行政院國家科學委員會專題研究計畫　期中進度報告

革蘭氏陽性菌細胞壁成分Peptidoglycan刺激巨噬細胞前發炎物質釋放之訊息傳遞探討(1/3)

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-038-059-
執行期間：92年08月01日至93年07月31日
執行單位：臺北醫學大學醫事技術學系

計畫主持人：林建煌
共同主持人：陳炳常
計畫參與人員：詹雅旋

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 5 月 31 日

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 ■期中進度報告

革蘭氏陽性菌細胞壁成分 Peptidoglycan 刺激巨噬細胞前發炎物質釋放之訊息傳遞探討(1/3)

計畫類別：■ 個別型計畫　　整合型計畫
計畫編號：NSC　92－2314－B－038－059
執行期間：　92 年　8 月　1 日至 93 年　7 月 31 日

計畫主持人：林建煌
共同主持人：陳炳常
計畫參與人員：詹雅璇

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：台北醫學大學醫事技術學系

中　華　民　國　93　年　5　月　31　日

英文摘要。

關鍵字：peptidoglycan (PGN); macrophages; NF-κB; Ras/Raf-1/ERK; signal transduction; cyclooxygenase-2 (COX-2)。

In this study, we investigated the signaling pathway involved in cyclooxygenase-2 (COX-2) expression caused by peptidoglycan (PGN), a cell wall component of the gram-positive bacterium, *Staphylococcus aureus*, in RAW 264.7 macrophages. PGN caused dose- and time-dependent increases in COX-2 expression, which was attenuated by a Ras inhibitor (manumycin A), a Raf-1 inhibitor (GW 5074), and a mitogen-activated protein kinase kinase (MEK) inhibitor (PD 098059). Treatment of RAW 264.7 macrophages with PGN caused time-dependent activations of Ras, Raf-1, and extracellular signal-regulated kinase (ERK). The PGN-induced increase in Ras activity was inhibited by manumycin A. Raf-1 phosphorylation at Ser338 by PGN was inhibited by manumycin A and GW 5074. The PGN-induced increase in ERK activity was inhibited by manumycin A, GW 5074, and PD 098059. Stimulation of cells with PGN activated IκB kinase α/β (IKKα/β), IκBα phosphorylation, IκBα degradation, and κB-luciferase activity. Treatment of macrophages with a NF-κB inhibitor (pyrrolidine dithiocarbamate, PDTC), an IκBα phosphorylation inhibitor (Bay 117082), and IκB protease inhibitors (L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and calpain inhibitor I) all inhibited PGN-induced COX-2 expression. The PGN-mediated increase in the activities of IKKα/β and κB-luciferase were also inhibited by the Ras dominant negative mutant (RasN17), manumycin A, GW 5074, and PD 098059. Further studies revealed that PGN induced the recruitment of p85α and Ras to Toll-like receptor 2 (TLR2) in a time-dependent manner. Our data demonstrate for the first time that PGN activates the Ras/Raf-1/ERK pathway, which in turn initiates IKKα/β and NF-κB activation, and ultimately induces COX-2 expression in RAW 264.7 macrophages.

中文摘要。

關鍵字：peptidoglycan (PGN);巨噬細胞;NF-κB 轉錄因子;Ras/Raf-1/ERK;訊息傳遞;環氧化酶-2。

在本計劃中，我們將探討革蘭氏陽性菌細胞壁成分peptidoglycan (PGN)誘導RAW 264.7巨噬細胞cyclooxygenase-2 (COX-2)表現之訊息傳遞路徑。PGN依劑量及時間相關曲線增加COX-2的表現，而這個作用可被Ras抑制劑(manumycin A)、Raf-1抑制劑(GW 5074)及mitogen-activated protein kinase kinase (MEK)抑制劑(PD 098059)所抑劑。在RAW 264.7巨噬細胞給予PGN可以依照時間曲線活化Ras、Raf-1及ERK。PGN所誘導增加Ras的活性可被manumycin A所抑制； PGN所誘導增加Raf-1的Ser-338磷酸化可被manumycin A及GW 5074所抑制； PGN所誘導增加ERK的活性可被manumycin A、GW 5074及PD 098059所抑制。RAW 264.7巨噬細胞給予PGN可以增加IκB kinase α/β (IKKα/β)和IBα磷酸化及IBα的降解及κB-luciferase的活性。巨噬細胞給予NF-κB抑制劑(pyrrolidine dithiocarbamate, PDTC)、IκBα磷酸化抑制劑(Bay 117082)及IκB protease抑制劑(L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and calpain inhibitor I)皆可抑制PGN誘導COX-2的表現。PGN誘導IKKα/β和κB-luciferase的活性也會被Ras dominant negative mutant (RasN17)、manumycin A、GW 5074和PD 098059所抑制。更進一步探討發現在PGN刺激可以依照時間作用使p85α及Ras結合至Toll-like receptor 2。因此在本計劃中首先發現PGN可以經由Ras/Raf-1/ERK的路徑來活化IKKα/β及NF-κB，進一步誘導巨噬細胞COX-2的表現。

報告內容[前言及文獻探討、研究目的、研究方法、結果與討論(含結論與建議)]

本研究計劃第一年的成果目前已經發表在The Journal of Biological Chemistry Vol. 279, No. 20, Issue of May 14, pp20889-20897的期刊中，請見所附之期刊內容。經由本計劃之執行與研究，必定能清楚明瞭在革蘭氏陽性菌感染時所產生的發炎現象。因此，藉由本計劃的完成將能尋找出治療革蘭氏陽性菌的治療方針。

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 20, Issue of May 14, pp. 20889–20897, 2004
Printed in U.S.A.

Peptidoglycan Induces Nuclear Factor-κB Activation and Cyclooxygenase-2 Expression via Ras, Raf-1, and ERK in RAW 264.7 Macrophages*

Received for publication, October 14, 2003, and in revised form, March 4, 2004
Published, JBC Papers in Press, March 8, 2004, DOI 10.1074/jbc.M311279200

Bing-Chang Chen‡, Ya-Sheng Chang§, Ju-Chiun Kang¶, Ming-Jen Hsu§, Joen-Rong Sheu¶, Ta-Liang Chen‖, Che-Ming Teng, and Chien-Huang Lin‡§‡‡**

*From the ‡School of Respiratory Therapy, §Graduate Institute of Biomedical Technology, the ¶Department of Pharmacology, School of Medicine, Taipei Medical University, the ‖Department of Anesthesiology, Taipei Medical University-Wan Fang Hospital, and **Pharmacological Institute, College of Medicine, National Taiwan University, Taipei 110, Taiwan*

In this study, we investigated the signaling pathway involved in cyclooxygenase-2 (COX-2) expression caused by peptidoglycan (PGN), a cell wall component of the Gram-positive bacterium *Staphylococcus aureus*, in RAW 264.7 macrophages. PGN caused dose- and time-dependent increases in COX-2 expression, which was attenuated by a Ras inhibitor (manumycin A), a Raf-1 inhibitor (GW 5074), and an MEK inhibitor (PD 098059). Treatment of RAW 264.7 macrophages with PGN caused time-dependent activations of Ras, Raf-1, and ERK. The PGN-induced increase in Ras activity was inhibited by manumycin A. Raf-1 phosphorylation at Ser-338 by PGN was inhibited by manumycin A and GW 5074. The PGN-induced increase in ERK activity was inhibited by manumycin A, GW 5074, and PD 098059. Stimulation of cells with PGN activated IκB kinase α/β (IKKα/β), IκBα phosphorylation, IκBα degradation, and κB-luciferase activity. Treatment of macrophages with an NF-κB inhibitor (pyrrolidine dithiocarbamate), an IκBα phosphorylation inhibitor (Bay 117082), and IκB protease inhibitors (L-1-tosylamido-2-phenylethyl chloromethyl ketone and calpain inhibitor I) all inhibited PGN-induced COX-2 expression. The PGN-mediated increase in the activities of IKKα/β and κB-luciferase were also inhibited by the Ras dominant negative mutant (RasN17), manumycin A, GW 5074, and PD 098059. Further studies revealed that PGN induced the recruitment of p85α and Ras to Toll-like receptor 2 in a time-dependent manner. Our data demonstrate for the first time that PGN activates the Ras/Raf-1/ERK pathway, which in turn initiates IKKα/β and NF-κB activation, and ultimately induces COX-2 expression in RAW 264.7 macrophages.

Peptidoglycan (PGN),[1] a cell wall component of Gram-positive bacteria, is an alternating β-linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan whose residues are cross-linked by short peptides (1, 2). Like lipopolysaccharide (LPS) as a cell wall component of Gram-negative bacteria, PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever, septic shock, etc. (3). Most of these effects are due to the activation of macrophages and generation of proinflammatory cytokines, such as tumor necrosis factor α, interleukin (IL)-6, and IL-8 (4–8). PGN binds CD14 and Toll-like receptor 2 (TLR2), which activates transcription factors and induces gene expression (4, 9). Even though it is well known that bacterial products have multiple and various effects on the regulation of host defense and immune responses by macrophages, little is known about how PGN regulates induction of the cyclooxygenase-2 (*cox-2*) gene.

