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Critical Roles of the p110β Subtype of Phosphoinositide 3-Kinase in Lipopolysaccharide-Induced Akt Activation and Negative Regulation of Nitrite Production in RAW 264.7 Cells

Ken Tsukamoto,* Kaoru Hazeki,[1]* Megumi Hoshi,* Kiyomi Nigorikawa,* Norimitsu Inoue,[†] Takehiko Sasaki,[‡] and Osamu Hazeki*

It has been suggested that PI3K participates in TLR signaling. However, identifying specific roles for individual PI3K subtypes in signaling has remained elusive. In macrophages from the p110$\gamma^{-/-}$ mouse, LPS-induced phosphorylation of Akt occurred normally despite the fact that the action of anaphylatoxin C5a was impaired markedly. In RAW 264.7 cells expressing short hairpin RNA that targets p110β, LPS-induced phosphorylation of Akt was significantly attenuated. In contrast, the LPS action was not impaired, but was rather augmented in the p110α-deficient cells. Previous pharmacologic studies have suggested that a PI3K-Akt pathway negatively regulates TLR-induced inducible NO synthase expression and cytokine production. In the p110β-deficient cells, inducible NO synthase expression and IL-12 production upon stimulation by LPS were increased, whereas LPS-induced expression of COX-2 and activation of MAPKs were unaffected. Together, the results suggest a specific function of p110β in the negative feedback regulation of TLR signaling. *The Journal of Immunology,* **2008, 180: 2054–2061.**

Innate immune reactions are triggered through TLRs. The mammalian family of TLRs includes 13 members that recognize a variety of microbial products, such as LPS, peptidoglycans, flagellin, and unmethylated CpG motifs in bacterial DNA (1, 2). TLR signaling, which leads the production of various cytokines and nitrite, begins in the Toll-IL-1R (TIR)[2] domain, a motif of ~200 aa found in the cytosolic regions of all TLRs and IL-1R (2). Adaptor molecules, which also contain the TIR domain, bind to the TIR domain of TLRs due to a TIR-TIR interaction upon stimulation (3). Currently, the TLR adaptor family is known to consist of four members (MyD88, TIRAP/Mal, TICAM-1/TRIF, and TICAM-2/TRAM) (3). Studies in mice lacking each of these individual adaptors have revealed their specific roles (3). MyD88 is shared by all TLRs, except TLR3, whereas TIRAP/Mal binds to TLR2 and TLR4 (3). Likewise, TICAM-1/TRIF binds to TLR3 and TLR4, whereas TICAM-2/TRAM binds only to TLR4 (4, 5). 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 major classes I, II, and III based on their primary sequences, mechanisms of regulation, and substrate specificities. Of the class I PI3Ks, class Ia subtypes are heterodimers that consist of a catalytic subunit (p110) and a regulatory subunit (p85) (6). These subtypes are thought to be the major in vivo source of phosphatidylinositol (3,4,5)-trisphosphate upon activation of the receptors possessing protein-tyrosine kinase activity or the receptors coupling to Src-type protein-tyrosine kinases (6). In mammals, there are multiple isoforms of class Ia PI3Ks (6). Different genes encode class Ia catalytic subunits, referred to as p110α, p110β, and p1110δ, whereas two genes encode the associating regulatory subunits, referred to as p85α and p85β. Targeted disruption of either p110α or p110β causes death at the early embryonic stage (7). Although the regulatory p85α and p85β subunits can compensate for each other during development, disruption of both p85α and p85β genes is lethal (8). For these reasons, the role of individual isoforms of PI3K of this class in signaling is poorly understood.

Class I PI3K includes another member, PI3Kγ, which is mainly expressed in hemopoietic cells (9–13). This subtype consists of a catalytic subunit (p110γ) and a regulatory subunit (p101), and is classified as class Ib. PI3Kγ can be activated by the $\beta\gamma$ subunits of G proteins, and thus mediates the signal from G protein-coupled receptors (14). Although this property was originally thought to be the specific function of PI3Kγ, this opinion has been challenged by the finding that p110β is also activated by the $\beta\gamma$ subunits (11, 15, 16). Mice lacking p110γ have been successfully created (12, 13, 17). Phagocytes from these mice have defects in their response to fMLP and the complement component, C5a, each of which are known to activate G protein-coupled receptors (12, 13, 17).

Activation of PI3K is the common event observed after stimulation with various TLR ligands (18). Recently, glycogen synthase kinase (GSK)-2 and GSK-3β was proposed as a key downstream target of PI3K to regulate TLR-mediated cytokine production (19, 20). PI3K-dependent activation of serine-threonine protein kinase Akt leads to the phosphorylation of GSK-3β, which is active in

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[2] Abbreviations used in this paper: TIR, Toll-IL-1R; GSK, glycogen synthase kinase; COX, cyclooxygenase; poly(I:C), polyinosinic-polycytidylic acid; IKK, IκB kinase; iNOS, inducible NO synthase; Malp, macrophage-activating lipopeptide; PTX, pertussis toxin; shRNA, short hairpin RNA.

