Opposite effects of wortmannin and LY294002 on TLR-mediated nitric oxide production: negative regulation of NF-κB by phosphoinositide 3-kinase

Kaoru Hazeki, Sachiko Kinoshita, Takayo Matsumura, Kiyomi Nigorikawa, Hiroshi Kubo, and Osamu Hazeki

Division of Molecular Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima 734-8551, Japan.
Running title: Negative Regulation of NF-κB by PI3-kinase

Correspondence: Kaoru Hazeki, Division of Molecular Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima 734-8551, Japan.
Fax: +81-82-257-5309, Tel: +81-82-257-5308
e-mail: khazeki@hiroshima-u.ac.jp

Text pages: 26
Tables: 0
Figures: 10
References: 30
Words in the Abstract: 187
Words in the Introduction: 696
Words in the Discussion: 893

Abbreviations: PI3K, Phosphoinositide 3-kinase; iNOS, inducible NO synthase; shRNA; short hairpin RNA; TNF, tumor necrosis factor; Poly (I:C), polyinosinic-polycytidylic acid; CpG DNA, non-methylated CpG oligodeoxynucleotide; PBMC, peripheral blood mononuclear cells; MAPK, mitogen-activated protein kinase,
ABSTRACT

A number of previous studies have suggested the involvement of phosphoinositide 3-kinase (PI3K) in TLR signaling. However, there have also been a number of conflicting reports. The PI3K inhibitor, wortmannin, greatly enhanced TLR-mediated inducible nitric oxide synthase (iNOS) expression and cytokine production in the mouse macrophage cell line, Raw264.7. The effect of wortmannin was common to TLR2, 3, 4, and 9, and was accompanied by activation of NF-κB and up-regulation of cytokine mRNA production. Surprisingly, another PI3K inhibitor, LY294002, strongly suppressed the production of iNOS and cytokines. This effect of LY294002 was based on its inhibitory effect on mRNA synthesis. Expression of dominant negative mutants of PI3K in macrophages augmented the LPS-induced expression of iNOS. Introduction of a pH1 vector producing short hairpin RNA (shRNA) that targets a catalytic subunit of PI3K (p110β) also enhanced the TLR-mediated responses. Thus, the augmentation of TLR signals by wortmannin was mediated through inhibition of PI3K, while the effect of LY294002 was not explained by its effect on PI3K. These discrepancies in the effects of pharmacological inhibitors in TLR-signaling may have caused confusion regarding the role of PI3K in innate immunity.
Introduction

Innate immune reactions are triggered through Toll-like receptors (TLRs) that recognize a variety of microbial products, such as lipopolysaccharide (LPS), peptidoglycans, flagellin, and unmethylated CpG motifs in bacterial DNA (Akira, 2003; Akira and Takeda, 2004). Recognition leads to production of various cytokines and nitric oxide (NO). The mammalian family of TLRs has now been expanded to 11 members (Akira, 2003; Akira and Takeda, 2004). TLR signaling begins from the Toll-interleukin-1 receptor (TIR)-domain, a motif of ~200 amino acids found in the cytosolic regions of all TLRs and the IL-1 receptor. Adaptor molecules, which also contain the TIR domain, bind to the TIR domain of TLRs due to TIR/TIR interaction on stimulation (Akira, 2003; Akira and Takeda, 2004). At present, the TLR adaptor family consists of 4 members: MyD88, TIRAP/Mal, TICAM-1/TRIF, and TICAM-2/TRAM. Studies in mice lacking each of these individual adaptors have revealed their specific roles. MyD88 is shared by all TLRs except TLR3, while TIRAP/Mal binds to TLR2 and TLR4 (Yamamoto et al., 2004). Likewise, TICAM-1/TRIF binds to TLR3 and TLR4, while TICAM-2/TRAM binds only to TLR4 (Oshiumi et al., 2003; Yamamoto et al., 2003). The diversity of the adaptor molecules may account, at least in part, for the variety of inflammatory responses to different ligands.

Mammalian PI3K can be grouped into three major classes based on primary sequence, mechanism of regulation, and substrate specificity (Wymann and Pirola, 1998). Class Ia PI3Ks are heterodimers consisting of a catalytic subunit (p110) and a regulatory subunit (p85). These enzymes are thought to be the major source of PI-3, 4, 5-P₃ in vivo on activation of receptor type protein tyrosine kinases. Mammals have multiple isoforms of class Ia PI3K (Fruman et al., 1998). Different genes encode class Ia catalytic subunits, termed p110α, p110β, and p110δ, while two
genes encode the associated regulatory subunits, termed p85α and p85β. Targeted disruption of either p110α or p110β causes death in the early stages of embryonic development. Likewise, targeted disruption of all p85α and p85β gene products is lethal (Brachmann et al., 2005). The regulatory p85α and p85β can compensate for each other because mice lacking either p85α or p85β have been generated successfully (Wymann et al., 2003).