COX is the key enzyme that synthesizes prostaglandins and thromboxane from arachidonic acid (10, 11). Two COX isozymes, COX-1 and COX-2, have been cloned and identified to have 60% homology in humans (12, 13). COX-1, which is constitutively expressed in most tissues, mediates physiological responses and regulates renal and vascular homeostasis. COX-2, an inducible gene for cell synthesis, can be up-regulated by inflammatory stimuli including IL-1β, lipoteichoic acid (LTA), and LPS to produce proinflammatory prostaglandins in inflammation (14–19). Several consensus sequences, including those for nuclear factor-κB (NF-κB), NF-IL6, and the activating transcription factor/cyclic AMP-responsive element in the 5′ region of the *cox-2* gene, have been identified as regulatory sequences that can induce COX-2 in response to various stimuli (20–22). NF-κB, the most important transcription factor for regulating COX-2 expression, is a dimer of the transcription factors p65 or p50 (23). In a resting state, this dimer is associated with IκBs to retain NF-κB in the cytosol (24). IκB kinase (IKK), which is activated through stimulation by cytokines and bacterial products, phosphorylates IκBα at Ser-32 and Ser-36 and IκBβ at Ser-19 and Ser-23 (25–27), to produce ubiquitination of IκBα/β at lysine residues and degradation by the 26 S proteasome (28). This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, to bind specific DNA enhancer sequences and to induce gene transcription (23). A previous report showed that PGN-induced NF-κB acti-

* This work was supported by Grant NSC 92-2314-B-038-059 from the National Science Council of Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: School of Respiratory Therapy, Taipei Medical University, 250 Wu-Hsing St., Taipei 110, Taiwan. Tel.: 886-2-27361661 (ext. 3510); Fax: 886-2-27397313; E-mail: chlin@tmu.edu.tw.

[1] The abbreviations used are: PGN, peptidoglycan; COX-2, cyclooxygenase-2; DMEM/Ham's F-12, Dulbecco's modified Eagle's medium/Ham's F-12; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IL, interleukin; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; MyD88, myeloid differentiation protein; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; Raf-1 RBD, Ras-binding domain for Raf-1; TIR, toll/IL-1 receptor; TLR2, Toll-like receptor 2; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone, TRAF6, tumor necrosis factor receptor-associated factor 6.

vation is mediated through TLR2-dependent multiple signaling molecules including myeloid differentiation protein (MyD88), IL-1 receptor-associated kinase (IRAK), tumor necrosis factor receptor-associated factor 6 (TRAF6), NF-κB-inducing kinase, and the IKK signaling pathway (4, 5), but little information is available about the role of Ras in regulating NF-κB signaling following PGN stimulation.

Ras has been found to couple with multiple effector systems to activate distinct physiological and pathological responses such as cell proliferation and release of proinflammatory mediators (22, 29–31). An important class of Ras effectors is the mitogen-activated protein kinase (MAPK) family. The classic Ras-mediated pathway involves the binding of Raf-1 and subsequent phosphorylation of Raf-1 at Ser-338 by many kinases (32, 33), which in turn activates extracellular signal-regulated kinases (ERKs) (34), and consequently phosphorylates many target proteins including transcription factors and protein kinases (35). Although a role for Ras in COX-2 induction has been implied by many cell types (22, 30, 31), however, PGN-induced COX-2 expression has not been investigated in macrophages. This study was intended to identify the signaling pathway of PGN-induced Ras activation and its roles in PGN-mediated NF-κB activation and COX-2 expression in RAW 264.7 macrophages. Our hypothesis is that PGN might induce TLR2, p85α, and Ras complex formation and subsequently activate the Ras/Raf-1/ERK pathway, which in turn increases IKKα/β activity and NF-κB activation and finally causes COX-2 expression in RAW 264.7 macrophages.

EXPERIMENTAL PROCEDURES

Reagents—Peptidoglycan (PGN derived from *Staphylococcus aureus*) was purchased from Fluka (Buchs, Switzerland). Manumycin A, PD 098059, and SB 203580 were obtained from Calbiochem. GW 5074 was obtained from Tocris (Bristol, UK). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/Ham's F-12), fetal calf serum, and penicillin/streptomycin were purchased from Invitrogen. Antibodies specific for α-tubulin and COX-2 were purchased from Transduction Laboratories (Lexington, KY). Protein A/G beads, IκBα protein (amino acids 1–317), antibodies specific for ERK, ERK phosphorylated at Tyr-204, IκBα, IκBα phosphorylated at Ser-32, IKKα, IKKβ, Raf-1, TLR2, Ras, p85α, and anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for Raf-1 phosphorylated at Ser-338 was purchased from Cell Signaling and Neuroscience (St. Louis, MO). Anti-mouse and anti-rabbit IgG-conjugated alkaline phosphatases were purchased from Jackson ImmunoResearch (West Grove, PA). pGL2-ELAM-Luc (which is under the control of a single NF-κB-binding site) and pBK-CMV-LacZ were kindly provided by Dr. W.-W. Lin (National Taiwan University, Taipei, Taiwan). A Ras dominant negative mutant (RasN17) and a Ras activity assay kit were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from Amersham Biosciences. Bay 117082 was obtained from Alexis (Lausen, Switzerland). GenePORTER™ 2 was purchased from Gene Therapy System (San Diego, CA). All materials for SDS-PAGE were purchased from Bio-Rad. All other chemicals were obtained from Sigma.

Cell Culture—The mouse macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (Livingstone, MT), and cells were maintained in DMEM/Ham's F-12 nutrient mixture containing 10% fetal calf serum, 100 units/ml of penicillin G, and 100 μg/ml streptomycin in a humidified 37 °C incubator. After reaching confluence, cells were seeded onto either 6-cm dishes for immunoblotting, co-immunoprecipitation, or kinase assays, or 12-well plates for transfection and κB luciferase assays.

Transfection and κB Luciferase Assays—For these assays, 2×10^5 RAW 264.7 cells were seeded onto 12-well plates, and cells were transfected the following day using GenePORTER™ 2 with 0.5 μg of pGL2-ELAM-Luc and 0.5 μg of pBK-CMV-LacZ. After 24 h, the medium was aspirated, replaced with fresh DMEM/Ham's F12 containing 10% FBS, and then stimulated with PGN (30 μg/ml) for another 24 h before harvest. To assess the effects of Ras, Raf-1, and ERK inhibitors, drugs were added to cells 20 min before PGN addition. To assay the effect of RasN17, cells were co-transfected with RasN17, pGL2-ELAM-Luc, and pBK-CMV-LacZ for 24 h and then treated with PGN. Luciferase activity was determined with a luciferase assay system (Promega) and was normalized on the basis of LacZ expression. The level of induction of luciferase activity was compared as a ratio to cells with and without stimulation.

Immunoblot Analysis—To determine the expressions of COX-2, α-tubulin, ERK phosphorylated at Tyr-204, ERK2, IKKα/β, IκBα phosphorylated at Ser-32, IκBα, Ras, Raf-1 phosphorylated at Ser-338, and Raf-1 in RAW 264.7 macrophages, proteins were extracted, and Western blotting analyses were performed as described previously (36). Briefly, RAW 264.7 macrophages were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle and PGN or pretreated with specific inhibitors as indicated followed by PGN. After incubation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (60 μg) were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane that was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.02% Tween 20 (pH 7.4)) containing 5% non-fat milk. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase- or alkaline phosphatase-conjugated second antibodies. Immunoreactivity was detected using enhanced chemiluminescence or nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate following the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Eastman Kodak Co.).

Immunoprecipitation and Protein Kinase Assays—RAW 264.7 cells were grown in 6-cm dishes. After reaching confluence, cells were treated with 30 μg/ml PGN for the indicated time intervals or pretreated with specific inhibitors as indicated followed by PGN. After incubation, cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate, and centrifuged. The supernatant was then immunoprecipitated with respective polyclonal antibodies against IKKα, IKKβ, or ERK2 in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, and 2 mM dithiothreitol. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 20 μM ATP and 3 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. To assess IKKα/β and ERK activities, 0.5 μg of GST-IκBα protein (amino acids 1–317) and 50 μg/ml of myelin basic protein (MBP) were respectively added as the substrates. The reaction mixtures were analyzed by 12 (IKKα/β) or 15% (ERK) SDS-PAGE followed by autoradiography.

Ras Activity Assay—Ras activity was measured using a Ras activity assay kit. The assay was performed according to the manufacturer's instructions. Briefly, cells were washed twice with ice-cold PBS, lysed in magnesium lysis buffer (MLB) (25 mM HEPES (pH 7.5), 150 mM NaCl, 5% Igepal CA-630, 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and centrifuged. An equal volume of lysate was incubated with 5 μg of Ras-binding domain for Raf-1 (Raf-1 RBD) at 4 °C overnight, and the beads were washed three times with MLB lysis buffer. Bound Ras proteins were then solubilized in 2× Laemmli sample buffer and quantitatively detected by Western blotting (10% SDS-PAGE) using mouse monoclonal Ras with the ECL system and by densitometry of corresponding bands using scientific imaging systems.

