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Switch activation of PI-PLC downstream signals in activated macrophages with wortmannin

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Abstract

Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) has been known to serve as a substrate for phosphatidylinositol 3-kinase (PI_3K) and phosphoinositide-specific phospholipase C (PI-PLC), which can produce PtdIns(3,4,5)P_3 and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG), respectively. In this study, we elucidated the role of PI-PLC during the LPS-activated mouse macrophages RAW264.7 treated with PI_3K inhibitor wortmannin. First, wortmannin treatment enhanced Ins(1,4,5)P_3 production and iNOS expression in LPS-activated macrophages. Inhibition of PI_3K by p85 siRNA also showed an enhancement of iNOS expression. On the other hand, overexpression of PI_3K by ras-p110 expression plasmid significantly decreased iNOS expression in LPS-activated macrophages. In addition, overexpression of wild-type or dominant-negative Akt expression plasmid did not affect the iNOS expression in LPS-activated macrophages. Second, treatment of PI-PLC inhibitor U73122 reversed the enhancement of iNOS expression, the increase of phosphorylation level of ERK, JNK and p38, and the increase of AP-1-dependent gene expression in wortmannin-treated and LPS-activated macrophages. However, NF-κB activity determined by EMSA assay and reporter plasmid assay did not change during LPS-activated macrophages with or without wortmannin. We propose that the inhibition of PI_3K by wortmannin in mouse macrophages enhances the PI-PLC downstream signals, and subsequently increases the LPS induction of iNOS expression independently of Akt pathway.

Keywords: Phosphatidylinositol 3-kinase; Phosphoinositide-specific phospholipase C; Inducible nitric oxide synthase; Wortmannin; Lipopolysaccharide

1. Introduction

The macrophage is a very important cell for development of an effective immune response to flight infection within the body. The important functions of macrophages are phagocytosis, antigen presentation to the T-helper cells, secretion of cytokines, and storage of iron. Bacterial endotoxin (lipopolysaccharide, LPS) is an outer membrane component of *Gram*-negative bacteria. When LPS binds with a toll-like receptor 4 complex of out membrane in macrophage, it induces the expression of many

Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PI_3K, phosphatidylinositol 3-kinase; PI-PLC, phosphoinositide-specific phospholipase C; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; iNOS, inducible nitric oxide synthase; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PKC, protein kinase C; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SHIP, SH2-containing inositol phosphatase; MAPK, mitogen activated protein kinase; PC-PLC, phosphatidylcholine-specific phospholipase C; siRNA, small interfering RNA; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction; EMSA, electrophoretic mobility shift assay

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proinflammatory mediators, including arachidonic acid metabolites (e.g. prostaglandins and leukotrienes), nitric oxide (NO), and several cytokines such as IL-1, TNF-α, IL-6, IL-8, and IL-12 [1,2]. Nitric oxide (NO) is a multifunctional free radical produced by the enzyme, nitric oxide synthase (NOS), and plays a major role in regulating vascular tone, neurotransmission, killing of microorganisms and tumor cells, and other haemostatic mechanisms [3]. Inducible NOS (iNOS) is one isoform that is mainly controlled at the transcriptional level in response to a wide range of proinflammatory cytokines and bacterial cell wall products, such as LPS [4]. The molecular mechanisms of LPS-induced macrophage activation involve several signal transduction pathways, such as protein kinase A, protein kinase C (PKC), src-related tyrosine kinases, mitogen-activated protein kinases (MAPKs): ERK, JNK and p38 [5], and G proteins [6], which play significant roles in the induction of proinflammatory gene expression.

The phosphoinositide phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) is an essential regulator of many cellular processes and is present in the Golgi, plasma membrane, and the nucleus. In addition, PtdIns(4,5)P_2 can, itself, interact with actin-regulatory proteins such as profiling and gelsolin to regulate actin polymerization. It is also present in the nuclear matrix, heterochromatin and the sites of active RNA splicing, and is implicated in regulatory RNA splicing, the regulation of chromatin remodeling, and histone-mediated transcriptional repression [7–9]. In mammalian cells, PtdIns(4,5)P_2 is a substrate for members of the phosphoinositide-specific phospholipase C (PI-PLC), phosphatidylinositol 3-kinase (PI_3K), and PtdIns(4,5)P_2 phosphatase [10], the products of which contribute to many cellular responses, including cell survival and inflammation [11,12]. Hydrolysis of PtdIns(4,5)P_2 is catalyzed by PI-PLC to produce the second-messenger molecules inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG), which in turn mobilize intracellular Ca^{2+} and activate protein kinase C (PKC), respectively. On the other hand, PtdIns(4,5)P_2 can be phosphorylated by PI_3K to generate PtdIns(3,4,5)P_3, which helps to activate downstream Akt [13,14].

Previous studies have shown that inhibition of PI_3K positively enhanced LPS-induced iNOS expression in mouse peritoneal macrophages [15] and TNF-α expression in human monocytic cells [16]. The possible mechanisms were that activation of PI_3K downstream Akt resulted in the negative regulation of NF-κB, AP-1, and Egr-1 transcriptional activity. PI_3K negatively regulated the stability of cyclooxygenase 2 mRNA in LPS-induced human alveolar macrophages [17]. Pahan et al. demonstrated that induction of NOS in C6 glial cells was also regulated negatively by the activation of PI_3K [18]. However, several studies also have found that activation of PI_3K promotes inflammatory signaling pathways. For example, activation of PI_3K-Akt pathway positively regulated p65 NF-κB transactivation activity in human hepatoma cells [19], and high Akt activity could increase NF-κB-dependent gene expression through the activation of IκB kinase and the p38 in mouse NIH3T3 fibroblasts [20]. These contrasting observations may result from cell type-specific influences of PI_3K or the cell type-specific PI_3K subtype. However, this is not yet fully understood. Recently, Yamaguchi et al. showed that PI_3K inhibitor LY294002 exhibited antitumorigenic properties by induction of Egr-1 phosphorylation and ATF3 expression in human colorectal cancer cells [21]. Another PI_3K inhibitor, wortmannin, and dominant-negative Akt had no effect on the induction of ATF3 expression. The results indicated that induction of ATF3 expression by LY294002 is independent of the PI_3K/Akt pathway.

Both PI-PLC and PI_3K are activated in response to LPS stimulation and have been shown to play roles in the signaling cascades triggered by LPS [22,23]. In this study, we examine the influence of the inhibition of PI_3K by wortmannin in LPS-induced activation of mouse macrophages. This inhibition occurs via the increased Ins(1,4,5)P_3 formation by PI-PLC and results in the activation of PKC, MAPKs, AP-1, and finally, iNOS expression. These effects are caused by the Akt- and NF-κB-independent pathways. Our study showed that inhibition of PI_3K may go through PI-PLC pathway leading to increase of iNOS expression, indicating that PI_3K and PI-PLC may counteract to regulate the metabolism of PtdIns(4,5)P2 depending on cells types or their conditions.

