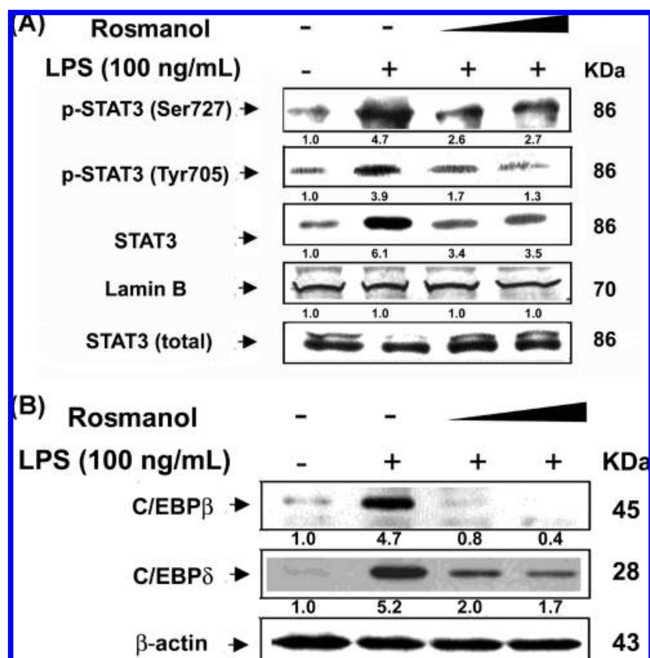

Figure 7. Effects of rosmanol on LPS-induced phosphorylation, nuclear translocation of STAT3 and C/EBPs protein expression in RAW 264.7 cells. The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μM) for 1 h. (**A**) Nuclear fractions were assayed for p-STAT3 (Ser727 and Tyr705) and STAT3 and (**B**) total cell lysates were determined for C/EBPβ and C/EBPδ by Western blotting analysis. Lamin B as an internal control for nuclear fraction. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

effects of rosmanol on the activation of p38, p44/42 MAPK and PI3K/Akt in LPS-stimulated macrophages. In cells treated with LPS alone, the phosphorylation of PI3K and Akt was observed at 30 min. After cotreatment with rosmanol and LPS, the phosphorylation of Akt and PI3K decreased as compared with LPS alone (**Figure 8A**). Activation of MAPK requires phosphorylation of threonine and tyrosine residues. Western blot analysis showed that LPS treatment markedly induced phosphorylation of ERK1/2 and p38 MAPK, whereas treatment of the cells with rosmanol significantly reduced the activation of ERK1/2 and p38 MAPK in a dose dependent manner. Specific kinase inhibitors were used to confirm the role of upstream kinases involved in LPS-induced iNOS and COX-2 expression and to further clarify the different inhibitory effects of rosmanol in RAW264.7 macrophage. As shown in **Figure 8C**, pretreatment with SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and LY294002 (PI3K/Akt inhibitor) strongly inhibited LPS-induced NO production as well as the protein levels of iNOS and COX-2. However, treatment with ERK inhibitor PD98059 only decreased the protein expression of COX-2 but with almost no effect on NO production and iNOS protein. In addition, as was observed in the rosmanol treated group, not only NO production but also the protein expression of iNOS and COX-2 were markedly reduced. These results of immunoblot analysis suggest that rosmanol might block LPS-induced NF-κB activation by inhibiting ERK1/2, p38 MAPK and PI3K/Akt /IKK pathways, which interrupt the degradation of IκBα and downstream iNOS and COX-2 gene expression.

DISCUSSION

In the present study we examined, for the first time, the effects and mechanisms of rosmanol on LPS-induced expression of iNOS and COX-2 in murine macrophage, and also provide molecular evidence for elucidation the function of rosmanol serving as potential anti-inflammatory drug. Overproduction of NO by iNOS occurred in various cell types after stimulation with cytokine and endotoxin, and are also involved in different inflammatory diseases and tumorigenesis (*5*, *23*). High levels of PGE_2 synthesized by COX-2 also occurred in various cancer tissues and implicated in proliferation, angiogenesis and tumor growth (*7*, *8*). Because there is a causal relationship between inflammation and cancer, iNOS and COX-2 are considered potential molecular targets for chemoprevention (*30*). In our present study, we found rosmanol dose-dependently inhibited LPS-induced NO and PGE_2 production by decreasing the gene expression of iNOS and COX-2, as well as protein levels but did not affect enzyme activity. Additional experiments were carried out to evaluate the effect of rosmanol on iNOS and COX-2 protein stability, and neither of these proteins were affected after rosmanol treatment. These results indicate that rosmanol might inhibit NO and PGE_2 production through regulating the transcription levels and upstream molecules of iNOS and COX-2 induced by LPS treatment.