Co-immunoprecipitation of TLR2 Complex Formation—RAW 264.7 cells were grown in 6-cm dishes. After reaching confluence, cells were treated with 30 μg/ml PGN for the indicated time intervals. The cells were harvested, lysed in 1 ml of PD buffer (40 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM dithiothreitol), and centrifuged. The supernatant was then immunoprecipitated with specific antibodies against TLR2, p85α, or Ras in the presence of protein A/G beads at 4 °C overnight. The immunoprecipitated beads were washed three times with PD buffer. The samples were fractionated on 15 (for Ras) or 8% (for TLR2 or p85α) SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblot analysis with antibodies specific for Ras, TLR2, or p85α.

Statistical Analysis—Results are presented as the mean ± S.E. from at least three independent experiments. One-way analysis of variance followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance of the difference between

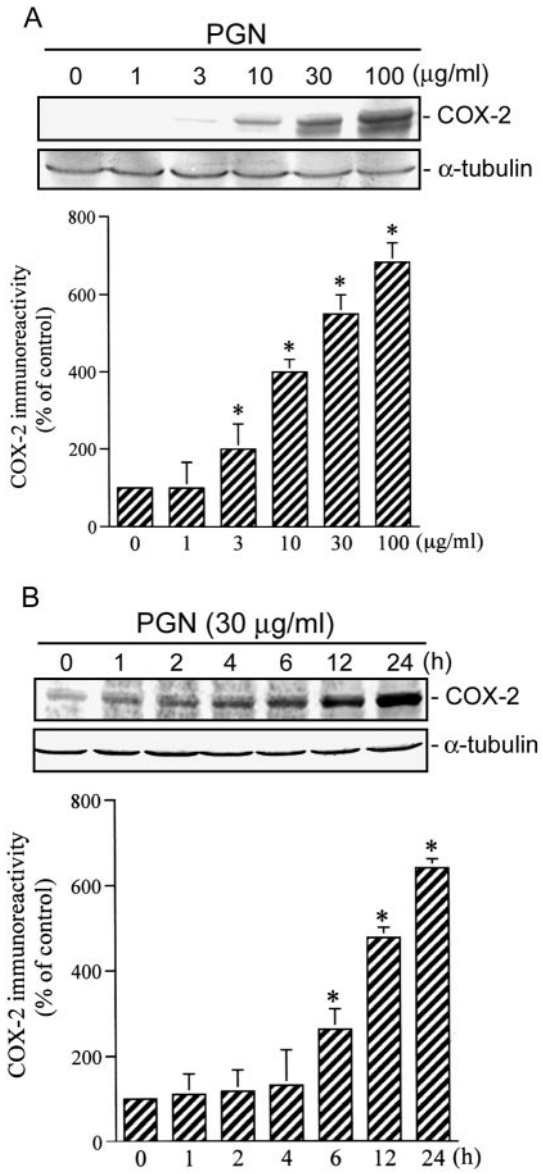

FIG. 1. **Concentration- and time-dependent increases in COX-2 expression by PGN in RAW 264.7 macrophages.** *A*, macrophages were incubated with various concentrations of PGN for 24 h, and then COX-2 or α-tubulin protein levels were determined. *B*, cells were incubated with 30 μg/ml PGN for the indicated time intervals, and then COX-2 or α-tubulin protein levels were determined. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± S.E. *, $p < 0.05$ as compared with the control group.

means. A p value of less than 0.05 was considered statistically significant.

RESULTS

PGN Induces Macrophage COX-2 Expression—Murine RAW 264.7 macrophages were chosen to investigate the signal pathways of PGN in COX-2 expression, an inflammatory response gene. Treatment with PGN (1–100 μg/ml) for 24 h induced COX-2 protein expression in a concentration-dependent manner (Fig. 1*A*); this induction also occurred in a time-dependent manner (Fig. 1*B*). After 24 h of treatment with 30 μg/ml PGN, the COX-2 protein had increased by ~640 ± 14% ($n = 3$) (Fig. 1*B*). To further confirm this stimulation-specific mediation by PGN without LPS contamination, polymycin B, an LPS inhibitor, was tested. We found that polymycin B (0.3 μg/ml) did not change PGN-induced COX-2 protein levels. In contrast, polymycin B completely inhibited LPS (1 μg/ml)-induced COX-2 protein expression (data not shown).

Augmentation of COX-2 Expression Occurred at the Level of Transcription—RAW 264.7 macrophages were pretreated with either actinomycin D (a transcriptional inhibitor) or cycloheximide (a translational inhibitor) and then treated with 30 μg/ml PGN. As a result, the PGN-induced elevation of COX-2 expression was inhibited by actinomycin D (30 nM) and cycloheximide (100 nM) by about 55 ± 4 and 72 ± 6%, respectively ($n = 3$) (Fig. 2*A*). The results suggest that the increase in COX-2 protein in RAW 264.7 macrophages responsive to PGN may have been due to COX-2 transcriptional expression.

Involvement of NF-κB in PGN-induced COX-2 Expression—As mentioned previously, NF-κB activation is necessary for COX-2 induction. Pyrrolidine dithiocarbamate (PDTC), an antioxidant, has been shown to inactivate NF-κB in macrophages (37). To examine whether NF-κB activation is involved in the signal transduction pathway leading to COX-2 expression caused by PGN, the NF-κB inhibitor PDTC was used. Fig. 2*B* shows that PGN-induced COX-2 protein levels were inhibited by PDTC (5–50 μM) in a concentration-dependent manner. When cells were treated with 50 μM PDTC, PGN-induced COX-2 expression was inhibited by 64 ± 4% ($n = 3$). Furthermore, macrophage pretreatment with an IκBα phosphorylation inhibitor (Bay 117082) (38) inhibited PGN-induced COX-2 protein expression in a concentration-dependent manner. Pretreatment of cells with 10 μM Bay 117082 inhibited the PGN response by 53 ± 3% ($n = 3$) (Fig. 2*C*). In parallel with the inhibition by PDTC and Bay 117082, IκB protease inhibitors (L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK, 3 μM) and calpain inhibitor I (10 μM)) (39, 40) also inhibited PGN-induced COX-2 protein expression by 35 ± 3 and 65 ± 5%, respectively ($n = 3$) (Fig. 2*D*). These results suggest that NF-κB activation is necessary for PGN-induced COX-2 expression in RAW 264.7 macrophages.

PGN Causes Increases in IKKα/β Activity, IκBα Phosphorylation, IκBα Degradation, and κB-Luciferase Activity—We further determined the upstream molecules of NF-κB in PGN-induced NF-κB activation. Stimulation of cells with 30 μg/ml PGN induced increases in IKKα/β activity and IκBα phosphorylation in a time-dependent manner, reaching maximums after 10 and 10–30 min of treatment, respectively (Fig. 3, *A* and *B*). In parallel with IKKα/β activity and IκBα phosphorylation, IκBα degradation was apparent after 10 min of treatment with 30 μg/ml PGN, and the IκBα protein was resynthesized after 60 min of treatment (Fig. 3*C*). To directly determine NF-κB activation after PGN treatment, RAW 264.7 macrophages were transiently transfected with pGL2-ELAM-κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 3*D*, macrophage treatment with PGN (1–100 μg/ml) for 24 h caused a concentration-dependent increase in κB luciferase activity.

Ras Is Involved in PGN-induced COX-2 Expression—To explore whether Ras might mediate PGN-induced COX-2 expression, manumycin A, a Ras inhibitor (41), was used. As shown in Fig. 4*A*, pretreatment of RAW 264.7 macrophages with manumycin A inhibited PGN-induced COX-2 expression in a concentration-dependent manner. When cells were treated with 3 μM manumycin A, PGN-induced COX-2 expression was inhibited by 58 ± 2% ($n = 3$). However, manumycin A had no effect on the basal level of COX-2 expression (Fig. 4*A*). Moreover, we found that 3 μM manumycin A also inhibited 1 μg/ml LPS-stimulated COX-2 expression by 72 ± 8% ($n = 3$) (Fig. 4*A*). Next, we directly measured the Ras activity in response to PGN and LPS. Fig. 4, *B* and *C*, shows that treatment of RAW 264.7 cells with 30 μg/ml PGN or 1 μg/ml LPS induced an increase in Ras activity in a time-de-