2. Materials and methods

2.1. Cell culture and treatments

The mouse macrophage cell lines RAW 264.7 (BCRC 60001, Food Industry Research and Development Institute, HsinChu, Taiwan) were cultured as previously described [24]. For all assays except the luciferase assay, cells were plated in 60-mm dishes at 5×10^6 cells/dish and allowed to grow for 18–24 h. LPS (*Escherichia coli* 0127:B8), wortmannin, and KN93 were purchased from Sigma Chemical Co. (St Louis, MO). D609, U73122, Chelerythrine chloride, SP600125, SB203580, U0126 and LY294002 were purchased from Tocris Bioscience (Ellisville, Missouri), and Bapta-AM was purchased from BIOMOL (Plymouth Meeting, PA).

2.2. Plasmids and transfection assays

The dominant-negative Akt (DN-Akt), ras-p110, and HA-Akt expression plasmids were generously provided by Professor Jen-Kun Lin (National Taiwan University) [25]. The mouse iNOS promoter plasmid (pGL2-iNOS) was generously provided by Professor Charles J. Lowenstein (Johns Hopkins University) [26].

For Western blot, cells were seeded in 60-mm dishes. The next day, the cells were transfected with DN-Akt, HA-Akt, ras-p110 expression plasmids, scramble control siRNA cocktail, or p85 siRNA cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After 36 h of transfection, the cells were treated with 50 ng/ml of LPS for another 18 h.

For luciferase reporter assay, cells were seeded in 12-well plates at a density of 2.5×10^4. The next day, the cells were replaced the serum-free Opti-MEM (Invitrogen Corporation, Carlsbad, CA) and transfected with the pGL2-iNOS, pNF-κB-Luc or pAP-1-Luc reporter plasmids (Stratagene, La Jolla, CA) and phRL-TK (Promega, Madison, WI) as an internal control plasmid using LipofectAMINE2000™ (Invitrogen, Carlsbad, CA) [27]. After transfection (9 h), the medium was replaced with complete medium and continually incubated for another 39 h. Transfected cells were then directly treated with drugs for various time periods. Each well was washed twice with cold PBS and harvested in 150 μl of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). One hundred μl of cell lysate was used to assay

luciferase activity by RenLite™ luciferase reporter gene assay kit (Packard Instrument Company, Meriden, CT). Luminescence was measured on a Plate Chameleon Multilabel plate reader. (HIDEX OY, Turku, Finland).

2.3. Western blot

Equal amounts of total cellular protein (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) as described previously [27]. The membrane was then incubated with an anti-iNOS antiserum (BD Biosciences, San Jose, CA), anti-IκB antiserum, anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK antiserum (Santa Cruz Biotechnology), anti-phospho-p38, anti-p38 antiserum (Cell Signaling Technology, Inc., Danvers, MA), or anti-α tubulin antiserum (Invitrogen Taiwan, Ltd., Taipei, Taiwan). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence kits. The band densities were quantitated by a computer densitometer (IS-1000 Digital Imaging System).

2.4. RT-PCR

Total RNA was isolated from both of control and tested cultured cells, and the mRNA level was detected by RT-PCR as described previously [21]. Four sets of primers were used to amplify the cDNA of iNOS and GAPDH: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′; TNFα, forward primer 5′-CGAAGGAGTTGGAGGTGTTTTCC-3′, reverse primer 5′-TTTATTGACT-GAGGCACTGGGG-3′; IL-1β, forward primer 5′-GATCTGGACACT-CAGGGTCTCATC-3′, reverse primer 5′-GCTCTGTCTTCTTGATGG-GACCTG-3′; GAPDH, forward primer 5′-ACATCAAGAAGGTGGT-GAAGC-3′, reverse primer 5′-CTTACTCCTTGGAGGCCATGT-3′. For measurement of mRNA stability, cells were pretreated with or without 100 nM wortmannin and stimulated with 50 ng/ml LPS for 6 h, followed by treatment with actinomycin D (4 μg/ml). Total RNA was prepared, and iNOS mRNA was measured as above. Band densities were quantitated with a computer densitometer (IS-1000 Digital Imaging System).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins and the ^{32}P-labeled double-stranded AP-1 and NF-κB oligonucleotide probe were prepared as described previously [24]. For DNA binding reaction, 2 μg of nuclear proteins was mixed with 3 μl of the labeled probe (approximately 5000 cpm) and 2 μg of poly(dI-dC) poly(dI-dC) in a reaction buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 5% glycerol, and 1 mM DTT) for 20 min at room temperature. The DNA–protein complex was separated on 5% non-denaturing acrylamide gels before vacuum drying and autoradiography. The specificity of NF-κB or AP-1 binding to its consensus motif has been proved by supershift experiment as described previously [24].

2.6. Measurement of Ins(1,4,5)P_3 levels

Cells were suspended in the PBS buffer, added with 0.2 volume of ice-cold 20% trichloroacetic acid, and kept on ice for 20 min. The protein sediment was removed by centrifugation at 2000×*g* for 15 min at 4 °C, and the supernatant was neutralized with ice-cold 10 M KOH to pH 7.5. The $KClO_4$ sediment was removed by centrifugation at 2000×*g* for 15 min at 4 °C. The supernatant was determined the Ins(1,4,5)P_3 level using Inositol-1,4,5-Trisphosphate [^{3}H] Radioreceptor Assay Kit (Amersham Biosciences, Piscataway, NJ).

2.7. Kinase assay

Equal amounts of total cellular protein (100 μg) were immunoprecipitated with JNK or p38 antibody (Santa Cruz Biotechnology) and protein A/G-PLUS agarose (Santa Cruz Biotechnology) for 16 h at 4 °C. The JNK or p38 kinase reaction was carried out in a final volume of 40 μl containing 1 μg Gst-ATF2 substrate, 10 μM cold ATP, 5 μCi [γ-32p] ATP (5000 Ci/mmol, Amersham) and incubated for 20 min at 25 °C. Each sample was mixed with 10 μl of 5× Laemmli's loading buffer to stop the reaction, heated for 10 min at 100 °C, and subjected to SDS-PAGE as described previously [24]. Gels were dried and visualized by autoradiography.

2.8. Statistical analysis

Data are presented as the mean of S.E. for the indicated number of independently performed experiments. Statistical analysis was done using one-way Student's *t* test.