There are a large number of binding sites for transcription factors in the region of iNOS and COX-2 promoters, including NFκB, activator protein-1 (AP-1), interferon regulatory factor 1 (IRF1), STATs and CCAAT/enhancer-binding protein (C/EBP), according to cell type and stimulation (*28*). Among the promoter regions of iNOS and COX-2, the transcription factor NF-κB is mainly responsible for their transcription by LPS stimulation (*10*). We also showed that rosmanol markedly inhibited nuclear translocation and transcriptional activity of NF-κB in LPS-stimulated RAW264.7 cells. The major mechanism of inhibition NF-κB activation by rosmanol might be through the suppression of phosphorylation and degradation of IκB. Moreover, decreased phosphorylation of NF-κB at Ser525 by rosmanol appears to be another action inhibiting NF-κB activation. In further experiments of *inos* and *cox-2* promoter analysis, rosmanol and NF-κB inhibitor PDTC also inhibited the LPS-induced transcriptional activity of both iNOS and COX-2. In comparison with PDTC,

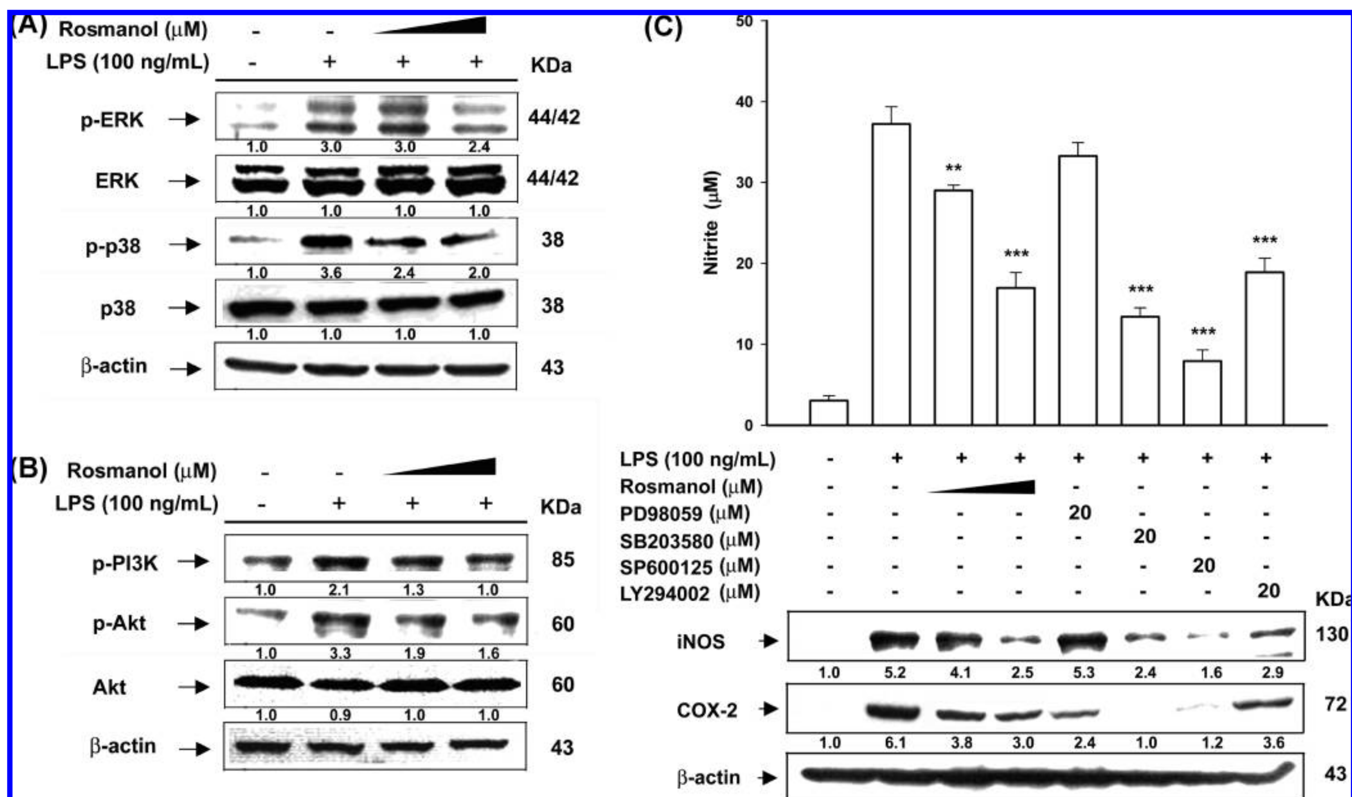

Figure 8. Inhibition of PI3K/Akt, ERK1/2, and p38 MAPK by rosmanol and the effects of kinase inhibitors on iNOS and COX-2 protein expression in LPS-activated macrophages. The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μM) for 30 min. Total cell lysates were then prepared for determination of (**A**) p-ERK, p-p38 and (**B**) p-PI3K and p-Akt by Western blot analysis. These experiments were repeated three times with similar results. (**C**) Cells were pretreated with rosmanol (2.5 or 5 μM) or different specific kinase inhibitors for 30 min, and then treated with LPS (100 ng/ mL) for another 12 h. The culture media were collected for nitrite assay, and total cell lysates were prepared for Western blot analysis. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

rosmanol shows great inhibition of *cox-2* promoter activity, indicating not only NF-κB but also other transcription factors involved in regulating the COX-2 gene could to be a target for rosmanol. These data together suggest that rosmanol might inhibit iNOS and COX-2 transcription through attenuating the activation of NF-κB, that leads to decreased NO and PGE_2 production.

