Protein Kinase C-α Regulates Toll-like Receptor 4-Mediated Inducible Nitric Oxide Synthase Expression

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Abstract

Purpose: The nitric oxide (NO) release by inducible nitric oxide synthase (iNOS) is the key events in macrophage response to lipopolysaccharide (LPS) which is suggested to be a crucial mediator for inflammatory and innate immune responses. NO is an important mediator involved in many host defense action and may also lead to a harmful host response to bacterial infection. However, given the importance of iNOS in a variety of pathophysiological conditions, control of its expression and signaling events in response to LPS has been the subject of considerable investigation. **Materials and Methods:** The Raw264.7 macrophage cell line was used to observe LPS-stimulated iNOS expression. The expression of iNOS is observed by Western blot analysis and real-time RT-PCR. Protein kinase C (PKC)-α overexpressing Raw264.7 cells are established to determine the involvement of PKC-α in LPS-mediated iNOS expression. NF-κB activity is measured by IκBα degradation and NF-κB luciferase activity assay. **Results:** We found that various PKC isozymes regulate LPS-induced iNOS expression at the transcriptional and translational levels. The involvement of PKC-α in LPS-mediated iNOS induction was further confirmed by increased iNOS expression in PKC-α overexpressing cells. NF-κB dependent transactivation by LPS was observed and PKC-α specific inhibitory peptide abolished this activation, indicating that NF-κB activation is dependent on PKC-α. **Conclusion:** Our data suggests that PKC-α is involved in LPS-mediated iNOS expression and that its downstream target is NF-κB. Although PKC-α is a crucial mediator in the iNOS regulation, other PKC isozymes may contribute LPS-stimulated iNOS expression. This finding is needed to be elucidated in further study.

Key words
Lipopolysaccharide, Inducible nitric oxide synthase, Protein kinase C, NF-κB

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria and has a dominant role in the host response to bacterial infection[1]. Macrophages, which are the cells in the front line of host defense, play a pivotal role in the cellular response to LPS[2]. Results from genetic and biochemical studies showed that toll-like receptor 4 is the LPS receptor and mediates LPS-induced activation of downstream signaling pathways and expression of inflammatory target genes[3]. Stimulation of macrophages with LPS results in the production of various cytokines such as tumor necrosis factor-α, interleukin-1 and interleukin-6, and pro-inflammatory lipid mediators such as prostaglandins and leukotrienes[4,5]. In addition, one of the key events in macrophage response to LPS stimuli is the expression of inducible nitric oxide synthase (iNOS) and the formation of NO, an important mediator involved in many host defense action in macrophages. These cytokines, lipid mediators, and NO participate in the innate immune response and may also lead to a harmful

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host response to bacterial infection. However, given the importance of iNOS in a variety of pathophysiological conditions, control of its expression and signaling events in response to LPS has been the subject of considerable investigation[6].

Protein kinase C (PKC) activation is one of the earliest events in a cascade leading to iNOS expression[7]. PKC is a family of phospholipids-dependent serine-threonine kinases which is subdivided into three categories. The classical calcium-dependent (PKC-α, βI/βII and γ) and novel calcium-independent (PKC-δ, -θ, -ε, and -η) isoforms of PKC are dependent on phosphatidylserine and diacylglycerol (DAG) for activation. PKCs have been implicated in LPS-dependent signaling and iNOS expression, although their role is somewhat confounding due to the potentially opposing effects of multiple PKC isozymes in the same cell type, and conflicting data resulting from alternate methods of inhibiting PKC activity[8,9]. Several lines of evidence suggest that PKC isozymes are involved in the LPS-induced iNOS expression. For example, using PKC isozyme specific antisense-oligonucleotides, it was revealed that PKC plays a key role in iNOS expression[10]. Macrophages treated with PKC-specific inhibitors have been shown to significantly lose their ability to make NO in response to LPS[9,11]. Furthermore, PKC-δ has been shown to mediate upregulation of iNOS expression in response to stimulation with LPS[12]. Which PKC isozymes are involved in iNOS expression and whether they play a positive or negative role in that regulation is most likely cell dependent and dependent on the complement of PKC isozymes present. Although it is known that LPS generates NO through iNOS during macrophage inflammatory response, the mechanism of NO production by PKC isozymes in macrophages remains to be further determined.