3. Results

3.1. Akt pathway was not involved in the increase of iNOS expression in wortmannin-treated macrophages

Wortmannin has been shown to inhibit PI_3K activity and result in lower downstream Akt phosphorylation levels. To test the effect of wortmannin on the inhibition of PI_3K activity in RAW 264.7 macrophage, we detected the phosphorylation level of Akt by Western blot. The cells were pretreated with wortmannin for 30 min, then stimulated with 50 ng/ml LPS for various time periods (0.5–4 h). As shown in Fig. 1A, 100 nM of wortmannin significantly inhibited the phosphorylation level of Akt in LPS-activated macrophages. Next, we found that wortmannin enhanced LPS-induced iNOS protein expression about 4.2-fold in the presence or absence of serum (Fig. 1B). In wortmannin-treated cells, the induction of iNOS protein expression occurred early, at 4 h after LPS treatment, peaked at 14 h, and was sustained for 36 h. However, iNOS protein expression occurred late at 9 h and terminated at 36 h in the cells treated with LPS alone (Fig. 1C). Further work was done to determine whether wortmannin enhanced iNOS expression at the transcription level. RT-PCR results showed that wortmannin indeed enhanced the iNOS mRNA expression (Fig. 1D upper panel); indicating that enhancement of iNOS expression by wortmannin was due to the increase of iNOS gene expression. Using actinomycin D (an RNA transcription inhibitor), we examined whether wortmannin could enhance iNOS mRNA stability and then increase the mRNA level and its translation product. Results indicated that wortmannin-enhanced iNOS expression did not result from enhancing iNOS mRNA stability (data not shown). In addition, wortmannin also enhanced another two proinflammatory genes TNFα and IL-1β expression in LPS-activated macrophage (Fig. 1D, lower panels). To further investigate the enhancement of iNOS expression by wortmannin, transient transfection was performed using mouse iNOS luciferase promoter construct. Both PI_3K inhibitor-wortmannin and LY294002 significantly enhanced iNOS promoter activity in a dose-dependent manner (Fig. 1E).

Wortmannin can inhibit PI_3K activity, and therefore limit Akt activity, but whether it is PI_3K, or Akt, or both which regulate iNOS expression is currently controversial. We used a ras-p110 expression plasmid that constantly expressed activated-p110 (PI_3K catalytic subunit) or a p85 siRNA

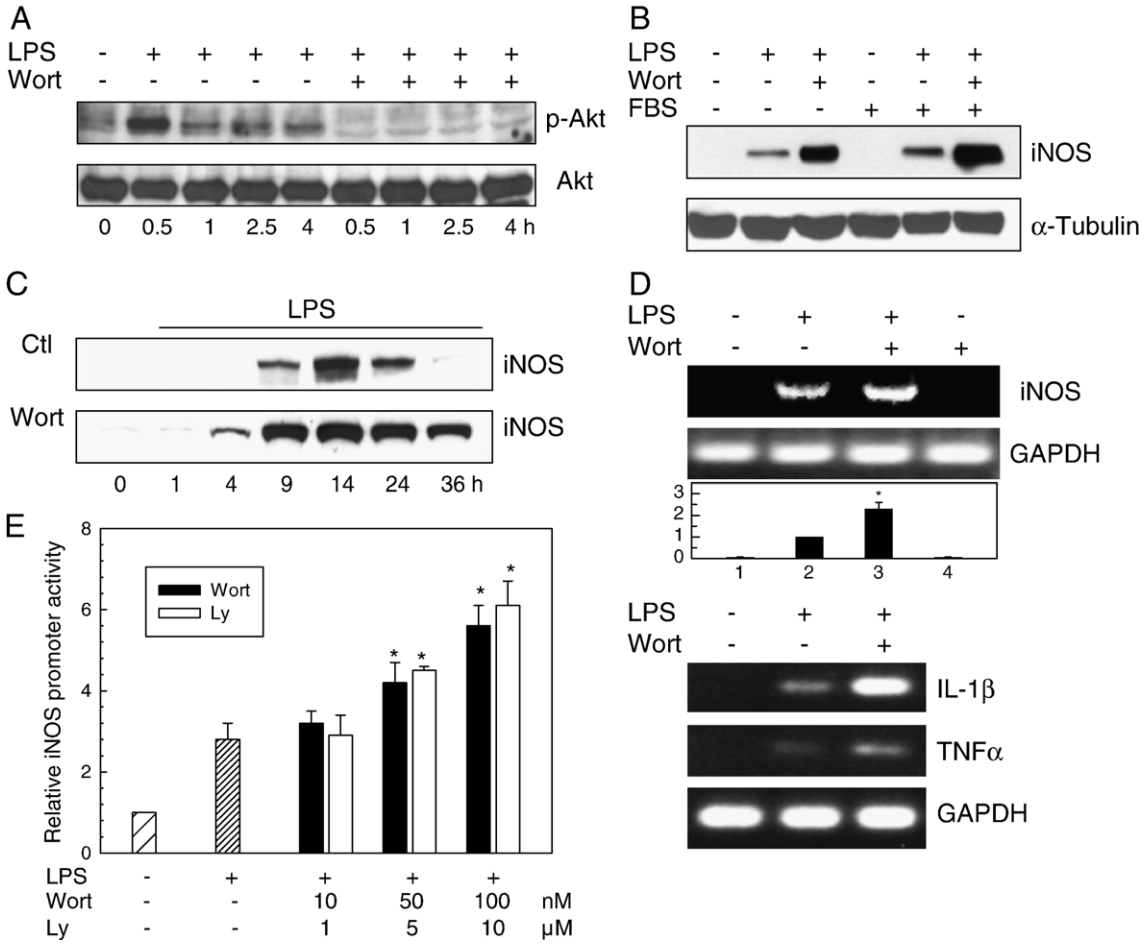

Fig. 1. Inhibition of PI_3K resulted in increase of iNOS expression in mouse macrophages. (A–C) Cells were pretreated with PI_3K inhibitor-wortmannin (Wort, 100 nM) for 30 min, then LPS (50 ng/ml) for various times indicated (A, C) or 18 h (B). Total cell lysates were analyzed by Western blot. FBS, fetal bovine serum. (D) Cells were treated with PI_3K inhibitor-wortmannin (100 nM) for 30 min and then LPS (50 ng/ml) for 6 h. Total mRNA was prepared, and the mRNA levels of iNOS, IL-1β, TNFα, and GAPDH were detected by RT-PCR. The relative fold of iNOS were quantitated and shown in graphical form (lower). *$p<0.01$ versus LPS treatment. (E) Cells were transfected with a mouse iNOS reporter plasmid and phRL-TK plasmid as an internal control. After transfection, the cells were treated with PI_3K inhibitor-wortmannin or LY294002 (Ly) for 30 min and then LPS (50 ng/ml) for 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Materials and methods. Values are expressed as the mean±S.E. of triplicate tests. *$p<$ 0.01 versus LPS treatment.

cocktail that decreased p85 (PI_3K regulatory subunit) expression and then downregulated p110 activity to evaluate the role of PI_3K itself in the regulation of iNOS expression. As shown in Fig. 2A, LPS induced iNOS expression, and wortmannin further enhanced the expression in mock expression cells; however, overexpression of ras-p110 significantly limited the induction of iNOS in LPS-activated cells with or without wortmannin. Transient transfection of iNOS reporter plasmid also showed that constantly-activated p110 limited iNOS promoter activity (Fig. 2B). On the other hand, transient transfection of p85 siRNA significantly enhanced LPS-induced iNOS expression in comparison to the cells transfected with scramble control siRNA cocktail (Fig. 2C, lanes 3 and 4). Next, we evaluated the role of Akt in the LPS-induced iNOS expression that accompanies overexpression of wild-type Akt or dominant-negative Akt (DN-Akt). Interestingly, iNOS expression did not change in the cell's overexpression of either Akt or DN-Akt in LPS-activated cells (Fig. 2D, lanes 3 and 5). However, activated p110 limited LPS-induced iNOS expression (Fig. 2D, lane 4). These results suggest that PI_3K, not Akt, was positively involved in the regulation of iNOS expression in LPS-activated macrophages.