resting cells but inactivated by phosphorylation (21). GSK-3β has been linked to the regulation, either positive or negative, of a number of transcription factors, including NF-κB, AP-1, NF-AT, and CREB (20). Therefore, the altered activity of GSK-3β is expected to cause diverse effects on cytokine expression. Generally, activation of PI3K results in the inhibition of proinflammatory events, such as the expression of IL-12 and TNF-α (19). Thus, PI3K plays a role as a negative feedback regulator of TLR-dependent inflammatory responses. However, it is not known what subtype of PI3K is activated upon TLR stimulation. We have previously suggested that class Ia PI3K may be involved in TLR-mediated signaling using the mutant form of p85 (22). In the present study, we used RAW 264.7 cells that were transfected with short hairpin RNA (shRNA) targeting p110α or p110β to further clarify the roles of the PI3K subtypes. The results demonstrated that the subtype involved in the negative regulation is in fact p110β. In p110β-deficient cells, TLR-mediated Akt activation was abolished, and in turn, TLR-induced inducible NO synthase (iNOS) expression was dramatically enhanced in the cells.

Materials and Methods

Reagents

Materials were obtained from the following sources: LPS (*Escherichia coli* serotype O111:B4), BSA (fatty acid-free), Con A, and C5a (Sigma-Aldrich); Griess Romijin Nitrite Reagent (Wako Biochemicals); wortmannin (Kyowa Medex); polyinosinic-polycytidylic acid (poly(I:C); Amersham Biosciences); CpG DNA (Cy3-oligonucleotides with sequence of TCC ATG ACG TTC CTG ATG CT; synthesized by Hokkaido System Science); RPMI 1640 medium (Invitrogen Life Technologies); protein assay kit (Bio-Rad); Abs against phosphorylated Akt (Ser^{473}), phospho-Erk1/2 (Thr^{202}/Tyr^{204}), phospho-p38MAPK, phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), phospho-IκB kinase (IKK)$\alpha\beta$ (Ser^{180}/Ser^{181}), and iNOS (Cell Signaling Technology); anti-Akt1/2, anti-p110β, anti-p110γ, and anti-cyclooxygenase (COX)-2 Abs (Santa Cruz Biotechnology); anti-$p67^{phox}$ and anti-p110α (BD Biosciences Clontech); and anti-p85α polyclonal Ab prepared by immunizing rabbits with full-length GST-p85α. A synthetic lipopeptide based upon the full-length macrophage activating lipopeptide of 2 kDa (Malp2) was prepared with dipalmitoyl-*S*-glyceryl cysteine (Pam_2Cys), as described (23).

Cells

Female C57/BL6 mice, 8- to 12-wk-old, were purchased from Japan SLC. PI3K$\gamma^{-/-}$ mice with a C57/BL6 background were bred at Akita University (Akita City, Japan); peritoneal macrophages were harvested from these mice. Briefly, mice were injected i.p. with 2 ml of 3% thioglycolate broth. After 3 days, the peritoneal exudate cells were harvested by washing the peritoneal cavity with ice-cold PBS. The cells were seeded at $\sim 5 \times 10^5$ cells/well in 24-well plates to adhere to dishes and incubated in humidified 5% CO_2 at 37°C for 1–2 h in RPMI 1640 medium supplemented with 10% FCS. Nonadherent cells were washed with PBS and the attached cells were designated as macrophages. The mouse macrophage-like cell line, RAW 264.7 cells, were maintained in RPMI 1640 medium fortified with 2.5 g/L glucose and 10% FCS in humidified 5% CO_2 at 37°C.

Stimulation of the cells

For short-term incubation (0–60 min), culture medium (RPMI 1640) of RAW 264.7 cells or mouse peritoneal macrophages in 24-well plates were aspirated and replenished with incubation buffer (RPMI 1640 without sodium bicarbonate, fortified with 20 mM HEPES). The cells were treated with or without 1 μM wortmannin for 15 min followed by stimulations with TLR ligands (100 ng/ml LPS, or stated otherwise), 50 ng/ml C5a, or 0.1 μM calyculin A in a water bath incubator at 37°C in ambient conditions. For the assessment of nitrite, iNOS, COX-2, or IL-12 production, the cells were incubated with or without 1 μM wortmannin for 15 min, and then with or without the additions of 1–100 ng/ml LPS in normal complete medium in humidified 5% CO_2 at 37°C. The periods of incubation were 6 h for COX-2 induction, 15 h for nitrite and iNOS productions, and 20 h for IL-12 production. When IL-12 production was determined, 50 ng/ml mouse recombinant IFN-γ (PeproTech) was added with LPS.