MATERIALS AND METHODS

Reagents and antibodies

LPS (*Escherichia. coli,* 0111:B4) was obtained from Sigma-Aldrich (St. Louis, MO); inhibitors of PKC isozymes and SN-50 were from Calbiochem (La Jolla, CA). For Western blot analysis, iNOS, IκBα and PKC-α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-PKC-α/βII antibody was from Cell Signaling Technology (Beverly, MA); β-actin antibody was from Sigma-Aldrich (St. Louis, MO). Peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody was used as secondary antibody.

Cell culture

The Raw264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 Medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 2 μM L-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37 ℃ in a humidified atmosphere under 5% CO_2. Cells were treated with LPS for the various times.

Western blot analysis

Macrophages were stimulated with LPS, washed twice in cold phosphate-buffered saline, and then lysed on ice with lysis solution (1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease inhibitor mixture). Sample protein concentrations were determined using the Bio-Rad protein assay. Proteins from the cell lysates were boiled at 95 ℃ in Laemmli SDS loading buffer, separated on 8% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were blocked for at least 30 min at room temperature in Tris-buffered saline plus 0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing primary antibody. For immunoblotting, incubation was performed for 4 h at room temperature. After five washes for 30 min in TTBS, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h, and after five washes for 30 min with TTBS, blots were detected by enhanced chemiluminescence using an image analyzer LAS-3000 (Fujifilm, Japan).

Total RNA isolation and RT-PCR

Total RNA was isolated from LPS-stimulated macrophages using Tri reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and was then reverse transcribed using a GeneAmp RNA PCR core kit (Applied Biosystems, Branchburg, NJ). The PCR was performed using the following primers: iNOS forward (5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′) and reverse (5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′, which generates a product of 497 bp), and the housekeeping gene β-actin forward (5′-TCC TTC

GTT GCC GGT CCA CA-3′) and reverse (5′-CGT CTC CGG AGT CCA TCA CA-3′, which generates a product of 509 bp).

Real-time RT-PCR

Assays for iNOS mRNA expression were done by real-time PCR using a Light Cycler 1.5 (Roche Diagnostics, Netherlands). Primers for murine iNOS were based on the sequence of the gene (Entrez-PubMed); forward primer, 5′-CAG CTG GGC TGT ACA AAC CTT-3′, reverse primer, 5′-CAT TGG AAG TGA AGC GTT TCG-3′. Synthesis of double-stranded DNA during PCR cycles was monitored using SYBR Green I (Roche Applied Science, Indianapolis, IN). The results of real-time PCR are presented as iNOS induction fold, and these were calculated using β-actin, which was amplified under the same conditions, as an internal control.

PKC-α overexpression

Using Lipofectamine 2000, PKC-α cDNA subcloned into the vector pMTH was transfected into macrophages along with pCIneo for selection against G418 containing medium. The parental vector pMTH/pCIneo was transfected as a negative control. Next day, the cells were moved in 400 μg/ml G418 containing medium for transfectant selection. After 7-10 days of culture, individual clones were isolated and examined for PKC-α expression by Western blotting.

Establishment of stable cell line containing NF-κB luciferase reporter construct

A promoter-reporter construct containing eight copies of the NF-κB element was transfected into macrophages using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection, cells were incubated in complete media for 24 h at 37 °C, and transfectants were selected under 400 μg/ml of G418. Established cells were stimulated with LPS for the indicated times at 37 °C. In some experiments, cells were preincubated with a specific inhibitor for 1 h at 37 °C prior to LPS stimulation. Cell lysates were assayed for luciferase activity using Promega buffer system (Promega, Madison, WI) and a luminometer according to the manufacturer's instructions.