3.2. PI-PLC pathway contributes to wortmannin-enhanced iNOS expression in macrophages

It is well known that $PtIns(4,5)P_2$ can either be phosphorylated to form $PtdIns(3,4,5)P_3$ by PI_3K or hydrolyzed to form DAG and $Ins(1,4,5)P_3$ by PI-PLC. $Ins(1,4,5)P_3$ can activate Akt, but Akt was not involved in the wortmannin-enhanced iNOS expression in the above experiment. In addition, previous studies have demonstrated that PI-PLC is involved in the activation of macrophages and other cell types by LPS treatment [23,28,29]. Our next experiment examined whether inhibition of PI_3K activity by wortmannin could increase $PtdIns(4,5)P_2$ to be hydrolyzed by PI-PLC. The $Ins(1,4,5)P_3$ level in the cells treated with various concentrations of wortmannin and/or LPS

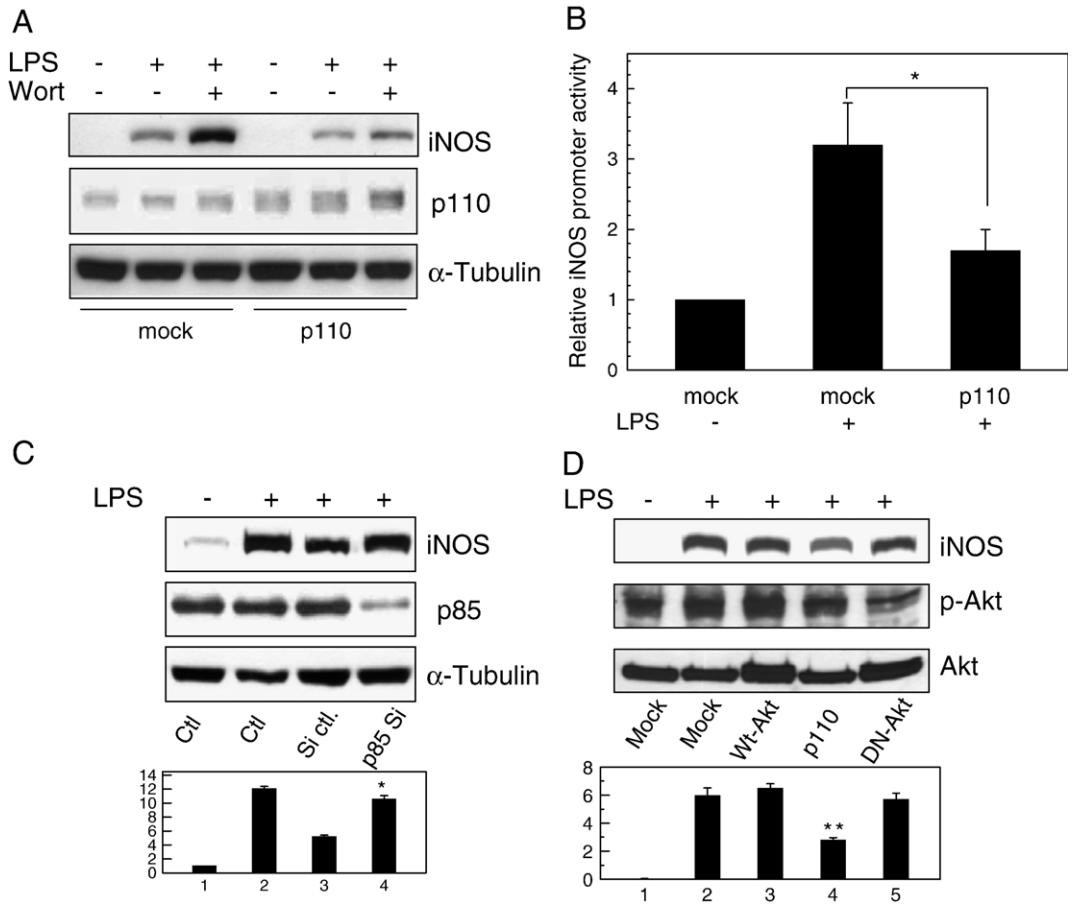

Fig. 2. Activation of PI_3K but not Akt resulted in inhibition of iNOS expression. (A, C, D) Cells were transfected with (A) ras-p110 expression plasmid, (C) scramble control siRNA cocktail (Si Ctl.) or p85 siRNA cocktail (p85 Si), or (D) Akt expression plasmid (wt, wild-type; DN, dominant-negative) for 36 h, and then treated with LPS (50 ng/ml) for 18 h. Total cell lysate was analyzed by Western blot. The relative fold of iNOS were quantitated and shown in graphical form (lower). *$p<0.01$ versus siRNA control (lane 3). **$p<0.01$ versus mock cells with LPS treatment (lane 2). (B) Cells were transfected with ras-p110 expression plasmid, a mouse iNOS reporter plasmid, and phRL-TK plasmid as an internal control. After transfection, cells were treated with LPS (50 ng/ml) for 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Materials and methods. Values are expressed as the mean±S.E. of triplicate tests. *$p<0.01$.

was determined. As shown in Fig. 3, LPS induced Ins(1,4,5)P_3 formation and wortmannin enhanced the Ins(1,4,5)P_3 formation in the presence of LPS in a dose-dependent manner. The results suggest that inhibition of PI_3K can increase the PtdIns(4,5)P_2 hydrolyzed by PI-PLC.