There is increasing evidence of the involvement of PI3K in TLR signaling (Fukao and Koyasu, 2003). Binding of tyrosine-phosphorylated TLR2 (Arbibe et al., 2000), TLR3 (Sarkar et al., 2004), MyD88 (Ojaniemi et al., 2003), and TRIF (Aksoy et al., 2005) to PI3K have been reported to cause activation of this lipid kinase and its downstream target, PKB/Akt. However, there have been conflicting reports on the physiological consequences of this phenomenon. The inhibition of PI3K was reported to result in the inhibition of TLR2-mediated activation of NF-κB (Arbibe et al., 2000) or TLR3-mediated activation of IRF-3 (Sarkar et al., 2004). Activation of PI3K is also needed for LPS-induced IL-1β expression (Ojaniemi et al., 2003). PI3K activation is indispensable for LPS- (Manna and Aggarwal, 2000) and CpG- (Ishii et al., 2002) induced activation of NF-κB. These reports indicated a positive regulatory role of PI3K in TLR signaling.

In contrast, several lines of evidence suggest negative regulation by PI3K. LPS-induced NO production is increased in the presence of PI3K inhibitors (Diaz-Guerra et al., 1999). Gene targeting of the regulatory subunit of PI3K (p85α), as well as treatment with PI3K inhibitors, resulted in augmentation of LPS-induced IL-12 production (Fukao et al., 2002). TRIF-dependent NF-κB activation and IFN-β synthesis are enhanced by PI3K inhibition (Aksoy et al., 2005). Most of these studies employed pharmacological tools, wortmannin and LY294002, to inhibit PI3K.

Although several studies have utilized mutants of PI3K and Akt to allow more specific analysis, most have determined the effects of the mutants by transcription factor reporter assay under
artificial conditions. Here, we determined the effects of shRNA probe targeting p110β on TLR signaling in a mouse macrophage cell line. We also examined the effects of dominant negative mutants of PI3K on TLR-mediated NO production in macrophages. The results indicated that PI3K is a negative regulator in TLR-mediated NF-κB activation. Pharmacological data with wortmannin supported the conclusion. In contrast, LY294002 inhibited the TLR signaling in a manner independent of its action on PI3K.

**Materials and Methods**

**Reagents.** Materials were obtained from the following sources: LPS (*E. coli* serotype 0111: B4), bovine serum albumin (fatty acid-free), lipoteichoic acid (*Staphylococcus aureus*), and protein inhibitor cocktail from Sigma; IL-12p40 and IL-6 ELISA kits from Genzyme (Minneapolis, MN) and Amersham Pharmacia Biotech (Buckinghamshire, UK), respectively; Griess Romijin Nitrite Reagent from Wako (Tokyo, Japan); LY294002 from Cayman Chemical (Ann Arbor, MI); wortmannin from Kyowa Medex (Tokyo, Japan); peptidoglycan (*Staphylococcus aureus*) from Fluka (Riedel-deHaën, Switzerland); Poly I:C from Amersham Biosciences (Piscataway, NJ); CpG DNA (Cy3-oligo with the sequence: TCC ATG ACG TTC CTG ATG CT) was synthesized by Hokkaido System Science (Sapporo, Japan); antibodies to phospho-Akt (Thr308), phospho-Erk1/2 (Thr202/Tyr204), and iNOS from Cell Signaling (Beverly, MA); anti-phospho-IκB (Ser32) from Santa Cruz (Santa Cruz, CA); rGM-CSF from Pepro Tech EC (London, UK); protein assay kit from Bio-Rad (Hercules, CA); a synthetic lipopeptide based on the full-length macrophage activating lipopeptide-2kDa (Malp-2) was prepared with dipalmitoyl-S-glyceryl cysteine, as described previously (Nishiguchi et al., 2001).
Cells. Human macrophages were prepared from peripheral blood monocytes as described elsewhere (Nishiguchi et al., 2001). Briefly, aliquots of 400 ml of citrate-phosphate dextrose-supplemented human blood were separated by methylcellulose sedimentation and density-gradient centrifugation with Ficoll-Paque to obtain peripheral blood mononuclear cells. Monocytes were isolated from these peripheral blood mononuclear cells with a magnetic cell sorting system using anti-CD14-coated microbeads and cultured for 7–8 days in RPMI1640 medium supplemented with 10% heat-inactivated FCS and 50 IU/ml of rGM-CSF. Raw 264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium fortified with glucose to a concentration of 4.5 g/l and 10% FCS. The cells were treated with or without inhibitors for 15 min except where otherwise specified and stimulated with various TLR ligands. 293T cells were maintained in Dulbecco’s modified Eagle’s medium (D’MEM; Nissui, Tokyo, Japan) supplemented with 10% FCS.