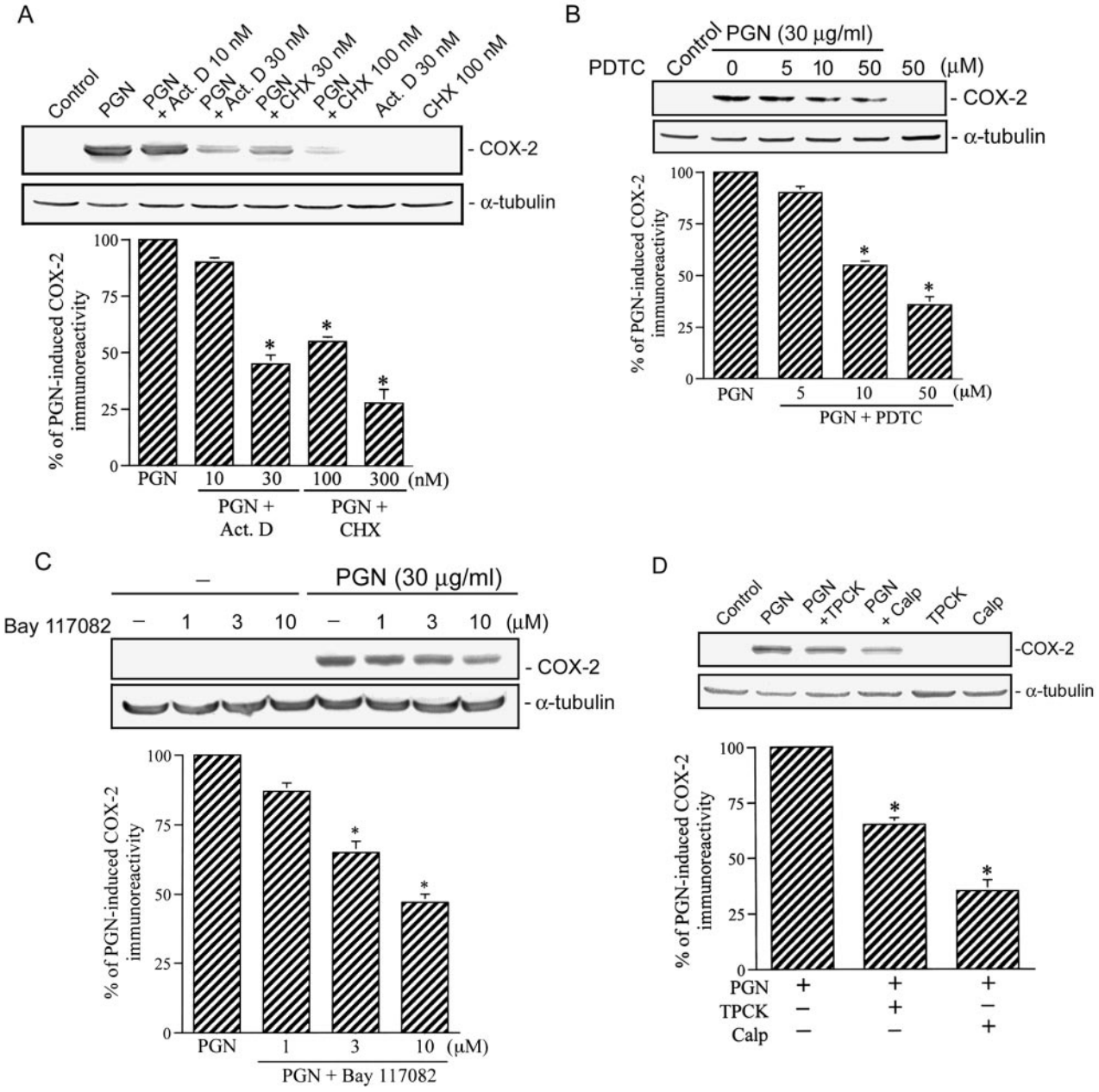

FIG. 2. **NF-κB is involved in PGN-mediated COX-2 induction in RAW 264.7 macrophages.** *A*, transcriptional activity is involved in PGN-induced COX-2 expression. Cells were pretreated for 30 min with vehicle, 10–30 nM actinomycin D (*Act. D*), and 30–100 nM cycloheximide (*CHX*) and then stimulated with 30 μg/ml PGN for 24 h. Cells were lysed and then immunoblotted for COX-2 or α-tubulin. *B–D*, the NF-κB signal pathway was necessary for PGN-induced COX-2 expression. Cells were pretreated for 30 min with 5–50 μM PDTC (*B*), 1–10 μM Bay 117082 (*C*), or 3 μM TPCK and 10 μM calpain inhibitor I (*Calp*) (*D*) and then stimulated with 30 μg/ml PGN for 24 h. Cell were lysed and then immunoblotted for COX-2 or α-tubulin. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Results are representative of three independent experiments. Traces represent results from three independent experiments, which are presented as the mean ± S.E. *, $p < 0.05$ as compared with PGN treatment.

pendent manner, as assessed by immunoblotting samples for Ras immunoprecipitated from lysates using Raf-1-RBD. Maximal activation was detected after 5 min of stimulation, and the response continued until 30 min after PGN stimulation (Fig. 4*B*). Similarly, the LPS-induced increase in Ras activity began at 3 min, peaked at 5 min, and then declined at 10 min after LPS treatment (Fig. 4*C*). Furthermore, the PGN-induced increase in Ras activity was markedly inhibited by pretreatment of cells for 30 min with manumycin A (1–3 μM) in a concentration-dependent manner (Fig. 4*D*). Taken together, these results imply that Ras activation is involved in PGN- or LPS-induced COX-2 expression.

Raf-1 Is Involved in PGN-induced COX-2 Expression—To examine whether Raf-1, a target protein for Ras, might play a crucial role in PGN-induced COX-2 expression, the Raf-1 inhibitor GW 5074 (42) was used. As shown in Fig. 5*A*, 1 μM of GW 5074 alone did not affect the basal COX-2 level, but it significantly inhibited PGN-induced COX-2 expression by 43 ± 3% ($n = 3$). Furthermore, we found that GW 5074 (1 μM) inhibited LPS-induced COX-2 expression by 76 ± 3% ($n = 3$) (Fig. 5*A*). Raf-1 is associated with Ras-GTP and then by additional modifications such as phosphorylation at Ser-338 to become the active form (33). The activated Raf-1 then triggers sequential activation of downstream molecules. Thus, phosphorylation of Raf-1 at Ser-338 is a critical step in Raf-1 activation. Next, we further examined Raf-1 Ser-338 phosphorylation by

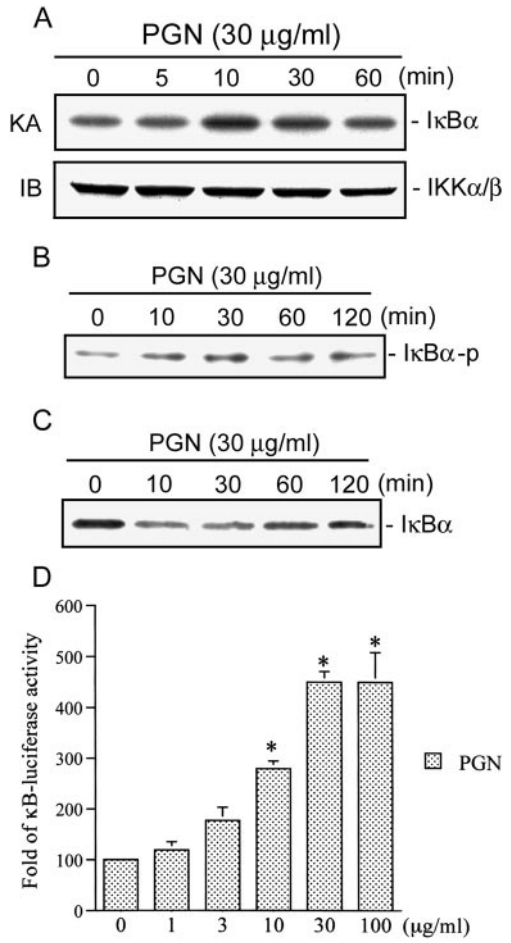

FIG. 3. **PGN induced IKKα/β activation, IκBα phosphorylation, IκBα degradation, and κB-luciferase activity in RAW 264.7 macrophages.** *A*, RAW 264.7 cells were incubated with 30 μg/ml PGN for 0–60 min, and cell lysates were then immunoprecipitated with antibodies specific for IKKα and IKKβ. One set of immunoprecipitates was subjected to the kinase assay (*KA*) described under "Experimental Procedures" using the GST-IκBα fusion protein as a substrate (*top panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and analyzed by immunoblotting (*IB*) with the anti-IKKα/β antibody (*bottom panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKKα/β. *B* and *C*, following incubation for 0–120 min with 30 μg/ml PGN, IκBα phosphorylation (*B*) and IκBα degradation (*C*) were determined by immunoblotting using phospho-IκBα- and IκBα-specific antibodies, respectively. Typical traces are representative of three experiments with similar results. *D*, RAW 264.7 macrophages were transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 0.5 μg of pBK-CMV-LacZ for 24 h, and then cells were incubated with 1–100 μg/ml PGN for another 24 h. Luciferase activities were determined as described under "Experimental Procedures." The level of induction of luciferase activity was compared with that of cells without PGN treatment. Data represent the mean ± S.E. of three to four experiments performed in duplicate. *, $p < 0.05$ as compared with the control without PGN treatment.