To further confirm the involvement of the PI-PLC pathway in the wortmannin-enhanced iNOS expression, the PI-PLC inhibitor U73122, PC-PLC inhibitor D609, calcium chelator Bapta-AM, and PKC inhibitor chelerythrine chloride were used. When cells were pretreated with wortmannin, LPS-induced iNOS expression was enhanced (Fig. 4A and B, lane 3); however, the enhancement of iNOS expression disappeared in U73122-treated cells but not in D609-treated cells (Fig. 4A and B, lane 5). Activation of PI-PLC can increase DAG and Ins (1,4,5)P_3 levels, and these events subsequently activate PKC and increase cytoplasmic calcium levels, respectively. As shown in Fig. 4C and D, the wortmannin-enhanced iNOS expression was decrease in LPS-activated cells with chelerythrine chloride (Fig. 4C, lane 5); however, Bapta-AM did not affect the iNOS expression in LPS-activated cells with or without wortmannin (Fig. 4D, lane 5). These results suggest that PI-PLC and downstream PKC positively contributed to wortmannin-enhanced iNOS expression in LPS-activated macrophages.

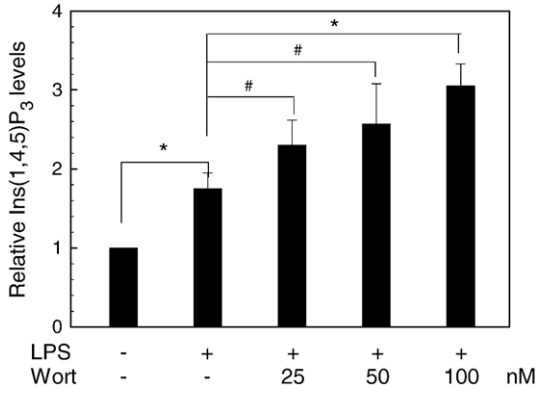

Fig. 3. Inhibition of PI_3K resulted in enhancement of Ins(1,4,5)P_3 production. Cells were treated with PI_3K inhibitor-wortmannin (25–100 nM) for 30 min and/or LPS (50 ng/ml) for 10 min, and total cell extract determined the Ins (1,4,5)P_3 content as described in Materials and methods. Values are expressed as the mean±S.E. of triplicate tests. *$p<0.01$. #$p<0.05$

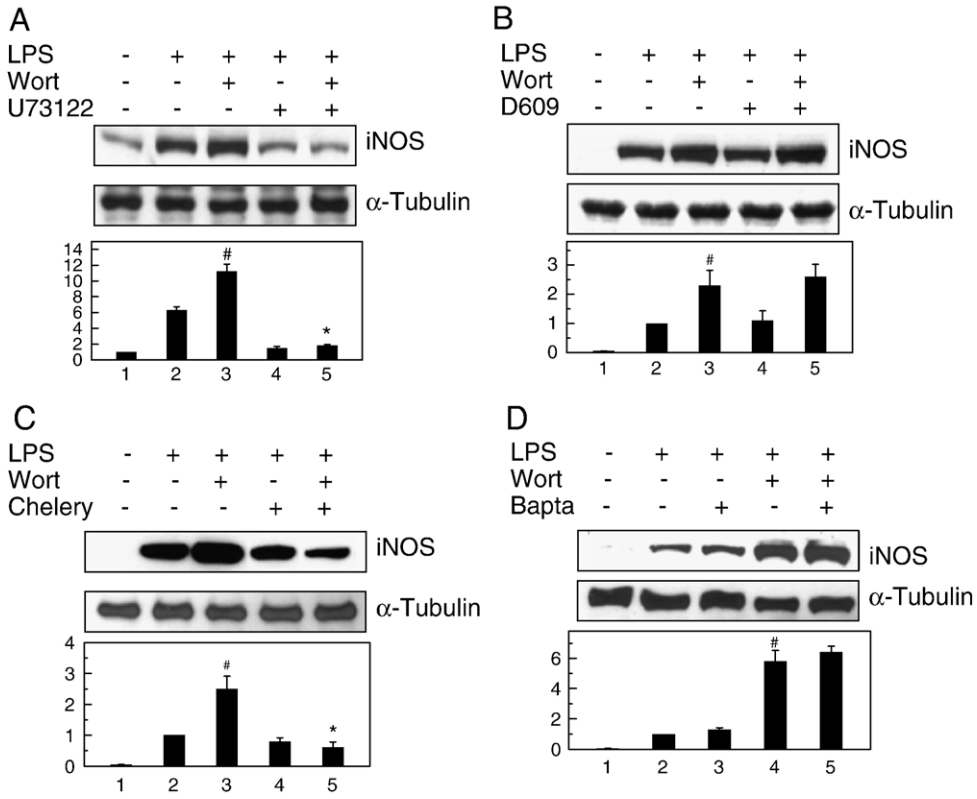

Fig. 4. PI-PLC and PKC involved in the iNOS expression of cells treated with PI_3K inhibitor. Cells were treated with wortmannin (100 nM) and (A) PI-PLC inhibitor U73122 (10 μM), (B) PC-PLC inhibitor-D609 (25 μM), (C) PKC inhibitor chelerythrine chloride (1 μM) or (D) calcium chelator Bapta-AM (10 μM) for 30 min and then LPS (50 ng/ml) for 18 h. Total cell lysates were analyzed by Western blot. In all cases, the relative fold of iNOS were quantitated and shown in graphical form (lower). $^{\#}p<0.01$ versus LPS treatment alone (lane 2). $^{*}p<0.01$ versus LPS and Wort treatment (lane 3).

3.3. AP-1 but not NF-κB mediates wortmannin-enhanced iNOS expression in macrophages

It is well known that MAPKs, MAPKs downstream AP-1 transcription factor, and NF-κB are the major activated signaling pathways in LPS-stimulated macrophages. To further understand the mechanisms by which PI-PLC downstream pathways positively regulate LPS-induced iNOS expression in wortmannin-treated cells, we evaluated the effects of wortmannin on the phosphorylation level of MAPKs and on the activation of AP-1 and NF-κB. Exposure of the cells to 50 ng/ml LPS resulted in a time-dependent phosphorylation of all three MAPK members, ERK, JNK, and p38 (Fig. 5A). In addition, wortmannin significantly enhanced the LPS-induced phosphorylation level of ERK, JNK, and p38 at 1 h and prolonged the phosphorylation level of ERK for 3 h. However, wortmannin did not enhance LPS-induced IκB degradation, indicating that the NF-κB pathway was likely not involved in wortmannin-enhanced iNOS expression (Fig. 5B). To determine whether wortmannin can enhance the DNA binding activity of AP-1 or NF-κB, nuclear extracts were characterized by EMSA assay. As shown in Fig. 5C, LPS induced AP-1 binding activity at 1 and 2 h, and wortmannin significantly enhanced the binding activity at 2 h (Fig. 5C, upper). LPS also induced NF-κB binding activity though wortmannin did not (Fig. 5C, down). The results approximated those of Western blot analysis, indicating wortmannin did not affect the activation of NF-κB in LPS-activated macrophages. The effect of wortmannin on gene expression mediated by AP-1 but not NF-κB was further confirmed in macrophages transfected with reporter plasmids. As shown in Fig. 5D, LPS induced NF-κB- and AP-1-dependent gene expression. Moreover, wortmannin significantly enhanced AP-1-dependent gene expression, but also did not affect the NF-κB-dependent gene expression in LPS-activated cells. These results suggest that the inhibition of PI_3K by wortmannin enhanced AP-1 activity but did not affect NF-κB activity. Because inhibition of PI_3K by wortmannin enhanced PI-PLC pathways, MAPKs phosphorylation, and AP-1 activity, we evaluated the effect of the PI-PLC inhibitor U73122 on reverse MAPKs phosphorylation and AP-1 activation in LPS-activated cells with wortmannin. LPS induced the phosphorylation of ERK, JNK and p38 (Fig. 6A) in a time-dependent manner. Pretreatment of cells with wortmannin enhanced the LPS-induced phosphorylation level of ERK, JNK, and p38. However, pretreatment of cells with U73122 significantly reversed the wortmannin-enhanced phosphorylation level of ERK, JNK, and p38 in LPS-activated cells. Next, we examined NF-κB- and AP-1-dependent gene expression by transient transfection with reporter plasmids. As shown in Fig. 6B and C, wortmannin enhanced AP-1-dependent gene expression, and U73122 significantly abrogated wortmannin-enhanced AP-1-dependent gene expression in LPS-activated