Plasmids. A plasmid encoding a dominant negative mutant of bovine p85 (pCMV5-flag-Δp85) and the pH1 vector (Sasai et al., 2005) for expression of short hairpin RNAs were kindly provided by Dr. M. Kasuga (Kobe University), and Dr. N. Inoue (Osaka Medical Center for Cancer and Cardiovascular Diseases), respectively. A deletion mutant of p85cSH2 (1495-end) and catalytically active PI3K p110α subunit (p110αCAAT) were generated by PCR. Mammalian expression vectors containing N-terminal flag-tagged TLR2 and TLR6 were from Dr. T. Seya (Hokkaido University).

Cytokine assay. Untreated human macrophages or those treated with inhibitors for 15 min in 96-well plates (5×10⁴ cells/well) were stimulated with LPS, peptidoglycan, or MALP-2 for 18 h. The concentrations of IL-12p40 and IL-6 in the supernatants were measured by ELISA.
**Western blotting.** Raw 264.7 cells in 24-well plates were treated with inhibitors or stimuli and lysed in 50 µl of lysis buffer containing 25 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na3VO4), 1 mM EDTA, 0.1% BSA, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 20 µM p-amidinophenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide. The cell lysates were centrifuged at 15,000 rpm for 10 min. Proteins in the resultant supernatants were determined using a Bio-Rad assay kit. Total cell lysates (100 µg protein) were mixed with 10 µl of ×5 sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue as final concentrations), and heated at 100°C for 3 min. The proteins were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked in 5% skim milk, and incubated with appropriate antibodies. The associated antibodies were located by enhanced chemiluminescence (Perkin-Elmer, Norwalk, CT).

**Nitric oxide production.** Raw 264.7 cells in 96-well plates were treated with inhibitors and stimulated with various TLR ligands. The culture supernatants (50 or 100 µl) were transferred to 96-well assay plates, mixed with Griess reagent, and incubated for 15 min at room temperature. The absorbance (520 nm) of the reaction in each well was measured using a plate reader.

**RNA isolation and RT-PCR.** Total RNA was isolated with an RNeasy kit (Qiagen, Hilden, Germany). Single-stranded cDNA was synthesized with M-MLV reverse transcriptase. The cDNAs for iNOS, β-actin, and IFN-β were amplified by a PCR-based method using the following specific primers: iNOS (sense) 5’-TGGGAATGGAGACTGT CCCAG-3’ and (antisense) 5’-GGGATCTGAATGTGATGTTTG-3’; β-actin (sense) 5’-atcatgtttgagaccttcaacacc-3’ and...
MOL21162

(antisense) 5’-gatgtcacgcagattccc-3’; IFN-β (sense) 5’-TTCCTGCTGTGCTTCTCCAC-3’ and
(antisense) 5’-GATTCGATTACCCAGTCCAGGTC-3’.

**Reporter gene assay for NF-κB.** HEK293T cells in 24-well plates were transfected with
25 ng of NF-κB reporter plasmid (Stratagene, La Jolla, CA), 2 ng of sea pansy luciferase reporter
plasmid (pRL-SV40 vector; Promega, Madison, WI) as an internal control, along with 2 ng of
TLR4 and 0.3 ng of MD-2 using FuGENE 6 (Roche, Indianapolis, IN). Twenty-four hours after
transfection, cells were treated with or without wortmannin for 15 min and stimulated with LPS for
6 h. Luciferase activity in the cell lysate was measured with Dual-Luciferase Reporter Assay
System (Promega).

**RNA Interference.** Two sequences, 5’-gaagcagccggtttat-3’ and
5’-GCAGGGATTAAAGTGTGAA-3’, were selected to interfere with p110β expression. For each
of these sequences, a pair of oligonucleotides were synthesized with sequences
5’-CCC(X)19TTCAAGAGA(Y)19TTTTTGAAA-3’
and
5’-CTAGTTTCCAAAA(Y)19TCTCTTGAA(X)19GGGTGCA, where (X)19 is the coding
sequence and (Y)19 is the complementary sequence. The oligonucleotide pairs were annealed and
ligated downstream of the H1-RNA promoter at the *Pst*I and *Xba*I sites of the pH1 vector. Both
vectors were transfected into Raw264.7 cells (5×10⁶ cells) by electroporation in a final volume of
300 µL at 250 V/950 µF (Gene Pulser II, Bio-Rad).