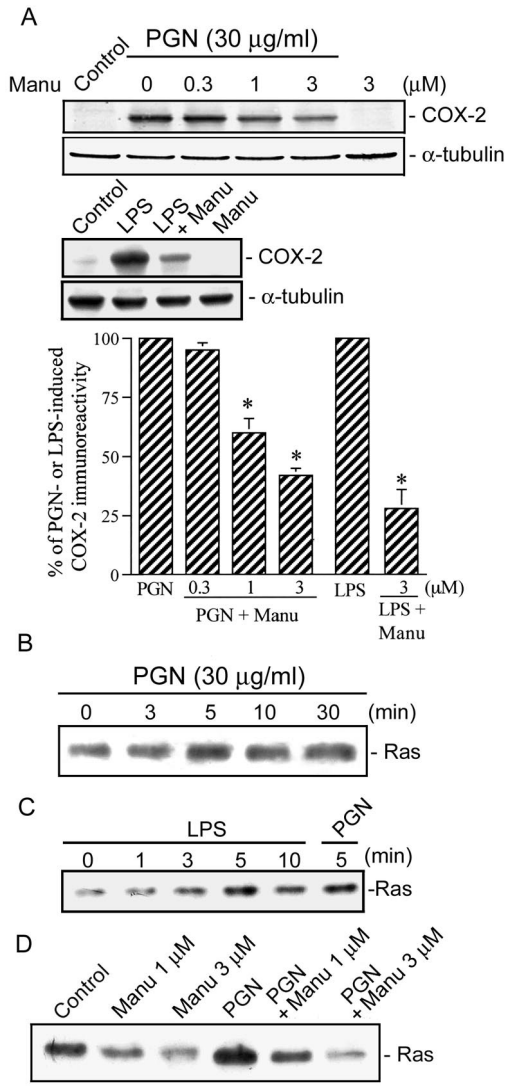

FIG. 4. **Effects of manumycin A on PGN-induced COX-2 expression and Ras activation in RAW 264.7 macrophages.** *A*, RAW 264.7 macrophages were pretreated with vehicle and 0.3–3 μM manumycin A (*Manu*) for 30 min followed by stimulation with 30 μg/ml PGN or 1 μg/ml LPS for another 24 h, and COX-2 expression was determined by immunoblotting with an antibody specific for COX-2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces are representative of three experiments with similar results, which are presented as the mean ± S.E. *, $p < 0.05$ as compared with PGN or LPS treatment. *B* and *C*, RAW 264.7 macrophages were incubated with 30 μg/ml PGN or 1 μg/ml LPS for indicated time intervals, and cell lysates were then immunoprecipitated with an antibody specific for Raf-1 RBD. The Ras activity assay is described under "Experimental Procedures." Typical traces represent three experiments with similar results. *D*, cells were pretreated with 1–3 μM manumycin A (*Manu*) for 30 min and then treated with 30 μg/ml PGN for another 10 min. Cells were then lysed for Ras activity assay as described above. Typical traces represent two experiments with similar results.

PGN stimulation in RAW 264.7 macrophages using the anti-phospho-Raf-1 antibody at Ser-338. When cells were treated with 30 μg/ml PGN for various time intervals, Raf-1 Ser-338 phosphorylation increased at 5 min and peaked at 10 min. After 20 min of treatment, the PGN-induced increase in Raf-1 Ser-338 phosphorylation declined (Fig. 5*B*). In addition, PGN-induced Raf-1 Ser-338 phosphorylation was also inhibited by treatment with 3 μM manumycin A and 1 μM GW 5074 (Fig. 5*C*). Results indicate that Raf-1 is a downstream molecule of Ras and is involved in Ras-mediated COX-2 protein expression.

PGN Mediates Phosphorylation of ERK—We next wished to determine whether PGN is able to activate ERK, a critical downstream target of Raf-1 (43), which has been shown to induce gene expression (44). We tested the role of ERK in mediating PGN-induced COX-2 expression by using the specific MEK inhibitor PD 098059. As shown in Fig. 6*A*, PGN-induced COX-2 expression was markedly attenuated by pretreatment of cells with PD 098059 (3–30 μM) in a concentration-dependent manner. Pretreatment of cells with 30 μM PD 098059 inhibited PGN-induced COX-2 expression by 85 ± 7% ($n = 3$) (Fig. 6*A*). Moreover, we also found that 30 μM PD 098059 inhibited LPS-induced COX-2 expression by 76 ± 5% ($n = 3$) (Fig. 6*A*). To directly confirm the crucial role of ERK in COX-2 expression, we determined ERK phosphorylation and kinase activity in response to PGN. As

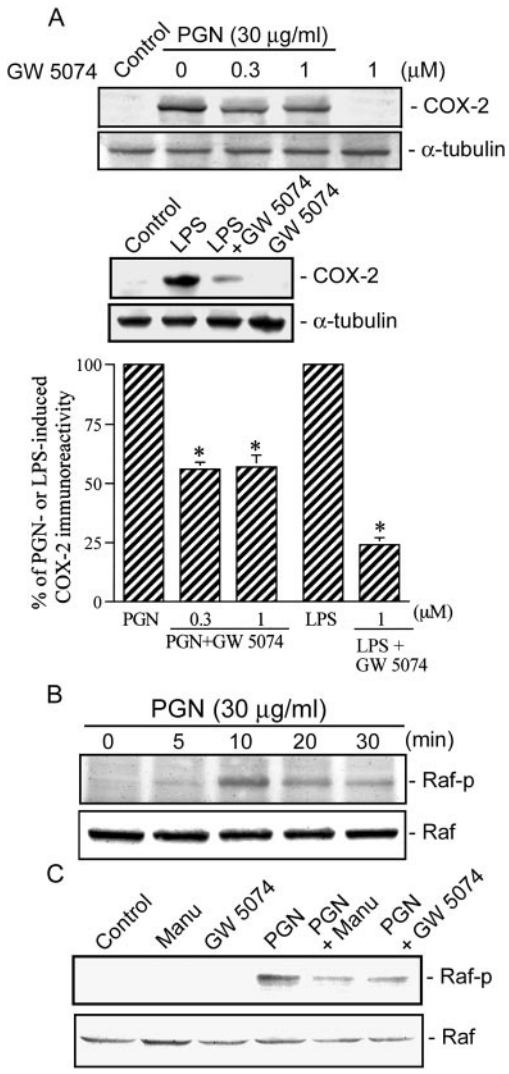

FIG. 5. **Effects of GW 5074 on PGN-induced COX-2 expression and Raf-1 phosphorylation in RAW 264.7 macrophages.** *A*, RAW 264.7 macrophages were pretreated with vehicle and GW 5074 (0.3–1 μM) for 30 min followed by stimulation with 30 μg/ml PGN or 1 μg/ml LPS for another 24 h, and COX-2 expression was determined by immunoblotting with an antibody specific for COX-2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces represent three experiments with similar results, which are presented as the mean ± S.E. *, $p < 0.05$ as compared with PGN or LPS treatment. *B*, RAW 264.7 macrophages were incubated with 30 μg/ml PGN for 0–30 min, and then Raf-1 phosphorylation (*upper panel*) and Raf-1 (*bottom panel*) protein levels were determined. The presence of equal loading in each lane is shown by the similar intensities of Raf-1. Traces represent results from three independent experiments. *C*, cells were pretreated with 3 μM manumycin A (*Manu*) or 0.3 μM GW 5074 for 30 min, and then treated with 30 μg/ml PGN for another 10 min. Cells were then lysed for Raf-1 phosphorylation (*upper panel*) and Raf-1 (*bottom panel*) protein levels as described above. Typical traces represent two experiments with similar results.

shown in Fig. 6*B*, treatment of RAW 264.7 macrophages with 30 μg/ml PGN resulted in a time-dependent phosphorylation of ERK. The ERK phosphorylation began at 10 min, peaked at 30–60 min, and then declined to 120 min after PGN treatment (Fig. 6*B*, *upper panel*). The protein level of ERK2 was not affected by PGN treatment (Fig. 6*B, bottom panel*). In parallel, using MBP as an ERK substrate, an increase in ERK activity was observed within 10 min and reached a peak 30 min after PGN stimulation, thereafter declining to 60 min after stimulation (Fig. 6*C*). To determine the relationships among Ras, Raf-1, and ERK in the PGN-mediated signaling pathway, we found that pretreatment of RAW 264.7 cells for 30 min with 3 μM manumycin A, 1 μM GW 5074, and 30 μM PD 098059 markedly inhibited the PGN-induced increase in ERK activity (Fig. 6*D*). Based on these results, we suggest that activation of Ras and Raf-1 occurs upstream of ERK in the PGN-induced signaling pathway.

Ras, Raf-1, and ERK Mediated PGN-induced IKKα/β and NF-κB Activation—We further examined whether the activation of IKKα/β and NF-κB occurs through the Ras/Raf-1/ERK signaling pathway. As shown in Fig. 7*A*, pretreatment of cells for 30 min with manumycin A (3 μM), GW 5074 (1 μM), and PD 098059 (30 μM) markedly attenuated the PGN-induced increase in IKKα/β activity. None of these inhibitors affected the basal IKKα/β activity (Fig. 7*A*). Similarly, the PGN-induced increase in κB-luciferase activity was also inhibited by 3 μM manumycin A, 1 μM GW 5074, and 30 μM PD 098059 by 74 ± 11, 71 ± 6, and 58 ± 13%, respectively ($n = 3$) (Fig. 7*B*). To confirm further the role of Ras in PGN-mediated NF-κB activation, RasN17 was tested. Transfection of RAW 264.7 cells with RasN17 inhibited the PGN-induced increase in κB-luciferase activity in a dose-dependent manner, with 1 μg of RasN17 reducing the basal κB-luciferase activity and PGN response by about 64 ± 15 and 82 ± 7%, respectively ($n = 3$) (Fig. 7*B*). Taken together, these data suggest that activation of Ras/Raf-1/ERK pathway is also required for PGN-induced IKKα/β and NF-κB activation in RAW 264.7 macrophages.