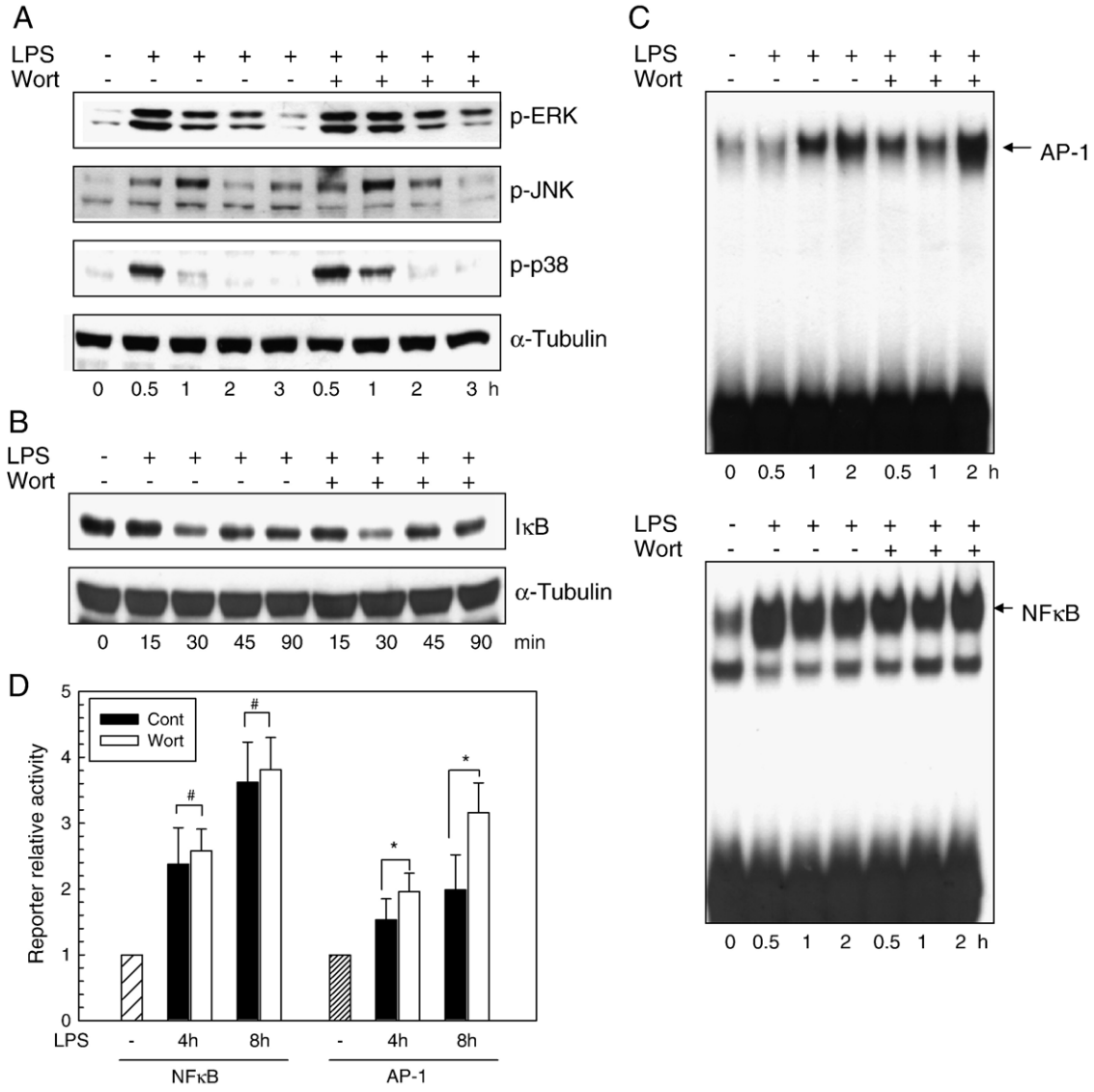

Fig. 5. Inhibition of PI_3K resulted in enhancement of MAPKs phosphorylation but not NF-κB activity. (A–C) Cells were treated with PI_3K inhibitor-wortmannin (100 nM) for 30 min and then LPS (50 ng/ml) for various times indicated. Total cell lysates were analyzed by Western blot (A and B), or nuclear proteins were extracted and analyzed by EMSA assay (C) as described in Materials and methods. (D) Cells were transfected with NF-κB or AP-1 reporter plasmid and phRL-TK plasmid as an internal control. After transfection, cells were treated with PI_3K inhibitor-wortmannin for 30 min and then LPS (50 ng/ml) for 4 h or 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Materials and methods. Values are expressed as the mean ± S.E. of triplicate tests. $^{*}p < 0.01$. $^{\#}p > 0.05$.

cells. In contrast, wortmannin and/or U73122 did not affect the NF-κB-dependent gene expression in LPS-activated cells. These results suggest that the enhancing effect of wortmannin on LPS-induced phosphorylation of ERK, JNK, and p38 and AP-1-dependent gene expression might be mediated by activation of PI-PLC-dependent signal pathways. To further understand which MAPKs members are involved in the enhancement of iNOS expression in wortmannin-treated cells, three MAPKs members' inhibitors were used. Western blot and kinase activity assay showed U0126 (1 and 5 μM), SP600125 (10 and 20 μM), and SB203580 (7.5 and 15 μM) dose-dependently inhibited ERK, JNK, and p38 kinase activity respectively, indicating these concentrations were effective to inhibit MAPKs members (Fig. 6D lower panels). Next, we examined these MAPKs inhibitors on the iNOS expression in wortmannin-treated and LPS-activated cells. As shown in Fig. 6D upper panel, wortmannin enhanced iNOS expression (lane 3) in LPS-activated cells, and U0126 and SP600125 significantly abrogated wortmannin-enhanced iNOS expression in a dose-dependent manner. However, SB203580 did not reverse the enhancement of iNOS expression in wortmannin- and LPS-treated cells. These results suggest that ERK and JNK involved in the wortmannin-enhanced iNOS expression in LPS-activated cells.