**Results**

**Effects of PI3K inhibitors on TLR-mediated IL-6 production in human macrophages.**

Human monocyte-derived macrophages were treated with increasing concentrations of
wortmannin or LY294002, followed by stimulation with TLR-ligands, and the accumulation of IL-6 in the culture medium was determined. As shown in Fig. 1, a TLR4 ligand, LPS, markedly increased the level of IL-6 production by the cells. Wortmannin augmented the effect of LPS in a dose-dependent manner with an EC$_{50}$ value around 50 nM (Fig. 1A). In contrast, another inhibitor of PI3K, LY294002, completely inhibited the TLR4-mediated event (Fig. 1B). TLR2 ligands, Malp2 and peptidoglycan, were effective in inducing IL-6 production by the cells, although their effects were weaker than that of LPS. The TLR2-mediated IL-6 production was again markedly increased in the presence of wortmannin (Fig. 1A). LY294002 completely suppressed the TLR2-mediated cytokine production (Fig. 1B). Similar results were obtained when the effects of the PI3K inhibitors on the TLR2- or TLR4-mediated IL12p40 production were examined (data not shown).

**Effects of PI3K inhibitors on LPS-induced iNOS expression in mouse macrophages.**

The mouse macrophage cell line, Raw 264.7, was treated with wortmannin, stimulated with various concentrations of LPS and the expression of iNOS was determined by Western blotting (Fig. 2A). Wortmannin markedly enhanced the LPS-induced iNOS expression. In the presence of 0.3 µM wortmannin, LPS-induced iNOS production saturated at LPS concentrations higher than 10 ng/ml. Wortmannin also enhanced LPS-induced nitrite accumulation well (Fig. 2B). Thus, wortmannin appeared to enhance TLR-mediated events in both human (Fig. 1) and mouse macrophages. The enhancing effect of wortmannin was prominent when lower concentrations of LPS were utilized (Fig. 2B). However, the effect was minimal at higher concentrations of LPS, because accumulation of nitrite saturated at 10 ng/ml LPS. Therefore, in subsequent experiments we used a suboptimal concentration of LPS to examine the enhancing effect of wortmannin

**Effects of wortmannin on iNOS expression induced by various TLR ligands.** The potentiation of iNOS expression by wortmannin was observed when macrophages were stimulated
with peptidoglycan (TLR1 and 2), Malp2 (TLR2 and 6), Poly I:C (TLR3), lipoteichoic acid (TLR4), or CpG (TLR9) (Fig. 3). These TLRs associate with different adaptors; TLR1, with MyD88; TLR2, with MyD88/Mal; TLR6, with MyD88; TLR3, with TRIF; TLR4 with MyD88/Mal/TRIF/TIRAM; and TLR9 with MyD88. Therefore, it is very likely that the negative feedback regulation by PI3K is common to most TLRs. PI3K may modify the function of a protein component common to all the adaptors.

**Effects of coexistence of wortmannin and LY294002 on LPS-induced iNOS expression.** The inhibitory effect of LY294002 was obvious in LPS-induced iNOS expression in Raw 264.7 cells (Fig. 4A, middle panel) indicating that the inhibition was also common to human and mouse macrophages. Interestingly, even when the cells were activated by both LPS and wortmannin, LY294002 effectively inhibited iNOS expression (Fig. 4A, lower panel). It is likely that the inhibitory action of LY294002 and the opposite action of wortmannin are exhibited via independent signaling pathways. It was also speculated that the target molecule of LY294002 functions downstream of the target of wortmannin, because the effect of wortmannin was abolished completely in the presence of LY294002. LPS-induced accumulation of phosphorylated Akt (pAkt), as well as the basal pAkt, was similarly inhibited by these PI3K inhibitors despite their opposite effects on iNOS expression (Fig. 4B).

**Effects of PI3K inhibitors on mRNA synthesis induced by LPS.** In agreement with iNOS protein expression, LPS-induced iNOS mRNA expression was markedly increased by wortmannin and inhibited by LY294002 (Fig. 5A). Similar results were obtained in experiments to examine LPS-induced expression of IFN-β mRNA (Fig. 5B). It has been suggested that both MyD88-dependent and TRIF-dependent pathways result in the activation of NF-κB, while only the
TRIF-dependent pathway is able to activate IFN-β gene expression. As wortmannin enhanced IL-6, IL-12, iNOS, and IFN-β production on LPS challenge, the inhibitor could modify both MyD88-dependent and TRIF-dependent pathways. LY294002 inhibited TRIF-dependent increase in IFN-β mRNA (Fig. 5B, left panels) as well as other TLR-mediated responses.