RESULTS

LPS-mediated iNOS induction in murine macrophage, Raw264.7 cells

We examined the effect of LPS on the induction of iNOS in murine macrophage Raw264.7 cells. Macrophages were exposed to the indicated doses (1~1000 ng/ml) of LPS for 24 h and whole cell lysates were prepared from those cells. Western blot analysis showed that a concentration of LPS as low as 10 ng/ml could induce iNOS expression and NO production (Fig. 1A). To determine the kinetics of iNOS induction, cells were stimulated with LPS for various times. As shown in Fig. 1B, the induction of iNOS protein was observed after 6 h of LPS stimulation. Taken together, we found that macrophage stimulation with LPS caused iNOS induction, in a dose- and time-dependent manner. Next, the level of iNOS mRNA in LPS-stimulated cells was observed using RT-PCR to determine whether the induction of iNOS protein occurred after this transcriptional up-regulation. Levels of iNOS mRNA were significantly increased by LPS, which was consistent with the expression of iNOS protein, and its induction was also dose- and time-dependently related to iNOS expression (Fig. 1C & 1D). These data confirm that in Raw264.7 cells, iNOS gene expression is up-regulated by LPS at the transcriptional as well as translational levels.

Effect of PKC isozyme-specific inhibitory peptide on LPS-mediated iNOS induction

Since PKC isozymes are known to be involved in a variety of cellular responses, we examined which PKC isozymes are involved with LPS-induced iNOS expression. Prior to LPS stimulation, cells were pretreated with inhibitory peptides specific for PKC-α/β, PKC-ϵ, PKC-η, or PKC-ζ. Western blot analysis revealed that PKC-α/β inhibitory peptide, which is a pseudosubstrate of PKC-α/β strongly reduced LPS-induced iNOS expression, while PKC-ϵ specific peptide had mild inhibitory effect (Fig. 2A). On the contrary, pretreatment with PKC-ζ inhibitory peptides did not affect LPS-induced iNOS expression. In addition, pretreatment of PKC-β inhibitory peptide also did not affect LPS response (data not shown). Interestingly, pretreatment with PKC-η inhibitory peptide increased LPS-induced iNOS expression. Real-time PCR showed that these inhibitory peptides