4. Discussion

Several studies have demonstrated Akt to be involved in the NF-κB signaling pathway in cells treated with PI_3K inhibitor. In the present study, we investigated the effect of a

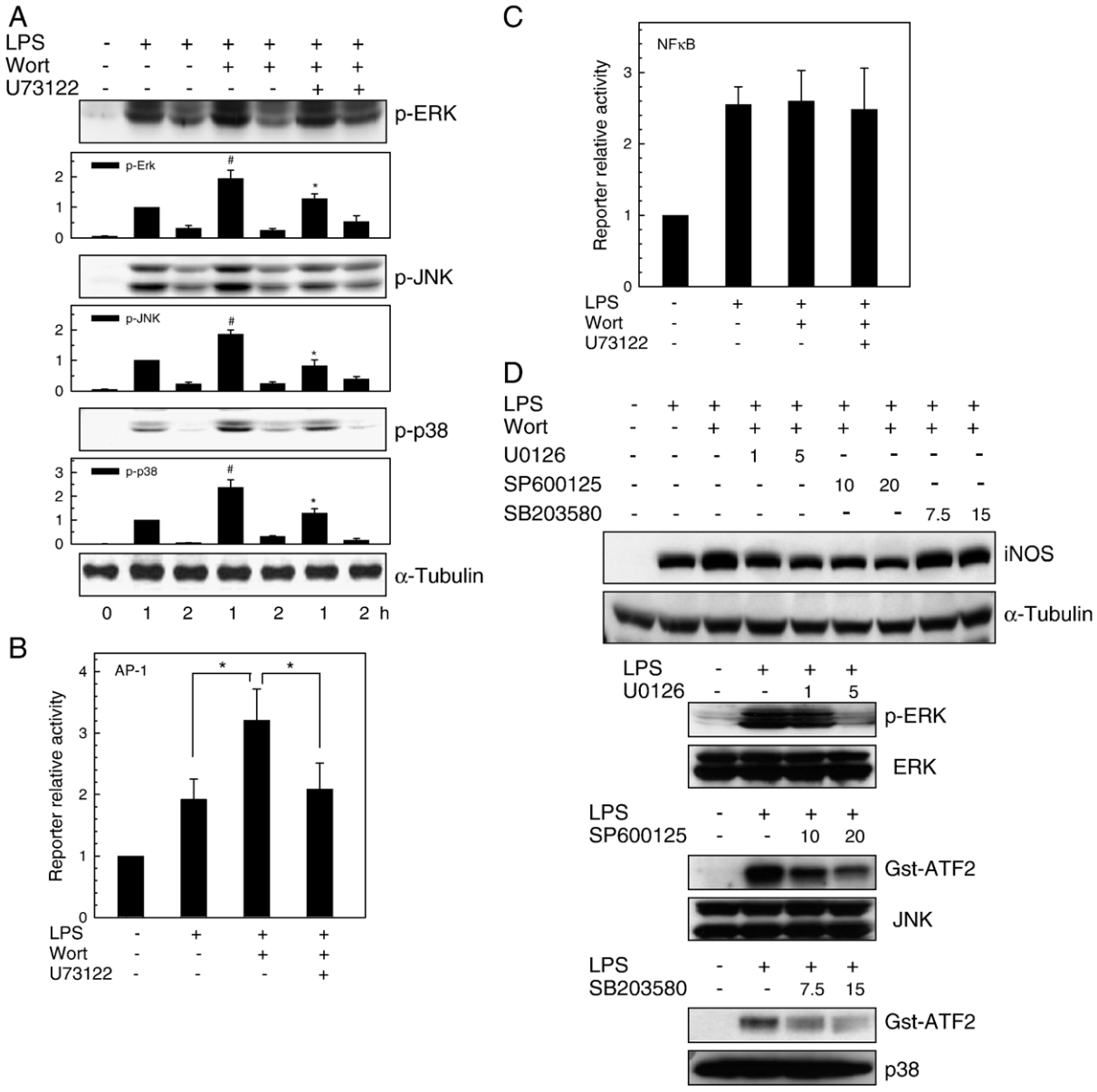

Fig. 6. Inhibition of PI-PLC resulted in blockade of the MAPKs phosphorylation and AP-1 activity in wortmannin-treated cells. (A) Cells were treated with PI_3K inhibitor-wortmannin (100 nM) and/or PI-PLC inhibitor U73122 (10 μM) for 30 min and then LPS (50 ng/ml) for various times indicated. Total cell lysates were analyzed by Western blot. $^{\#}p<0.01$ versus LPS treatment alone (lane 2). $^{*}p<0.01$ versus LPS and Wort treatment (lane 4). (B and C) Cells were transfected with AP-1 or NF-κB reporter plasmid and phRL-TK plasmid as an internal control. After transfection, cells were treated with PI_3K inhibitor wortmannin (100 nM) and/or PI-PLC inhibitor U73122 (10 μM) for 30 min and then LPS (50 ng/ml) for 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Materials and methods. Values are expressed as the mean ± S.E. of triplicate tests. $^{*}p<0.01$. (D) Upper panel, cells were treated with PI_3K inhibitor-wortmannin (100 nM) and MEK1/2 kinase inhibitor U0126 (1 or 5 μM), JNK kinase inhibitor SP600125 (10 or 20 μM), or p38 kinase inhibitor SB203580 (7.5 or 15 μM) for 30 min and then LPS (50 ng/ml) for 18 h. Total cell lysates were analyzed for iNOS expression by Western blot. Lower panels, cells were treated with MEK1/2 kinase inhibitor U0126 (1 or 5 μM), JNK kinase inhibitor SP600125 (10 or 20 μM), or p38 kinase inhibitor SB203580 (7.5 or 15 μM) for 30 min and then LPS (50 ng/ml) for 45 min. Phosphor-ERK levels were detected by Western blot, and JNK and p38 kinase activities were detected by kinase activity assay with Gst-ATF-2 as the substrate, and ^{32}P-labeled Gst-ATF-2 is shown.