Enhancement of TLR-mediated iNOS expression by PI3K mutants. We next investigated the effects of dominant negative mutants of PI3K to elucidate the role of PI3K in TLR signaling. Both p110α and p110β are known to be complexed with the common regulatory subunit, p85, the absence of which markedly impairs the stability of the catalytic subunits in cells (Brachmann et al., 2005; Yu et al., 1998). Binding of tyrosine-phosphorylated proteins to SH2 domains in p85 is one of the mechanisms leading to p110 activation. It has been demonstrated in several cell lines that Δp85, a mutant p85 regulatory subunit lacking the binding site to p110, inhibits class Ia PI3K-dependent cellular events (Hara et al., 1994). Likewise, a truncated form of p85 (p85cSH2; 1495-end), possessing the SH2 domain but lacking all other functional domains, is also expected to behave as a dominant negative mutant. The mutants were prepared as flag-tagged constructs, and transfected into Raw264.7 cells by electroporation. Expression of the mutants was first checked using anti-flag antibody. The expression of p85cSH2 was easily detected (about 35 kDa, Fig. 6A), but the expression of Δp85 was hardly detectable when the whole-cell lysates were used for the assay. However, the expression of Δp85 was clearly detected when the lysates were subjected to immunoprecipitation with anti-p85 antibody and the immune complex was examined with anti-flag antibody (Fig. 6B upper panel).

Transfection of Δp85 or p85cSH2 impaired the Akt activation induced by LPS (Fig. 6C, upper panel). In contrast, the effect of calyculin A (CA), a pan-inhibitor of Ser/Thr phosphatases, was not susceptible to expression of the dominant negative mutants of PI3K (Fig. 6C, upper panel).
Transfection of the empty vector did not cause any effects on the LPS-induced Akt phosphorylation (data not shown). The effects of the mutants were specific to the PI3K-dependent pathway in the sense that LPS-induced Erk1/2 activation was not affected by the mutants (Fig. 6C, lower panel). The amounts of p67phox protein were almost unchanged among the samples, showing that equal amounts of cellular protein were applied to SDS-PAGE (Fig. 6C, lower panel). Interestingly, transfection of the dominant negative PI3K resulted in enhancement of LPS-induced iNOS expression (Fig. 6D, E). Wortmannin, which greatly enhanced the action of LPS in normal untransfected cells, produced no further increase in the effects of mutants. Similar results were observed in LPS-induced nitrite production (data not shown). It is likely that the endogenous PI3K was severely impaired even in the absence of wortmannin in cells transfected with the dominant negative mutants. These results suggested that LPS-induced iNOS expression was suppressed by endogenous PI3K in normal cells.

The role of PI3K was further examined by introduction of shRNA vectors targeting p110β to Raw264.7. The cell lysates were immunoprecipitated with anti-p85 antibody and detected by Western blotting with anti-p110β antibody (Fig. 6B, lower panel). The amount of p110β was decreased by introduction of the shRNA vectors. Depletion of p110β resulted in enhancement of the LPS-mediated iNOS expression as observed in cells transfected with the dominant negative mutants (Fig. 6D). Again, the effect was not further increased by the addition of wortmannin (Fig. 6D). Thus, class Ia PI3K, at least p110β, play a role in the negative regulation of TLR signaling.

**Effects of wortmannin on stability of iNOS mRNA.** To elucidate the mechanism by which PI3K inhibits the LPS-induced iNOS expression, wortmannin was added at several times before and after LPS challenge (Fig. 7). Wortmannin was highly effective even when added at 60 min before LPS. The effect decreased gradually when added after LPS challenge until almost no
increase was observed. It is important to note that the enhancement was still remarkable when wortmannin was added several minutes after stimulation with LPS. It is likely that the inhibition of PI3K may not contribute to strengthening of the proximal signal of TLR. The inhibition of LPS-induced iNOS expression by LY294002 was also observed even when the inhibitor was added 90 min after LPS addition (data not shown).

It has been reported that LPS increases cyclooxygenase 2 production, which is enhanced by inhibition of PI3K (Monick et al., 2002). In this case, the inhibition of PI3K has been reported to stabilize cyclooxygenase 2 mRNA. Therefore, we next measured the rate of iNOS mRNA degradation with respect to wortmannin treatment. For these experiments, cells were first treated with or without wortmannin for 15 min, followed by addition of LPS, and cultured for 6 h. At 6 h, actinomycin D was added to the culture medium and the total RNA was harvested at 0 (without actinomycin), 6, and 12 h after addition. The initial level (time 0) of iNOS mRNA was higher in the wortmannin-treated cells (Fig. 8), but the degradation rate was the same regardless of the wortmannin treatment (Fig. 8). Thus, wortmannin-induced increases of mRNA levels were not due to inhibition of degradation.

**Effects of PI3K inhibitors on NF-κB activation.** iNOS mRNA synthesis is increased by activation of NF-κB. To examine the effect of wortmannin in NF-κB activation, we performed a reporter assay with 293T cells. The cells were transfected with TLR2, TLR6 along with NF-κB-luciferase reporter plasmid and control plasmid. NF-κB-dependent induction of the luciferase reporter gene was activated by 7-fold upon addition of Malp2, which was further increased in the presence of wortmannin (Fig. 9A). An NF-κB-independent expression of seapansy luciferase from the control plasmid was unaffected at all at 0.1 µM wortmannin, but the expression was decreased by around 50 % at 1 µM (data not shown). LY294002 impaired dramatically the
transcriptional activity of the NF-κB reporter plasmid. The inhibitor was considered to possess a general inhibitory effect on promoter activities, because it impaired also the transcriptional activity of NF-κB-independent plasmids including phRL-TK, phRL-SV40 and phRL-NF-E2 (data not shown).