STAT3 is important for production of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-10 that play a major role in inflammatory disease (*31*). In previous studies, it was shown that the promoter region of iNOS gene in murine macrophage contains the STAT-binding gamma activated sites (GAS) site (*12*). STAT3 is involved in the response of LPS-induced IL-1β production in macrophage, and partly dependent on the phosphorylation at tyrosine 705 which is required for dimerization and nuclear translocation (*32*). In addition, phosphorylation at Ser 727 is also crucial for nuclear translocation and DNA binding (*32*). In our study, we show that treatment with LPS alone markedly increased the phosphorylation of STAT3 at Tyr 705 and Ser 727 in nuclear levels, whereas rosmanol strongly decreased the phosphorylation of STAT3 both at Tyr 705 and Ser 727, as well as nuclear translocation. However, it is interesting to mention that the phosphorylation of STAT3 at Tyr 705 and Ser 727 is targeted by a different kinase. It has been reported that phosphorylation on Tyr 705 is mainly by receptor associated kinases (JAKs) (*33*), and Ser 727 phosphorylation is regulated by ERK1/2 and mTOR (*34*). This information indicates that rosmanol may inhibit phosphorylation of STAT3 at Tyr 705 and Ser 727 through multiple signaling pathways. However, the detailed mechanism remains to be elucidated.

Recently, C/EBPs have also been shown to play an important role in the transcriptional activation of iNOS and COX-2 promoters. Among the members of C/EBP transcription factors, C/EBPβ has been implicated in inducing the expression of COX-2 stimulated by endotoxin and cytokines (*35*). Furthermore, both C/EBPβ and C/EBPδ are also involved in induction of glial iNOS (*36*) as well being the inflammatory mediator production in RAW264.7 macrophage (*37*). Here, we demonstrate that rosmanol reduces the protein levels of C/EBPβ and C/EBPδ stimulated by LPS, indicating the possible mechanism of rosmanol on inhibition of iNOS and COX-2 gene transcription may also be through targeting these two C/EBP members. Moreover, according to the role of C/EBPβ on the COX-2 promoter binding site, it could be another major target of rosmanol that is compareable to the PDTC-treated group in the results of COX-2 promoter activity (**Figure 5D**).

Phosphorylation plays an important role in activating protein tyrosine kinase. ERK and p38 MAPK and are known to be involved in the LPS-mediated expression of iNOS and COX-2 in mouse macrophages (*11*). Activation of PI3K/Akt plays an important role in the expression of iNOS and COX-2 in vascular smooth muscle cells, peritoneal macrophages, and mesangial cells (*38*). Here we also explore the effects of these upstream kinases on LPS-indcued iNOS and COX-2 expression in RAW264.7 macrophage. We found blockage of p38, JNK and PI3K activities by specific inhibitors resulted in suppression both of LPS-induced iNOS and COX-2 protein expression as well as NO production, whereas inhibition of ERK only suppressed COX-2 protein levels (**Figure 8C**). In the results of rosmanol treatment, our findings also show that treatment with rosmanol

significantly blocked the activation of PI3K/Akt, ERK1/2, p38 MAPK (**Figures 8**A and **8B**) and subsequent decreased the protein levels of iNOS and COX-2 (**Figure 8C**), indicating rosmanol suppresses iNOS and COX-2 expression by mediating activation of these kinases . As mention above, LPS can activate the PI3K/Akt, NF-κB, STATs and MAPKs pathways. Our results demonstrate that the treatment of rosmanol can disrupt those LPS-induced signaling pathways in RAW 264.7 cells (**Figures 6** and **7**). In addition, PI3 kinase has been suggested to be upstream regulator of the NF-κB, STATs and MAPKs pathways (*29*). Therefore, it suggested that loss of function PI3K may play a key role for the rosmanol-inhibited LPS effect, including the disruption of the activities of transcription factors on iNOS and COX-2 promoter in RAW 264.7 cells. These results suggest that rosmanol might suppress LPS-induced NFκB translocation through inhibiting the activation of these intracellular signaling cascades and subsequently suppressing the gene transcription of iNOS and COX-2.

In conclusion, in the present study we observed the anti-inflammatory effects of rosmanol on LPS-induced iNOS and COX-2 expression in murine macrophage by regulating the signaling pathways, particularly affecting the activation of PI3K/Akt, pERK1/2 and p38 MAPK, the degradation and phosphorylation of IκBα, and the phosphorylation and translocation of NFκB. This study also demonstrates that rosmanol might block the phosphorylation and nuclear translocation of STAT3 as well as the expression of C/EBPβ and C/EBPδ protein, then subsequently inhibits the iNOS and COX-2 expression induced by LPS. Based on these findings, we suggest rosmanol shows great potential as a novel chemopreventive agent for the treatment of a variety of inflammatory diseases.

ABBREVIATIONS USED

iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; COX-2, cycoloxygenase-2; PGE_2, prostaglandin E_2; IκB, inhibitor κB; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; C/EBP, CCAAT/enhancer-binding protein, STAT3; signal transducer and activator of transcription 3.

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