specific inhibitor of PI_3K, wortmannin on the LPS-induced iNOS expression in mouse macrophages. Our results showed that the inhibition of PI_3K by wortmannin did suppress Akt phosphorylation, but also increased iNOS expression. However, the overexpression of wild-type Akt or DN-Akt did not change the iNOS expression in LPS-activated cells. On the other hand, inhibition of PI_3K by wortmannin could increase PtdIns(4,5)P2 to be hydrolyzed by PI-PLC, leading to subsequent enhancement of iNOS expression. The results suggest that this enhancement by wortmannin took place in an Akt-independent and PI-PLC-dependent manner in LPS-activated mouse macrophages. Both wortmannin and LY294002 inhibit PI_3K, but they are structurally distinct compounds. Several reporters have indicated that LY294002 can affect some targets besides PI_3K. For example, LY294002 decreased calcium signaling by serotonin in

airway smooth muscle cells independently of PI_3K inhibition [30]. ATF3 induction by LY294002, but not wortmannin, occurred partially through Egr-1 transcription factor and independent of the PI_3K pathway in human colorectal cancer cells [17]. Another report demonstrated that LY294002 bound with estrogen receptor and inhibited the estrogen response gene expression induced by 17β-estradiol [31]. Monocyte chemoattractant protein-1 expression was also inhibited by LY294002 but through a PI_3K-independent pathway [32]. These results suggest that, unlike wortmannin, LY294002 has target molecules besides PI_3K, and wortmannin is a more specific PI_3K inhibitor. Interesting, we found that wortmannin-enhanced iNOS expression was also independent of the PI_3K/Akt pathway and dependent on the PI-PLC pathway. Knockdown of PI_3K gene by p85 siRNA enhanced iNOS expression in LPS-activated macrophages. In contrast, activation of PI_3K by transfection of ras-p110 expression plasmid limited the enhancement of iNOS expression. Furthermore, we found that wortmannin enhanced $Ins(1,4,5)P_3$ formation in LPS-activated macrophages, and inhibition of PI-PLC limited the wortmannin-enhanced iNOS expression. This suggests that the PI-PLC pathway mediates, at least in part, wortmannin-enhanced iNOS expression in mouse macrophages.

Although several reports have demonstrated the involvement of the PI-PLC and PC-PLC in LPS-induced iNOS expression, which one participates in wortmannin-enhanced iNOS expression is still unknown. In the present study, both PI-PLC inhibitor U73122 and PC-PLC inhibitor D609 inhibited LPS-induced iNOS expression, but only U73122 markedly limited wortmannin-enhanced iNOS expression (Fig. 4A and B). Another result showed that wortmannin increased $Ins(1,4,5)P_3$ production, further suggesting that PI-PLC was indeed involved in wortmannin-enhanced iNOS expression. A recent report indicated that PI-PLC activation might be mediated by protein tyrosine kinase and that PI-PLC might have led to PtdIns(4,5) hydrolysis in LPS-activated macrophages [33]. Since it has been demonstrated that calcium had a opposed role in the regulation of iNOS expression, that either stimulatory [34,35] or inhibitory [36,37] effects. Unexpectedly, calcium seems to be uninvolved in the iNOS expression of mouse RAW264.7 macrophages in this study. When RAW264.7 macrophages were treated with calcium chelator Bapta-AM, iNOS expression showed no significant change in LPS-activated cells with or without wortmannin (Fig. 4D). The results suggest that $Ins(1,4,5)P_3$ might be mediated by signaling pathways besides calcium to induce iNOS expression.

To clarify which signal pathways were associated with wortmannin-enhanced iNOS expression, the phosphorylation level of MAPKs and IκB degradation, as well as AP-1 and NF-κB activities, were assessed by Western blot, EMSA assay, and reporter luciferase assay, respectively. We found that wortmannin enhanced the phosphorylation level of MAPKs-ERK, JNK, p38, and AP-1 binding and reporter activities (Fig. 5). However, IκB degradation and NF-κB binding and reporter activities were unchanged by wortmannin treatment. Previous studies have shown that both AP-1 and NF-κB transcription factors play a role in PI_3K-Akt downstream pathways, but NF-κB signaling is controversial in the regulation of downstream gene expression. Several reports have demonstrated that PI_3K-Akt positively or negatively regulates NF-κB-dependent gene expression, but that may be due to different cell types and different inducers. Other studies have also shown that the antiapoptotic effects of PI_3K-Akt are independent of NF-κB in human hepatocytes [38]. In this study, we demonstrated that the PI_3K/Akt pathway did not affect the NF-κB activity in wortmannin-treated cells as determined by IκB degradation, NF-κB binding assay (EMSA), and reporter plasmid assay. On the other hand, we showed that the MAPKs pathway and AP-1 transcription factor positively regulated iNOS expression in wortmannin-treated cells. The phosphorylation level of MAPKs and AP-1-dependent gene expression fell in wortmannin-treated cells with U73122. In addition, ERK inhibitor and JNK inhibitor reversed the enhancement of iNOS expression in wortmannin- and LPS-treated cells. A model of LPS induction of iNOS in mouse macrophages was shown in Fig. 7. The current study suggests that LPS stimulation of macrophages leads to activation of both the PI_3K and PI-PLC pathways, and that inhibition of PI_3K pathway by wortmannin leads to enhanced PI-PLC downstream signals. Enhancement of PI-PLC increases the formation of DAG and $Ins(1,4,5)P_3$, which then stimulates PKC, ERK and JNK, AP-1 and other unknown signaling

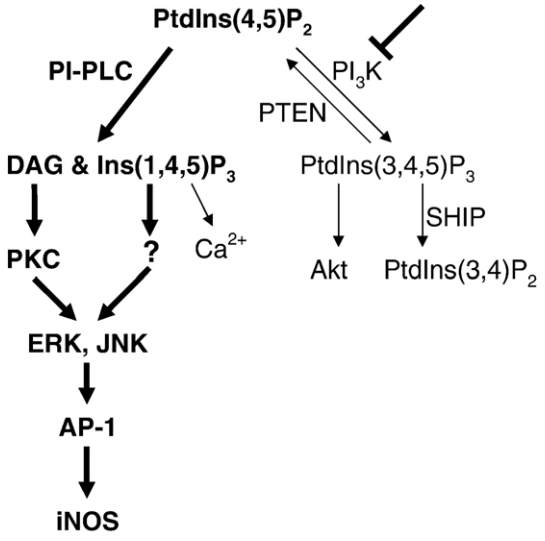

Fig. 7. The possible mechanisms for the increase of iNOS expression by inhibiting PI_3K. First, inhibition of PI_3K may lead to accumulation of $PtdIns(4,5)P_2$, resulting in increased conversion of $PtdIns(4,5)P_2$ into DAG and $Ins(1,4,5)P_3$ by PI-PLC. Second, DAG may activate PKC, and $Ins(1,4,5)P_3$ may activate unknown signals but not calcium, which in turn induces iNOS expression. $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PI_3K, phosphatidylinositol 3-kinase; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PtdIns(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SHIP, SH2-containing inositol phosphatase.

pathways, all of which cooperatively regulate iNOS gene expression.

Acknowledgments

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