We also carried out reporter assays by overexpressing PI3K mutants. A dominant negative form of PI3K, Δp85, increased Malp2-induced and NF-κB-dependent induction of reporter gene (Fig. 9B, left) while catalytically active p110α subunit inhibited the NF-κB activation (Fig. 9B, right). The results supported the idea that PI3K negatively regulates TLR-mediated NF-κB activation.

**Effects of PI3K inhibitors on IκB degradation.** Activation of NF-κB is known to accompany the degradation of IκB, which binds NF-κB in the cytoplasm and thereby inhibits nuclear translocation of the transcription factor. Exposure of Raw264.7 to LPS led to degradation of IκB, which was enhanced by wortmannin (Fig. 10). Interestingly, LY294002 also increased the IκB degradation. These observations support the suggestion that PI3K negatively regulates TLR-mediated IκB degradation because both LY294002 and wortmannin showed similar inhibitory effects on PI3K (Fig. 4B). The inhibitory effect of LY294002 on nitrite production and cytokine production (Figs. 1, 4, 5) may be due to its effects on some protein(s) functioning downstream of the transcription factor.

**Discussion**

There is increasing evidence for the involvement of PI3K in TLR signaling. However, the role of PI3K is still unclear. Here, we presented direct evidence that PI3K, at least p110β, negatively regulates LPS-induced iNOS expression (Fig. 6). Our results using dominant negative mutants of
the regulatory subunit of PI3K also supported the suggestion that class Ia PI3K negatively regulates LPS-induced iNOS expression (Fig. 6). Pharmacological observations suggested that the negative feedback regulation by PI3K is common to most TLRs (Fig. 3).

Using pharmacological methods with dendritic cells and reporter assay with HEK293T cells, Aksoy et al. showed that inhibition of PI3K enhances TLR3- and TLR4-dependent NF-κB activation and IFN-β synthesis (Aksoy et al., 2005). They concluded that PI3K negatively regulates TRIF-dependent events because both TLR3 and TLR4 associate with TRIF to activate IFN-β synthesis. In the present study, we showed that inhibition of PI3K increased TLR2, 3, 4, and 9-mediated iNOS expression (Fig. 3). These receptors interact with different adaptors—MyD88/Mal, TRIF, MyD88/Mal/TRIF/TRAM, and MyD88, respectively. The results suggested that PI3K is involved in both MyD88-dependent and TRIF-dependent pathways. The conclusion was supported by the observations that IFN-β mRNA synthesis (Fig. 5B) as well as IL-6 (Fig. 1), IL-12p40 (data not shown), and iNOS expression were increased by wortmannin where IFN-β gene expression is entirely TRIF-dependent and others were produced mainly through the MyD88-dependent pathway. There is another clue to elucidate the signaling pathway where PI3K is involved. We showed that increased NF-κB activity in wortmannin-treated cells is the result of accelerated IκB degradation (Fig. 9, 10). The increase in IκB degradation was dependent on the inhibition of PI3K because LY294002 exhibited a similar effect (Fig. 10). Therefore, we speculated that PI3K regulates protein(s) functioning upstream of IκB and commonly involved in the MyD88-dependent and TRIF-dependent pathways. Based on this consideration, TRAF6 or its associated proteins are possible candidates that may be regulated by PI3K. We showed that the enhancing effect of wortmannin could be observed even added after LPS stimulation (Fig. 7). The result suggests that a putative target of wortmannin functions in the
termination of the LPS signal. Since TRAF6 possesses E3 ubiquitin ligase activity, it is intriguing to consider that degradation of a protein playing an indispensable role in TLR signaling is under the control of PI3K.

We observed the inhibition of various actions of LPS by LY294002: nitrite production and IFN-β mRNA synthesis in Raw264.7 cells, and IL-6 and IL-12p40 production in human macrophages. The inhibition was not based on the inhibition of PI3K because dominant negative mutants of PI3K and also the shRNA probe targeting PI3K did not inhibit but rather increased the LPS-induced nitrite production (Fig. 6). These effects of the mutants and PI3K depletion were similar to those of wortmannin treatment. As LY294002 antagonized the effect of wortmannin on TLR-mediated responses (Fig. 4), the inhibitor may modulate the target protein functioning downstream of PI3K. Our data are in good agreement with those of Kim et al., who reported that LY294002 inhibits the LPS-induced nitrite production of Raw264.7 cells by a mechanism independent of the inhibition of PI3K using LY303511, an inactive analog of LY294002 (Kim et al., 2005). Contrasting with our data, they observed only a slight increase in LPS-induced iNOS expression by wortmannin. This seeming discrepancy may be because they used higher concentration of LPS to stimulate cells. We observed that the enhancing effect of wortmannin was minimized when the iNOS expression is fully stimulated by LPA (Fig. 2). Thus, we used submaximal concentration of LPS throughout this study.