Ras Is Associated with TLR2 by p85α upon PGN Stimulation—The rapid activation of Ras by PGN stimulation suggested that Ras activation might occur close to TLR2 in the PGN signal pathway. Therefore, we investigated whether PGN can induce the interaction between Ras and TLR2. As shown in Fig. 8*A*, treatment of RAW 264.7 macrophages with 30 μg/ml PGN led to the rapid association of Ras and TLR2, as detected by immunoblotting using the antibody to Ras after immunoprecipitation of TLR2. The association of TLR2 and Ras occurred at 1 min and peaked at 3–5 min (Fig. 8*A*). Control experiments using an unrelated polyclonal antibody for immunoprecipitation showed no Ras binding (Fig. 8*A*). The interaction between TLR2 and Ras was further confirmed by converse experiments in which the TLR2 and Ras complex was immunoprecipitated with the Ras antibody and immunoblotted with the TLR2 antibody (Fig. 8*B*). Previous reports (45) showed that p85 can interact with TLR2 and then induces Rac activation. Therefore, we further examined the role of p85α involvement in TLR2 and Ras complex formation by PGN stimulation. Fig. 8*C* shows that PGN (30 μg/ml) treatment rapidly increased the formation of the TLR2 and p85α complex at as early as 30 s and then it declined at 1–5 min. We also found that treatment of macrophages with PGN induced the association of p85α and Ras within 1 min, and this was sustained for 5 min (Fig. 8*D*). These results suggested that PGN induces Ras activation by interacting with TLR2 and p85α in macrophages.

DISCUSSION

The findings of this study showed that PGN induces COX-2 expression through activation of the Ras/Raf-1/ERK/IKKα/β/NF-κB signal pathway in RAW 264.7 macrophages. In mice and humans, the COX-2 promoter has many transcription factors including NF-κB in the 5′ region of the *cox-2* gene (20–22). Transcription factor NF-κB has been reported to induce macrophage COX-2 in response to various inflammatory stimuli (46). Previous studies have demonstrated that NF-κB activation is necessary for LPS-induced COX-2 induction in RAW 264.7 macrophages (47–50). Hwang *et al.* (47, 48) reported that in LPS-treated RAW 264.7 macrophages, NF-κB plays a crucial role in TLR4-induced *cox-2* gene expression. The results of this study showed that NF-κB activation also contributed to PGN-induced COX-2 induction in RAW 264.7 macrophages (Fig. 2, and that all inhibitors of the NF-κB-dependent signaling path-

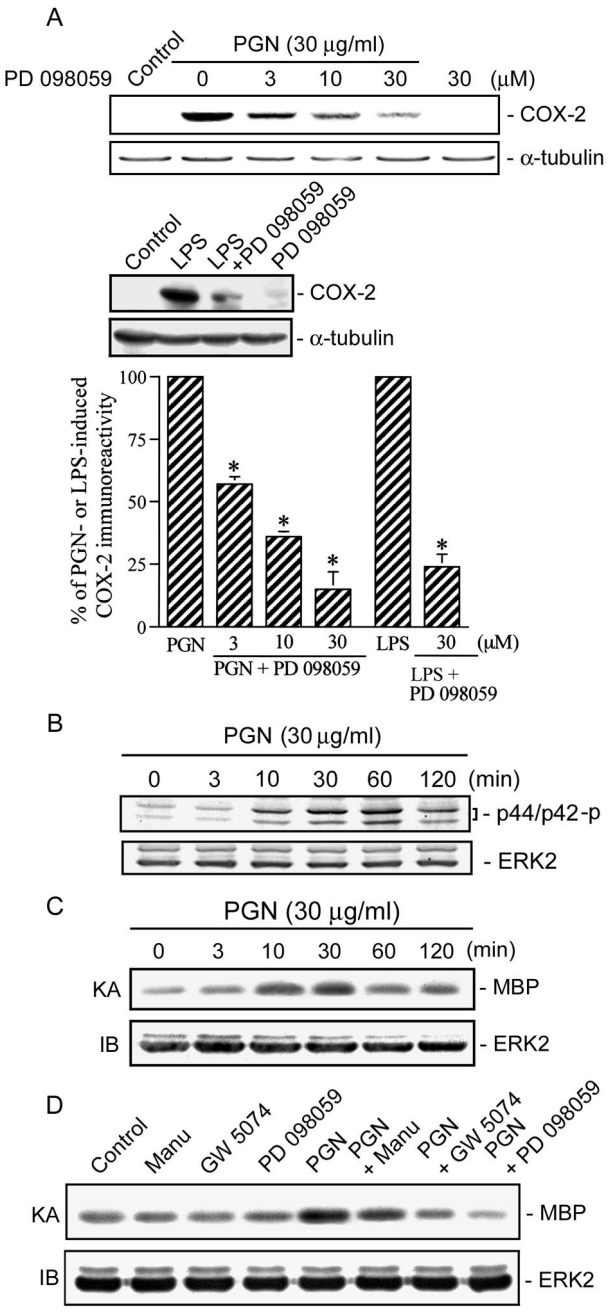

FIG. 6. **Effects of PD 098059 on PGN-induced COX-2 expression, ERK phosphorylation, and ERK activity in RAW 264.7 macrophages.** *A*, RAW 264.7 macrophages were pretreated with vehicle and PD 098059 (3–30 μM) for 30 min before treatment with 30 μg/ml PGN or 1 μg/ml LPS for another 24 h, and COX-2 expression was determined by immunoblotting with an antibody specific for COX-2. Equal loading in each lane is shown by the similar intensities of α-tubulin. Typical traces represent three experiments with similar results, which are presented as the mean ± S.E. *, $p < 0.05$ as compared with PGN or LPS treatment. *B*, after cells were treated with 30 μg/ml PGN for different intervals, ERK phosphorylation was shown by immunoblotting with an antibody specific for phosphorylated ERK (*upper panel*). Equal loading in each lane is shown by the similar intensities of ERK2 (*bottom panel*). Typical traces represent three experiments with similar results. *C*, for ERK kinase activity, cell lysates were immunoprecipitated with ERK2 antibody. One set of immunoprecipitates was subjected to the kinase assay (*KA*) using MBP as a substrate. The other set of immunoprecipitates was subjected to 12% SDS-PAGE and analyzed by immunoblotting (*IB*) with an anti-ERK2 antibody. Equal amounts of the immunoprecipitated kinase complexes present in each kinase assay were confirmed by immunoblotting for ERK2. Shown are representative results from three independent experiments. *D*, cells were pretreated with 3 μM manumycin A (*Manu*), 0.3 μM GW 5074, and 30 μM PD 098059 for 20 min and then incubated with 30 μg/ml PGN for another 10 min. Cell lysates were immunoprecipitated with an antibody specific for ERK2. The kinase assay (*KA*) and immunoblotting (*IB*) were as described above. Equal amounts of the immunoprecipitated kinase complexes present in each kinase assay were confirmed by immunoblotting for ERK2. The data shown represent three experiments.

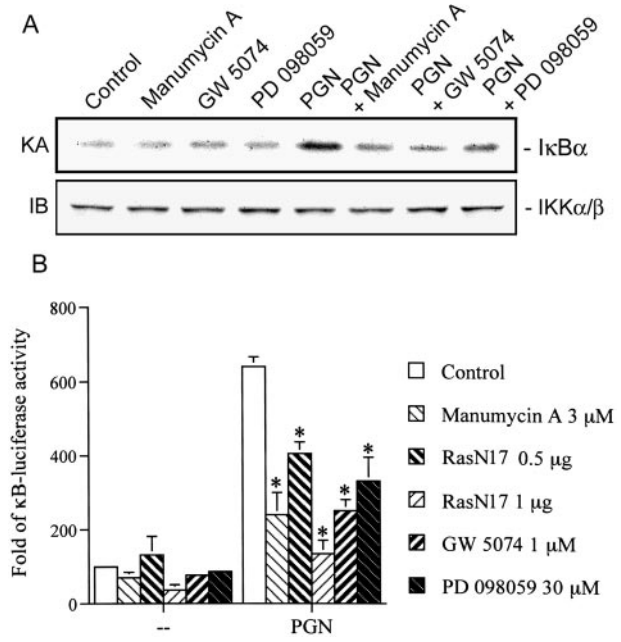

FIG. 7. **The Ras/Raf-1/ERK signal pathway is involved in PGN-mediated IKKα/β and NF-κB activation in RAW 264.7 macrophages.** *A*, RAW 264.7 macrophages were pretreated with vehicle, 3 μM manumycin A (*Manu*), 0.3 μM GW 5074, and 30 μM PD 098059 for 30 min followed by stimulation with 30 μg/ml PGN for another 20 min; cell lysates were then immunoprecipitated with antibodies specific to IKKα and IKKβ. One set of immunoprecipitates was subjected to the kinase assay (*KA*) by using the GST-IκBα fusion protein as a substrate (*top panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and analyzed by immunoblotting (*IB*) with anti-IKKα/β antibody (*bottom panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKKα/β. *B*, RAW 264.7 macrophages were either transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 1 μg of pBK-CMV-LacZ for 24 h and were co-transfected with 0.5 and 0.5 μg of RasN17, or were pretreated with 3 μM manumycin A, 0.3 μM GW 5074, and 30 μM PD 098059 for 20 min and then stimulated with 30 μg/ml PGN for another 24 h. Cells were harvested for luciferase assay as described in Fig. 4*C*. Data represent the mean ± S.E. of three experiments performed in duplicate. *, $p < 0.05$ as compared with the corresponding control response of PGN treatment.