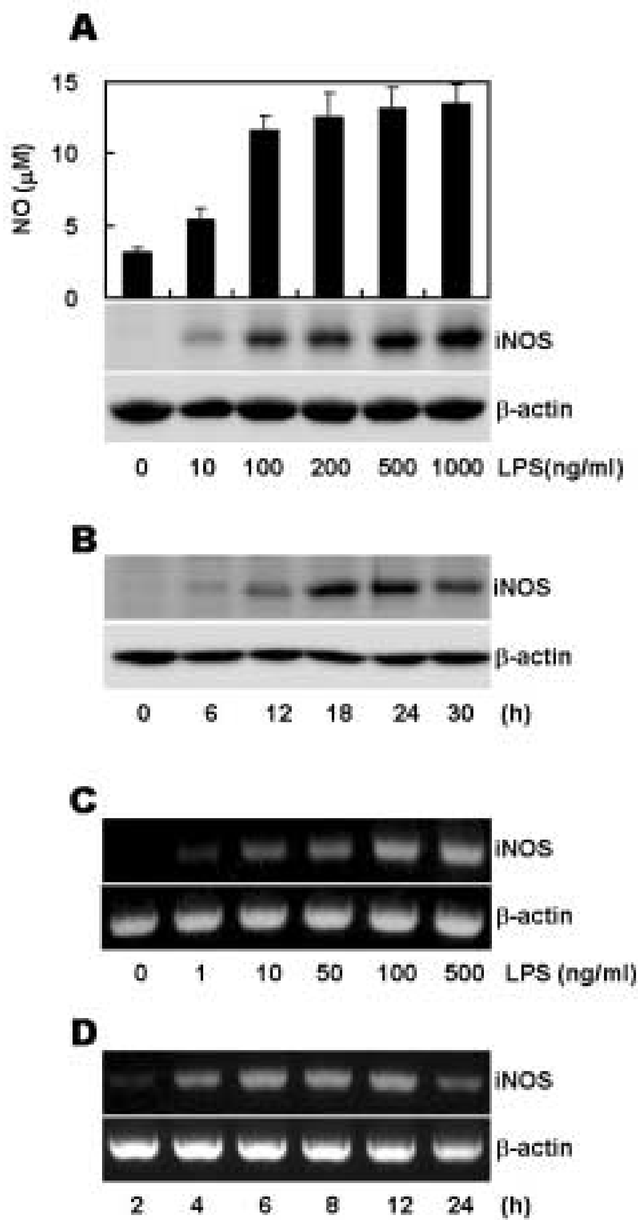

Fig. 1. LPS stimulates iNOS protein and mRNA expression in a dose- and time-dependent manner. A, Raw264.7 cells were cultured in a 6-well plate, subjected to overnight serum starvation, and stimulated with the indicated doses of LPS for 24 h. Whole cell lysates were analyzed by Western blot using anti-iNOS and anti-β-actin antibodies. B, Cells were stimulated with 100 ng/ml LPS for the indicated times and lysates were analyzed for iNOS and β-actin as in A. C & D, Total RNA was isolated from LPS-stimulated cells as indicated, and subjected to RT-PCR with specific primers for iNOS and β-actin.

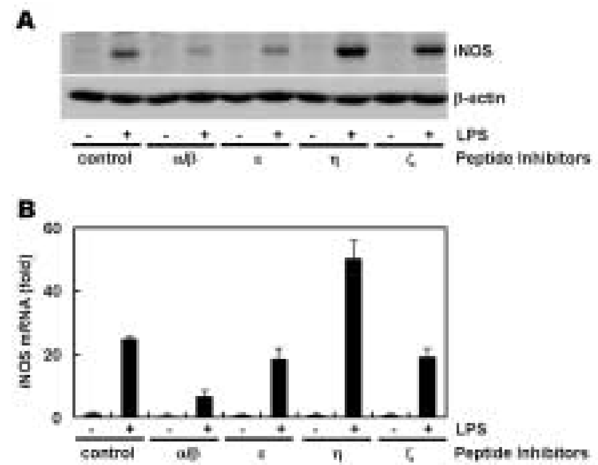

Fig. 2. PKC-α specific inhibitory peptide abolishes LPS-stimulated iNOS expression. A, Raw264.7 cells were pretreated with inhibitory peptides of each PKC isoform for 1 h and then stimulated with 100 ng/ml LPS for 24 h. Whole cell lysates were subjected to the Western blot analysis using anti-iNOS and anti-β-actin antibodies. B, Cells were pretreated with inhibitory peptides of each PKC isoform for 1 h and then stimulated with 100 ng/ml LPS for 6 h. Total RNA was isolated and subjected to quantitative real-time RT-PCR to determine iNOS mRNA levels calculated by normalizing versus β-actin.

had similar effects on the transcriptional regulation of the iNOS gene (Fig. 2B). PKC-α/β specific inhibitory peptide blocked the transcriptional up-regulation of LPS-mediated iNOS gene induction, whereas PKC-η inhibitory peptide increased iNOS mRNA levels, which were as same as the result of Western blot analysis. Therefore, we decided to focus on PKC-α and its role in LPS-mediated iNOS induction. Next, we examined LPS-mediated PKC-α phosphorylation to confirm that LPS elicits PKC-α activation to leading iNOS expression. Whole cell lysates from LPS-stimulated Raw264.7 cells for the indicated times were subjected to Western blot analysis using anti-phospho-PKC-α antibody. Phosphorylation of PKC-α was detected as early as 10 min, peaked at 15 min, and then declined to the baseline level (Fig. 3A). In addition, we used another approach to test for the involvement of PKC-α in LPS-stimulated iNOS induction by establishing PKC-α overexpressing Raw264.7 cells which were transfected with PKC-α cDNA encoding plasmid and then selected against G418 containing medium. Compared to control cells, which were transfected with pMTH and pCIneo plasmid only, PKC-α overexpressing cells

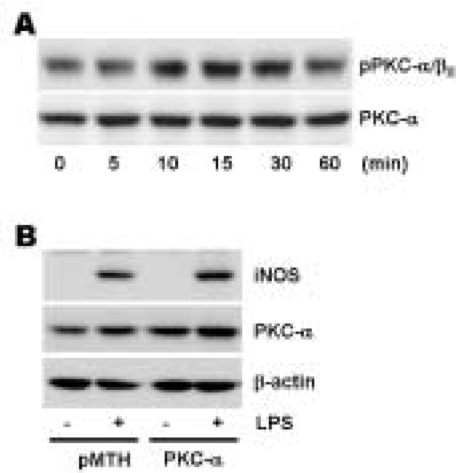

Fig. 3. The activation of PKC-*α* mediates LPS-induced iNOS expression. A, Cells were treated with LPS for the indicated times. To detect PKC-*α* phosphorylation, equals amount of cell extracts were analyzed by Western blotting using phospho-specific PKC-*α* antibody. As a loading control, the same blots were re-probed with anti-PKC-*α* antibody. B, Vector-transfected control cells or PKC-*α* overexpressing cells were stimulated with LPS for 24 h. Whole cell lysates were subjected to Western blotting using anti-iNOS and PKC-*α* antibodies.