The inhibition of TLR-mediated responses by LY294002 has been observed in many other studies (Arbibe et al., 2000; Dahle et al., 2004; Ojaniemi et al., 2003; Sarkar et al., 2004). In contrast, LY294002, as well as wortmannin, have been reported to enhance TLR-mediated cell events including cytokine production: IL-12p40 production in human monocytes (Martin et al., 2005), IFN-β production in human dendritic cells (Aksoy et al., 2005), cyclooxygenes 2
production in alveolar macrophages (Monick et al., 2002), and TNF-α production in PBMCs (Guha and Mackman, 2002). The reason for the discrepancy is currently unknown. However, as LY294002 increased the LPS-induced IkB degradation, similarly to wortmannin (Fig. 10), the compound may enhance NF-κB activity under certain conditions. It is always difficult to estimate the specificity of inhibitors especially when the effects of the inhibitors are determined by long-term incubation. We incubated the cells with the inhibitors for at least several hours to determine their effects on cytokine production or activation of transcription factors. The effects on autocrine/paracrine factors should be considered in addition to their non-specific actions. In addition, some TLR ligands are particles or insoluble materials, which are incorporated into the cells by pinocytosis or phagocytosis at the first step. PI3K is indispensable for these processes regardless of the target particle (Araki et al., 1996; Ninomiya et al., 1994; Sun et al., 2003). Therefore, the role of PI3K in TLR signaling is further obscured in such cases; the function of PI3K in engulfment must be distinguished from that in TLR signaling.

In conclusion, our results suggest caution regarding the use of PI3K inhibitors in studying TLR signaling. It is very likely that PI3K is a negative feedback regulator of a wide variety of TLR responses but further studies are needed to obtain a definitive answer regarding how PI3K is integrated into the system of TLR signaling.

Acknowledgement

We thank Dr. N Inoue (Osaka Medical Center for Cancer and Cardiovascular Diseases) for the pH1 vector for shRNA.
References


Legends to figures

**Fig. 1.** Effects of PI3K inhibitors on TLR-mediated IL-6 production. Human macrophages were treated with increasing concentrations of wortmannin (A) or LY294002 (B) for 15 min followed by stimulation with 50 ng/ml LPS (closed circles), 0.1 µg/ml Malp2 (closed squares), 5 µg/ml peptidoglycan (closed triangles), or vehicle (open circles) for 18 h. The culture supernatants were collected and the IL-6 levels were determined by ELISA.

**Fig. 2.** Enhancement by wortmannin of LPS-induced nitrite production. Raw264.7 cells were untreated or treated with wortmannin for 15 min followed by stimulation with increasing concentrations of LPS. (A) The amounts of iNOS after 6-h treatment with LPS were determined by Western blotting with specific antibody (upper panel). The membrane used for the blot was reprobed with anti-p67phox antibody to establish that equal amount of cellular protein had been applied on SDS-PAGE (lower panel). The concentration of wortmannin was 0.3 µM. (B) Nitrite accumulation in the supernatants after 20-h treatment with LPS were determined using Griess reagent. The concentrations of wortmannin were 0 (open circles), 0.1 (gray circles), or 1 µM (black circles).

**Fig. 3.** Enhancements of iNOS production by various TLR ligands. Raw264.7 cells were treated with the indicated concentrations of wortmannin (Wort) followed by stimulation with 5 µg/ml peptidoglycan (PGN), 0.2 µM Malp2, 50 µg/ml Poly I:C, 10 ng/ml LPS, 5 µg/ml lipoteichoic acid (LTA), or 0.1 µM CpG DNA for 6 h. Total cell lysates were analyzed by Western blotting with anti-iNOS antibody.
**Fig. 4.** Effects of PI3K inhibitors on LPS-induced iNOS expression. Raw264.7 cells were treated with various concentrations of wortmannin (Wort) and/or LY294002 (LY) for 15 min followed by stimulation with 50 ng/ml of LPS. (A) Expression of iNOS after 6-h stimulation with LPS was determined by Western blotting with anti-iNOS antibody. Anti-p67phox antibody was used for loading control. (B) Phosphorylation of Akt (pAkt, upper panel) after 15-min stimulation with LPS was determined using anti-phospho-Akt antibody. Also shown is the total amount of Akt (Akt, lower panel) determined by Western blotting with anti-Akt antibody.