way, including PDTC, Bay 117082, TPCK, and calpain inhibitor I, inhibited PGN-induced macrophage COX-2 expression. Furthermore, PGN induced IKKα/β activation, IκBα phosphorylation, and IκBα degradation, as well as an increase in κB-luciferase activity. These findings are in agreement with those from studies on LTA (16, 51), another cell wall component of Gram-positive bacteria. NF-κB activation is required to induce COX-2 transcription in epithelial cells. The results of this study also showed that Ras, Raf-1, and ERK are involved in NF-κB activation through an increase in IKKα/β activity. A previous report showed that in transformed liver epithelial cells, Ras and Raf-1 lead to constitutive activation of NF-κB through the IKKα/β complex (52). These pathways may mediate PGN effect. As shown in Fig. 7, manumycin A, GW 5074, and PD 098059 blocked PGN-induced IKKα/β activity. In addition, RasN17 and these inhibitors attenuated induction of NF-κB reporter activity or *cox-2* gene expression, implying a role in the pathway of transactivation by PGN stimulation.

The TLR family now consists of 10 different TLRs (TLR1-TLR10), but only TLR2 and TLR4 are known to have biological

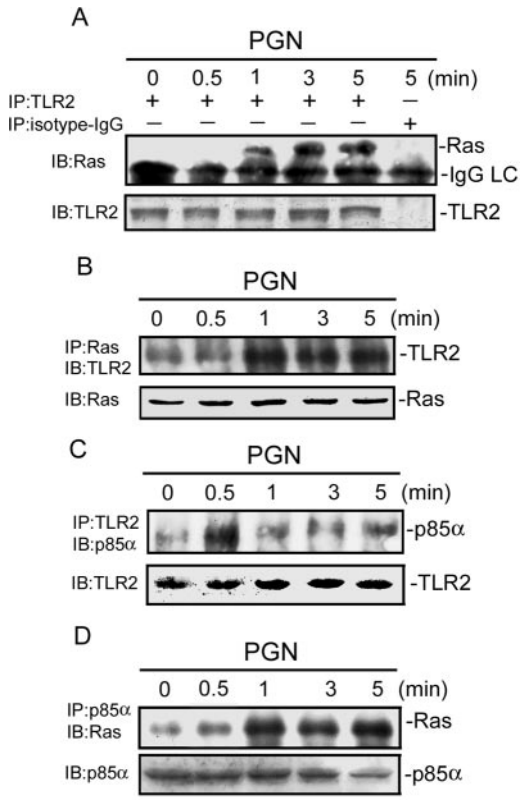

FIG. 8. **Ras is associated with p85α and TLR2 upon PGN stimulation in RAW 264.7 macrophages.** *A*, RAW 264.7 macrophages were stimulated with 30 μg/ml PGN for 0–5 min and were then lysed. Cells extracts were immunoprecipitated (*IP*) with anti-TLR2 or anti-isotype IgG antibody and then immunoblotted (*IB*) with anti-Ras antibody. IgG LC (IgG light chain). *B*, RAW 264.7 macrophages were treated with PGN for the indicated time intervals. Cells extracts were immunoprecipitated with the anti-Ras antibody and then immunoblotted with the anti-TLR2 antibody. *C*, cells were treated with PGN for 0–5 min, then immunoprecipitated with the anti-TLR2 antibody, and immunoblotted with the anti-p85α antibody. *D*, cells were stimulated with PGN for 0–5 min, then immunoprecipitated with the anti-p85 α antibody, and immunoblotted with the anti-Ras antibody. Typical traces are representative of three experiments with similar results.

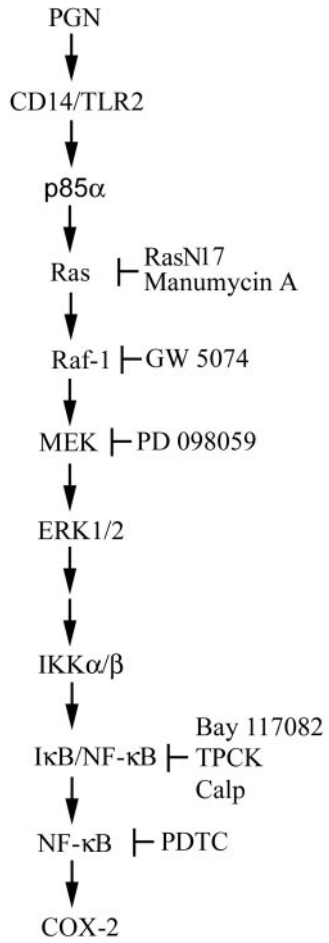

FIG. 9. **Schematic summary of signal transduction by PGN induces COX-2 expression in RAW 264.7 macrophages.** PGN-induced Ras activation may occur through the recruitment of p85α and Ras to TLR2 in RAW 264.7 macrophages. The PGN-induced activation of the Ras/Raf-1/ERK cascade results in an increase in IKKα/β activity, IκBα degradation, and NF-κB activation, which leads to expression of the macrophage COX-2.

and pathological functions (53, 54). The cytoplasmic portion of TLRs shows high similarity to that of the IL-1 receptor family and is now called the Toll/IL-1 receptor (TIR) domain (53). Upon recognizing respective ligands, the TIR domain recruits MyD88/IRAK/TRAF6 and activates downstream signaling molecules such as MAPK and NF-κB (53). In addition to the common MyD88/IRAK/TRAF6 pathway, the TIR domain family can activate downstream signal components through small G proteins such as Rac and Ras to mediate NF-κB activation and COX-2 expression (55, 56). To date, how the TIR domain family activates Ras, especially TLR2, is still not clear. Recently, Ras activation has been shown to be involved in the signaling pathway of IL-1-induced p38 MAPK activation and occurred through associating with IRAK, IRAK2, TRAF6, and TAK1 multiprotein complex in EL4.NOB-1 cells and HeLa cells (57, 58). Furthermore, Ras was found to participate in CpG oligonucleotide-induced COX-2 expression through association with TLR9 and to activate ERK and NF-κB in RAW 264.7 macrophages (56). In addition, a previous study also showed that the TIR domain of TLR2 contains a p85-binding motif (Y*XX*M), which contributes to Rac mediation of TLR2-induced NF-κB activation via the recruitment of p85 and Rac to the TLR2 (45). The findings of our experiments showed that PGN can rapidly induce TLR2 association with p85α as early as 30 s, and subsequently Ras interacted with TLR2 at 1 min following PGN treatment. Simultaneously, we also found that PGN induced the association of Ras and p85α during the interaction of Ras and TLR2. Based on these findings, we suggest that PGN-induced Ras activation may occur through the recruitment of p85α and Ras to TLR2 in RAW 264.7 macrophages.

Ras, an oncogenic protein, plays a critical role in the induction of COX-2 protein (59, 60). Ras might activate a number of signal pathways, including the Raf-1/MEK/ERK pathway and the phosphatidylinositol 3-kinase/Akt/NF-κB pathway (34, 59, 60). In RAW 264.7 macrophages, LPS induces tumor necrosis factor gene expression through the Ras/Raf-1/MEK/ERK pathway (61). In murine fibroblasts, oncogene- and growth factor-induced COX-2 transcription requires Ras-dependent Raf-2/MAPKK/ERK activation (60). In this study, we found that treatment of RAW 264.7 macrophages with PGN caused sequential activations of Ras, Raf-1, and ERK, and that manumycin A, GW 5074, and PD 098059 all inhibited PGN-induced ERK activation and COX-2 expression. These results suggested that the Ras/Raf-1/ERK signal pathway is important for PGN-induced COX-2 expression. This suggestion is further supported by our previous report that LTA induces COX-2 expression through the ERK pathway to induce NF-κB activation in human lung epithelial cells (62). However, the contribution of Ras or ERK to LPS-mediated RAW 264.7 macrophages COX-2 expression is controversial. Most reports (47, 50, 63–65) have shown that LPS-induced RAW 264.7 macrophages *cox-2* gene

expression is dependent on the ERK signaling pathway. However, Wadleigh *et al.* (66) showed that overexpression of a dominant negative mutant of Ras or ERK did not inhibit LPS-induced COX-2 luciferase reporter activity. Nevertheless, they did not directly measure Ras or ERK activity. In the present study, we found that LPS induced an increase in Ras activity and that manumycin A, GW 5074, and PD 098059 all attenuated LPS-mediated COX-2 expression. Taken together, our results indicate that the Ras/Raf-1/ERK signaling pathway is also necessary for LPS-induced COX-2 expression in RAW 264.7 macrophages.