showed substantially elevated iNOS expressions in response to LPS treatment (Fig. 3B), indicating that PKC-*α* is an important mediator of up-regulation of iNOS expression.

Requirement of NF-*κ*B activation in LPS-induced iNOS expression

Since NF-*κ*B has been proposed to be a key transcription factor in iNOS expression, we hypothesized that LPS might activate NF-*κ*B and result in the induction of iNOS expression. To examine NF-*κ*B activation by LPS, I*κ*B*α* degradation was observed by Western blot analysis using Raw264.7 cells stimulated with LPS for the indicated times. As shown in Fig. 4A, I*κ*B*α* phosphorylation and degradation was observed after 20 min of LPS stimulation and then paeked at 60 min. We also found that LPS-induced an increase in NF-*κ*B-dependent transactivation as determined by NF-*κ*B luciferase reporter assay. Raw264.7 cells that had been stably transfected with luciferase reporter construct containing eight consecutive copies of NF-*κ*B responsible elements were stimulated with different concentrations of LPS as indicated.

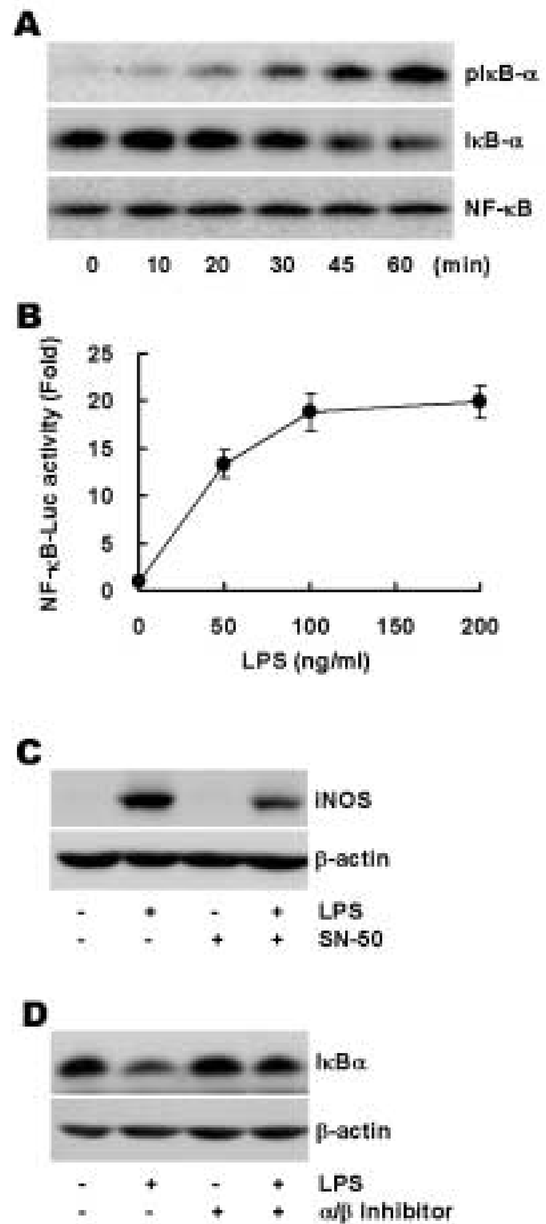

Fig. 4. LPS-induced iNOS expression depends on NF-*κ*B activation in macrophages. A, Cells were stimulated with LPS for the indicated times. Whole cell lysates were applied to Western blot analysis to detect I*κ*B*α* degradation. B, Cells stably transfected with NF-*κ*B luciferase reporter gene were stimulated with the indicated doses of LPS for 5 h. Luciferase activity was measured using whole cell lysates. C, Macrophages were pretreated with SN-50 for 1 h and stimulated with LPS for 18 h. Whole cells were analyzed by Western blotting using anti-iNOS and anti-*β*-actin antibodies. D, Cells were pretreated with PKC*α*/*β* inhibitor for 1 h and then stimulated with LPS for 30 min. Whole cells were analyzed by Western blotting using anti-I*κ*B*α* and anti-*β*-actin antibodies.