**Fig. 5.** Effects of PI3K inhibitors on LPS-induced expression of iNOS mRNA and IFN-β mRNA. (A) Raw264.7 cells were treated with 0.1 µM wortmannin (Wort), 30 µM LY294002 (LY), or vehicle (-) for 15 min followed by stimulation with 0.1 µg/ml LPS for 6 h. Total RNA was extracted and RT-PCR analysis was performed with the primers described in the Materials and Methods section. (B) Raw264.7 cells were treated with the inhibitors as in (A) followed by stimulation with 0.1 µg/ml LPS (left panels) or 10 ng/ml LPS (right panels) for 6 h. Total RNA was extracted and RT-PCR with 25 cycles was performed in the left panels while with 20 cycles in the right panels.

**Fig. 6.** Increases in iNOS production by expression of dominant negative mutants of PI3K or by depletion of p110β in LPS-treated cells. Raw264.7 cells (5×10⁶/300 µl) were transfected with 25 µg of flag-∆p85 (∆p85), 25 µg of flag-p85cSH2 (cSH2), or two shRNA constructs targeting p110β (15 µg each, ∆p110β) by electroporation. Mock control (-) was subjected to electroporation with similar condition. The cells were plated in 6-well (A, B) or 24-well (C, D) plates and cultured for about 40 h. (A) Total cell lysates were subjected to Western blotting with anti-flag antibody. (B)
Aliquots of the lysate were immunoprecipitated with anti-p85 antibody and subjected to Western blotting with anti-flag antibody (upper panel) or anti-p110β antibody (lower panel). (C) The cells were incubated with or without 1 µM wortmannin (WT) for 15 min, followed by stimulation with 10 ng/ml LPS or 0.1 µM calyculin A (CA) for 15 min. Cell lysates were subjected to Western blotting with anti-phospho-Akt (pAkt), anti-p67phox, or anti-phospho-Erk (pErk) antibodies. (D) The cells were incubated with or without 1 µM wortmannin (WT) for 15 min, followed by stimulation with 10 ng/ml LPS for 6 h. Cell lysates were subjected to Western blotting with anti-iNOS antibody. (E) The data in (D) were analyzed by a densitometer and the density was plotted on the graph.

**Fig. 7.** Effects of wortmannin added at different time points with respect to LPS stimulation. Raw264.7 cells were incubated with 50 ng/ml LPS for 6 h before determination of the iNOS expression by Western blotting with anti-iNOS antibody. Wortmannin (WT, 0.1 µM) was added at various times before and after addition of LPS.

**Fig. 8.** Effects of wortmannin on iNOS mRNA stability. Raw264.7 cells were incubated with or without 0.1 µM wortmannin (Wort) for 15 min before stimulation with 10 ng/ml LPS for 6 h. The cells were then harvested, or incubated for a further 6 or 12 h with the addition of 0.5 µg/ml actinomycin D before harvest. (A) The mRNA levels of iNOS and β-actin were evaluated by RT-PCR. (B) The amounts of iNOS mRNA (iNOS mRNA/β-actin mRNA) in the wortmannin-treated (closed columns) and untreated (open columns) cells are shown, as percentages of the values at the time of addition of actinomycin D (0 h).
Fig. 9. Inhibition of PI3K activity enhances NF-κB promoter activity downstream of TLR. (A) 293T cells were transfected with 25 ng of the NF–κB luciferase reporter gene plasmid and 2 ng of the seapansy luciferase gene along with 10 ng each of TLR2 and TLR6. The following day, cells were treated with wortmannin for 15 min and stimulated with 0.1 μM of Malp2. After 6 h, cell extracts were prepared and the expression levels of the firefly luciferase, which were normalized for those of control seapansy luciferase, were determined. Results are the means±SD of triplicate determination. (B) 293T cells were transfected with NF-κB luciferase, seapangy luciferase, TLR2 and TLR6 as in (A) along with pCMV5-Δp85 (left panel) or pCMV5-p110αCAAT (right panel) for 6 h. The total amount of DNA transfected was kept constant by supplementation with control pCMV5. After 24 h, cells were stimulated with 0.1 μM Malp2 for 6 h and NF-κB luciferase activities were determined as in (A).

Fig. 10. Enhancement by PI3K inhibitors of LPS-induced IκB degradation. Raw264.7 cells were treated with increasing concentrations of LY294002 or wortmannin for 15 min followed by stimulation with 10 ng/ml LPS for 30 min. Total cell lysates were analyzed by Western blotting with anti-IκB antibody (upper panel) or anti-p67phox antibody (lower panel).
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6, (A), (B)
Fig. 6, (C), (D), (E)
Fig. 7
Fig. 8

(A) Actinomycin D (hr) 0 6 12

iNOS β-actin

Wort - + - + - +

LPS

(B) iNOS mRNA Remaining (%) 0 6 12
Fig. 9
Fig. 10