In conclusion, the findings of our study for the first time showed that PGN induced COX-2 transcription through the Ras/Raf-1/ERK signaling pathway to increase in IKKα/β activity, NF-κB activation, and COX-2 expression in RAW 264.7 macrophages. This is the first study showing PGN-induced Ras activation may occur through the recruitment of p85α and Ras to TLR2 in RAW 264.7 macrophages. Fig. 9 is a schematic representation of the signaling pathway of PGN-induced COX-2 expression in RAW 264.7 macrophages. With an understanding of these signal transduction pathways, we can design therapeutic strategies to reduce inflammation caused by Gram-positive organisms.

REFERENCES

1. Bone, R. C. (1994) *Arch. Intern. Med.* **154,** 26–34
2. Ulevitch, R. J., and Tobias, P. S. (1995) *Annu. Rev. Immunol.* **13,** 437–457
3. Schleifer, K. H., and Kandler, O. (1972) *Bacteriol. Rev.* **36,** 407–477
4. Wang, Q., Dziarski, R., Kirschning, C. J., Muzio, M., and Gupta, D. (2001) *Infect. Immun.* **69,** 2270–2276
5. Xu, Z., Dziarski, R., Wang, Q., Swartz, K., Sakamoto, K. M., and Gupta, D. (2001) *J. Immunol.* **167,** 6975–6982
6. Bhakdi, S., Klonisch, T., Nuber, P., and Fischer, W. (1991) *Infect. Immun.* **59,** 4614–4620
7. Mattson, E., Verhage, L., Rollof, J., Fleer, A., Verhoef, J., and van Dijk, H. (1993) *FEMS Immunol. & Med. Microbiol.* **7,** 281–287
8. Wang, Z.-M., Lui, C., and Dziarski, R. (2000) *J. Biol. Chem.* **275,** 20260–20267
9. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) *J. Biol. Chem.* **273,** 8680–8690
10. Vane, J. R., Bakhle, Y. S., and Botting, R. M. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38,** 97–120
11. DeWitt, D. L. (1991) *Biochim. Biophys. Acta* **1083,** 121–134
12. Xie, W. L., Chipman, J. G., Roberstone, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2692–2696
13. Mitchell, J. A., Larkin, S., and Williams, T. J. (1995) *Biochem. Pharmacol.* **50,** 1535–1542
14. Chiang, L. L., Kuo, C. T., Wang, C. H., Lee, H. M., Ho, Y. S., Kuo, H. P., and Lin, C. H. (2003) *J. Pharm. Pharmacol.* **55,** 115–123
15. Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. J., and Vane, J. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11693–11697
16. Lin, C. H., Kuan, I. H., Lee, H. M., Lee, W. S., Sheu, J. R., Ho, Y. S., Wang, C. H., and Kuo, H. P. (2001) *Br. J. Pharmacol.* **134,** 543–552
17. Maier, J. A., Hla, T., and Maciag, T. (1990) *J. Biol. Chem.* **265,** 10805–10808
18. Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V., and Woolf, C. J. (1994) *Nature* **410,** 471–475
19. Hinz, B., and Brune, K. (2002) *J. Pharmacol. Exp. Ther.* **300,** 367–375
20. Inoue, H., Yokoyama, C., Hara, C., Tone, Y., and Tanabe, T. (1995) *J. Biol. Chem.* **270,** 24965–24971
21. Kim, Y., and Fischer, S. M. (1998) *J. Biol. Chem.* **273,** 27686–27694
22. Reddy, S. T., Wadleigh, D. J., and Herschman, H. R. (2000) *J. Biol. Chem.* **275,** 3107–3113
23. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14,** 649–683
24. Thompson, J. L., Phillips, R. J., Bromage, H. E., Tempst, P., and Ghosh, S. (1995) *Cell* **80,** 573–582
25. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554
26. Maniatis, T. (1997) *Science* **278,** 818–819
27. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Geoddel, D. (1997) *Science* **278,** 866–869
28. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597
29. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) *Oncogene* **17,** 1395–1413
30. Blaine, S., Wick, M., Dessev, C., and Nemenoff, R. A. (2001) *J. Biol. Chem.* **276,** 42737–42743
31. Subbaramaiah, K., Norton, L., Gerald, W., and Dannenberg, A. J. (2002) *J. Biol. Chem.* **277,** 18649–18657
32. Marshall, C. J. (1996) *Curr. Opin. Cell Biol.* **8,** 197–204
33. Zang, M., Hayne, C., and Luo, Z. (2002) *J. Biol. Chem.* **277,** 4395–4405
34. Zhang, X.-F., Settleman, J. M., Takeuchi-Suzuki, E., Ellsdge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) *Nature* **364,** 308–313
35. Whitmarsh, A. J., and Davis, R. J. (1998) *Trends Biochem. Sci.* **23,** 481–485
36. Lin, C. H., Sheu, S. Y., Lee, H. M., Ho, Y. S., Lee, W. S., Ko, W. C., and Sheu, J. R. (2000) *Mol. Pharmacol.* **57,** 36–43
37. Chen, B.-C., Chou, C.-H., and Lin, W.-W. (1998) *J. Biol. Chem.* **273,** 29754–29763
38. Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T., and Gerritsen, M. E. (1997) *J. Biol. Chem.* **272,** 21096–21103
39. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsura, M., Katz, D., FitzGerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. (1996) *EMBO J.* **15,** 4682–4690
40. Musial, A., and Eissa, N. T. (2001) *J. Biol. Chem.* **276,** 24268–24273
41. Hara, M., and Han, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3333–3337
42. Varga, E. V., Rubenzik, M., Grife, V., Sugiyama, M., Stropova, D., Roeske, W. R., and Yamamura, H. I. (2002) *Eur. J. Pharmacol.* **451,** 101–102
43. Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E., and Cobb, M. H. (1997) *EMBO J.* **16,** 6426–6438
44. Lee, H.-W., Ahn, D.-H., Crawley, S. C., Li, J.-D., Gun, J. R., Jr., Basbaum, C. B., Fan, N. Q., Szymkowaski, D. E., Han, S.-Y., Lee, B. H., Sleisenger, M. H., and Kim, Y. S. (2002) *J. Biol. Chem.* **277,** 32624–32631
45. Arbibe, L., Mira, J. P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowiski, P. J., Ulevitch, R. J., and Knaus, U. G. (2000) *Nat. Immun.* **1,** 533–540
46. Baeuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179
47. Hwang, D., Jang, B. C., Yu, G., and Boudreau, M. (1997) *Biochem. Pharmacol.* **54,** 87–96
48. Rhee, S. H., and Hwang, D. (2000) *J. Biol. Chem.* **275,** 34035–34040
49. Heiss, E., Herhaus, C., Klimo, K., Bartsch, H., and Gerhauser, C. (2001) *J. Biol. Chem.* **276,** 32008–32015
50. Yadav, P. N., Liu, Z., and Rafi, M. M. (2003) *J. Pharmacol. Exp. Ther.* **305,** 925–931
51. Elgavish, A. (2000) *J. Cell. Physiol.* **182,** 232–238
52. Arsura, M., Mercurio, F., Oliver, A. L., Thorgeirsson, S. S., and Sonenshein, G. E. (2000) *Mol. Cell. Biol.* **20,** 5381–5391
53. O'Nell, L. (2000) *Biochem. Soc. Trans.* **28,** 557–563
54. Vasselon, T., and Detmers, P. A. (2002) *Infect. Immun.* **70,** 1033–1041
55. Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) *Nature* **420,** 329–333
56. Yeo, S.-J., Yoon, J.-G., and Yi, A.-K. (2003) *J. Biol. Chem.* **278,** 40590–40600
57. Palsson, E. M., Popoff, M., Thelestam, M., and O'Nell, A. J. (2000) *J. Biol. Chem.* **275,** 7818–7825
58. McDermott, E. P., and O'Neill, L. A. (2002) *J. Biol. Chem.* **277,** 7808–7815
59. Sheng, H., Shao, J., and DuBois, R. N. (2001) *Cancer Res.* **61,** 2670–2675
60. Xie, W., Fletcher, B. S., Andersen, R. D., and Herschman, H. R. (1994) *Mol. Cell. Biol.* **14,** 6531–6539
61. Geppert, T. D., Whitehurst, C. D., Thompson, P., and Beutler, B. (1994) *Mol. Med.* **1,** 93–103
62. Lin, C. H., Kuan, I. H., Wang, C. H., Lee, W. S., Sheu, J. R., Hsiao, G., Wu, C. H., and Kuo, H. P. (2002) *Eur. J. Pharmacol.* **450,** 1–9
63. Frost, J, A., Swantek, J. L., Stippec, S., Yin, M. J., Gaynor, R., and Cobb, M. H. (2000) *J. Biol. Chem.* **275,** 19693–19699
64. Pahan, K., Sheikh, F. G., Namboodiri, A. M., and Singh, I. (1997) *J. Clin. Investig.* **100,** 2671–2679
65. Paul, A., Cuenda, A., Bryant, C. E., Murray, J., Chilveers, E. R., Cohen, P., Gould, G. W., and Plevin, R. (1999) *Cell. Signal.* **11,** 491–497
66. Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., and Herschman, H. R. (2000) *J. Biol. Chem.* **275,** 6259–6266