Luciferase activity at up to 200 ng/ml LPS was found to increase in a dose-dependent manner (Fig. 4B). NF-κB activation during LPS-stimulated iNOS induction was further determined using NF-κB inhibitor SN-50. Macrophages were preincubated with SN-50 and then stimulated with LPS. Western blotting showed that pretreatment with SN-50 significantly reduced LPS-induced iNOS expression at the translational levels (Fig. 4C), suggesting that NF-κB activation is required for LPS-induced iNOS expression. Since our data shows that LPS elicits both PKC-α and NF-κB activation, we investigated the possibility of a relationship between PKC-α and NF-κB, and found that PKC-α inhibition affected NF-κB activation. Macrophages were stimulated with LPS in the absence or presence of PKC-α specific inhibitory peptide. Western blot analysis showed that LPS-induced IκBα degradation was blocked by this pretreatment (Fig. 4D), indicating that NF-κB is a downstream target of PKC-α.

DISCUSSION

The studies presented here indicate that the administration of LPS increased iNOS expression in macrophages. The results of the present study suggest that LPS treatment increases the phosphorylation of PKC-α and the activation of NF-κB in macrophages. This suggestion is further supported by the finding that the effect of LPS on iNOS expression is inhibited by pretreating cells with selective PKC or NF-κB inhibitors. Taken together, these results show that LPS treatment increases iNOS expression, and suggest that PKC-α has a critical function in the regulation of signaling pathways that mediate iNOS expression and aspects of macrophage activation.

In the present study, we found that several kinds of PKC, i.e., PKC-α/βII, -δ, and -ζ/ι are phosphorylated in LPS-treated cells (data not shown). PKC belongs to a group of serine/threonine kinases and is known to play an integral role in many different signaling systems[13]. Many studies have provided data implicating the involvement of PKC in iNOS expression and NO release. For example, PKC was found to be required for LPS- or fMLP-induced iNOS expression and subsequent NO production in macrophages[9,14]. Pham et al[15]. reported that elevated serum NO levels in patients with inflammatory arthritis are associated with the co-expressions of iNOS and PKC-α in peripheral blood monocyte-derived macrophages. In addition, the significance of PKC was evident from the phenotype of PKC-α-/- mice, which exhibit both deficient iNOS induction and macrophage activation[16]. To address the involvement of PKC and identify the PKC isoform(s) involved in LPS-induced iNOS expression, we examined the effects of several inhibitory peptides of the different PKC isozymes; these inhibitory peptides are known to be highly specific and to have low side effects. Western blot analysis and real-time RT-PCR results showed that PKC-α/β inhibitory peptide strongly blocked LPS-induced iNOS expression and PKC-ε inhibitory peptide had mild inhibitory effect, while PKC-ζ inhibitory peptides did not affect LPS-induced iNOS expression. On the contrary, PKC-η inhibitory peptide increased LPS-induced iNOS expression.

PKC regulates various inflammatory responses in an isoenzyme-specific manner[17,18]. The regulation of cell signaling events by one PKC isoenzyme have also been shown to differ between cell types[19]. Distribution of PKC isozymes is cell type-specific. Among them, PKC-α, -βI, -βII, and PKC-δ seem to be ubiquitous enzymes, and are found in most cells including macrophages[18]. In the present study, various PKC inhibitors with different PKC isozyme profile were used to study the role of PKC, especially PKC-α, in LPS-induced iNOS expression and NO production in macrophages. The involvement of PKC-α in LPS-induced iNOS expression was confirmed by the observed overexpression of PKC-α in Raw264.7 cells. The induction of iNOS by LPS stimulation was increased, which were established to determine the constitutive expression of PKC-α. Consistent with this result, the level of iNOS mRNA and protein were also found to be increased in the same cells. Western blot analysis revealed that overexpression of PKC-α increased iNOS induction in response to LPS stimulation, suggesting that PKC-α is required for LPS-induced iNOS expression. Studies directed at examining the PKC isozymes activated in response to various stimulants suggest that the profile of PKC isozyme activation depends on tissue or cell type. However, our study suggests that the LPS-stimulated activation of PKC-α may play a critical role in the mediation of iNOS induction in macrophages.

We next investigated the events downstream of PKC-α activation. The iNOS promoter region has been extensively studied and is known to have several responsive elements for various transcription factors, thus suggesting that many factors may regulate iNOS expression. Moreover, several studies have reported that the activation of NF-κB can mediate the transcription of iNOS, and

that NF-κB comprises inducible transcription factors that serve as important regulators in inflammation[20]. Since two NF-κB binding sites have been identified in the iNOS promoter region, we examined the possibility of NF-κB involvement in the induction of iNOS gene expression. In unstimulated cells, NF-κB is found as an inactive heterodimer bound to its inhibitory protein, I?B. Upon stimulation, IκB is phosphorylated on specific serine residues, which are targets for IκB degradation in an ubiquitin-dependent manner. The liberated and activated NF-κB is then translocated to the nucleus for gene regulation[21]. To address NF-κB activation, we first examined the phosphorylation and the following degradation of IκBβ in LPS-stimulated Raw264.7 cells. Our finding that LPS induces NF-κB activity under these conditions makes the hypothesis that NF-κB regulates iNOS expression plausible, and concurs with previous studies[22,23]. Ghosh and Karin[24] demonstrate that NF-κB activation and provide information about its phosphorylation status. In conjunction with the results of NF-κB luciferase activity and IκBβ degradation, the above results indicate that NF-κB plays a critical role in the regulation of iNOS induction in LPS-activated macrophages. Moreover, stable cells harboring NF-κB luciferase reporter construct showed NF-κB dependent transactivation, and the luciferase activities of LPS-stimulated Raw264.7 cells were increased in dose-dependent manner. The NF-κB inhibitor SN-50 also exerted a suppressive effect on LPS-induced iNOS protein and mRNA expressions, confirming the requirement of NF-κB in the induction of iNOS gene expression. Our next question was whether there exists a relationship between PKC-α and NF-κB, since both mediators are activated in response to LPS stimulation. We found that pretreatment with the PKC-α specific inhibitor blocked LPS-induced IκBβ degradation, indicating that PKC-α is upstream module of NF-κB.

In conclusion, the present study shows that LPS treatment can result in the activation of PKC-α and subsequently induce NF-κB-dependent iNOS expression in macrophage. This increase in iNOS is further associated with macrophage activation to inflammatory responses. Although PKC-α plays a central role in the iNOS regulation, a number of PKC isozymes such as PKC-ε and PKC-η may be involved in LPS-induced iNOS expression. This finding is needed to be elucidated in further study.

REFERENCES

1. Yu L, Ding JL, Ho B, Wohland T: Investigation of a novel artificial antimicrobial peptide by fluorescence correlation spectroscopy: an amphipathic cationic pattern is sufficient for selective binding to bacterial type membranes and antimicrobial activity. Biochim Biophys Acta 2005;1716:29-39.
2. Kakishima K, Shiratsuchi A, Taoka A, Nakanishi Y, Fukumori Y: Participation of nitric oxide reductase in survival of Pseudomonas aeruginosa in LPS-activated macrophages. Biochem Biophys Res Commun 2007; 355:587-91.
3. Butchar JP, Parsa KV, Marsh CB, Tridandapani S: Negative regulators of toll-like receptor 4-mediated macrophage inflammatory response. Curr Pharm Des 2006;12:4143-53.
4. Huang H, Park CK, Ryu JY, Chang EJ, Lee Y, Kang SS, et al.: Expression profiling of lipopolysaccharide target genes in RAW264.7 cells by oligonucleotide microarray analyses. Arch Pharm Res 2006;29:890-7.
5. Rossi A, Acquaviva AM, Iuliano F, Di Paola R, Cuzzocrea S, Sautebin L: Up-regulation of prostaglandin biosynthesis by leukotriene C4 in elicited mice peritoneal macrophages activated with lipopolysaccharide/interferon-{gamma}. J Leukoc Biol 2005;78:985-91.
6. Kleinert H, Schwarz PM, Forstermann U: Regulation of the expression of inducible nitric oxide synthase. Biol Chem 2003;384:1343-64.
7. Ramzy D, Rao V, Tumiati LC, Xu N, Sheshgiri R, Miriuka S, et al.: Elevated endothelin-1 levels impair nitric oxide homeostasis through a PKC-dependent pathway. Circulation 2006;114:I319-26.
8. Greenberg SS, Jie O, Zhao X, Wang JF: Role of PKC and tyrosine kinase in ethanol-mediated inhibition of LPS-inducible nitric oxide synthase. Alcohol 1998;16:167-75.
9. Salonen T, Sareila O, Jalonen U, Kankaanranta H, Tuominen R, Moilanen E: Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages. Br J Pharmacol 2006;147:790-9.
10. Chen CC, Wang JK, Lin SB: Antisense oligonucleotides targeting protein kinase C-alpha, -beta I, or -delta but not -eta inhibit lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages: involvement of a nuclear factor kappa B-dependent mechanism. J Immunol 1998;161:6206-14.
11. Hamel C, Millette E, Lamontagne D: Role of nitric oxide and protein kinase C in the tachyphylaxis to vasopressin in rat aortic rings. Life Sci 2005;77:1069-81.
12. Tepperman BL, Chang Q, Soper BD: Protein kinase C mediates lipopolysaccharide- and phorbol-induced nitric-oxide synthase activity and cellular injury in the rat colon. J Pharmacol Exp Ther 2000;295:1249-57.
13. Ron D, Kazanietz MG: New insights into the regulation of protein kinase C and novel phorbol ester receptors. Faseb J 1999;13:1658-76.
14. Sodhi A, Biswas SK: fMLP-induced in vitro nitric oxide production and its regulation in murine peritoneal macrophages. J Leukoc Biol 2002;71:262-70.
15. Pham TN, Rahman P, Tobin YM, Khraishi MM, Hamilton SF, Alderdice C, et al.: Elevated serum nitric oxide levels in patients with inflammatory arthritis associated with co-expression of inducible nitric oxide synthase and protein kinase C-eta in peripheral blood monocyte-derived macrophages. J Rheumatol 2003;30:2529-34.
16. Littler CM, Wehling CA, Wick MJ, Fagan KA, Cool CD, Messing RO, et al.: Divergent contractile and structural responses of the murine PKC-epsilon null pulmonary circula-

tion to chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 2005;289:L1083-93.

17. Nechushtan H, Razin E: Studies of different aspects of the role of protein kinase C in mast cells. Int Arch Allergy Immunol 2001;124:130-2.
18. Tan SL, Parker PJ: Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem J 2003;376:545-52.
19. Kanashiro CA, Khalil RA: Signal transduction by protein kinase C in mammalian cells. Clin Exp Pharmacol Physiol 1998;25:974-85.
20. Feng X, Guo Z, Nourbakhsh M, Hauser H, Ganster R, Shao L, et al.: Identification of a negative response element in the human inducible nitric-oxide synthase (hiNOS) promoter: The role of NF-kappa B-repressing factor (NRF) in basal repression of the hiNOS gene. Proc Natl Acad Sci U S A 2002;99:14212-7.
21. Wang T, Zhang X, Li JJ: The role of NF-kappaB in the regulation of cell stress responses. Int Immunopharmacol 2002;2:1509-20.
22. Maestre N, Bezombes C, Plo I, Levade T, Lavelle F, Laurent G, et al.: Phosphatidylcholine-derived phosphatidic acid and diacylglycerol are involved in the signaling pathways activated by docetaxel. J Exp Ther Oncol 2003;3:36-46.
23. Plo I, Lautier D, Levade T, Sekouri H, Jaffrezou JP, Laurent G, et al.: Phosphatidylcholine-specific phospholipase C and phospholipase D are respectively implicated in mitogen-activated protein kinase and nuclear factor kappaB activation in tumour-necrosis-factor-alpha-treated immature acute-myeloid-leukaemia cells. Biochem J 2000;351 Pt 2:459-67.
24. Ghosh S, Karin M: Missing pieces in the NF-kappaB puzzle. Cell 2002;109 Suppl:S81-96.