RNA interference

Sets of oligonucleotides were cloned into the pH1 vector downstream of the human H1 RNA promoter to express p110α or p110β siRNA hairpins, as previously described (24). The following sequences were used: 5′-A-A-G-A-A-C-A-A-G-G-G-C-G-A-G-A-T-A-T-A-T-3′ for p110α and both 5′-A-A-G-A-A-G-C-A-G-C-C-G-T-G-T-T-A-T-T-A-T-3′ and 5′-A-A-G-T-G-G–A-A-T-A-A-A-C-T-T-G-A-A-G-A-T-3′ for p110β. RAW 264.7 cells (5–10 $\times$ 10^6 cells) were transfected by electroporation in a 300 μl of final volume at 250 V/950μF (Gene Pulser II; Bio-Rad). Twenty-four hours after transfection, puromycin (5 μg/ml) was added to the cells for selection, and incubation was continued for several more days. The resistant colonies were replated to 96-well plates at 0.5–1.0 cell/well and cultured for an additional week to obtain monoclonal p110α- or p110β-deficient cells. To determine the efficiency of gene silencing, total RNA was isolated with RNeasy (Qiagen) and mRNA was assessed by RT-PCR. Control cells were prepared in the same way with pH1 vector containing a 400 bp staffer sequence instead of probe sequence.

Western blotting

Cells were washed with PBS 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 (Na_3VO_4), 1 mM EDTA, 0.1% BSA, 20 mM sodium fluoride, 1 mM PMSF, 2 μM leupeptin, 20 μM *p*-amidino-PMSF, and 1 mM DTT. The cell lysates were centrifuged at 15,000 rpm for 10 min. Protein concentrations in the resultant supernatants were determined with a Bio-Rad assay kit. Total cell lysates (100 μg of protein) were mixed with 10 μl of 5$\times$ sample buffer (62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, 5% 2-ME, and 0.02% bromophenol blue) and heated at 100°C for 3 min. The proteins were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% skim milk and incubated with appropriate Abs. The associated Abs were detected by ECL (PerkinElmer). Quantification of the data was performed with NIH image and the intensity of bands was calculated as a relative value to the intensity in calyculin A-treated cells.

Immunoprecipitation

Cell lysates were prepared as described, and incubated with the appropriate antiserum for at least 60 min on ice. A slurry of prewashed protein A-Sepharose CL-4B (30% slurry, 50 μl) was added to each sample and incubated on a rotator at 4°C for an additional 2 h. The samples were spun briefly in a microfuge and washed four times with ice-cold lysis buffer.

Nitrite production

RAW 264.7 cells in a 96-well plate were treated or untreated with 1 μM wortmannin, then stimulated with various concentrations of LPS for 15 h. The culture supernatants (50 or 100 μl) were transferred to a 96-well assay plate, mixed with Griess reagent, and incubated for 15 min at room temperature. The absorbance (520 nm) of the mixture was measured by a plate reader.

IL-12 production

RAW 264.7 cells in a 96-well plate were treated with 100 ng/ml LPS and 50 ng/ml mouse recombinant IFN-γ for 20 h. The culture supernatants were harvested and IL-12 was determined by ELISA kit (BioSource International).

Results

TLR-mediated activation of PI3K

Incubation of RAW 264.7 mouse macrophages with a TLR4 ligand, LPS, resulted in Akt phosphorylation, which peaked at 15 min and then gradually declined (Fig. 1*A*). Phosphorylation of IKK also peaked at 15 min. Akt phosphorylation was susceptible to the PI3K inhibitor wortmannin, whereas IKK phosphorylation was not (Fig. 1*A*). Ligation of TLR2 with Malp2 or peptidoglycan also induced Akt phosphorylation (Fig. 1*B*). A TLR3 ligand, poly(I:C), showed a similar effect (Fig. 1*B*). The effects of these TLR ligands on Akt were inhibited by wortmannin, whereas IKK phosphorylation remained unaffected (data not shown). These results confirmed the previous reports indicating that activation of the PI3K-Akt pathway is the common event observed after stimulation of a variety of TLRs (18, 25).

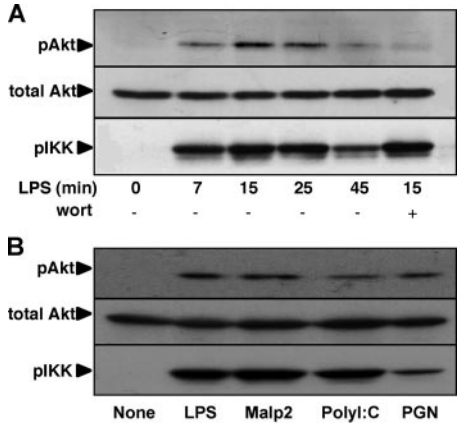

FIGURE 1. Phosphorylation of Akt in response to TLR ligands. *A*, RAW 264.7 cells were untreated (−) or treated (+) with 1 μM wortmannin (wort) for 15 min, followed by stimulation with 100 ng/ml LPS for the indicated times. *B*, RAW 264.7 cells were incubated with 100 ng/ml LPS for 15 min, 0.2 μM Malp2 for 10 min, 50 μM poly(I:C) for 30 min, or 50 μg/ml peptidoglycan (PGN) for 10 min. Whole cell lysates were analyzed by Western blotting with the Ab against the phosphorylated Akt (pAkt). The membranes used for the blotting were reprobed with anti-Akt (total Akt). The same membranes were also reprobed with anti-phospho-IKKαβ (pIKK). The experiments were repeated three times with similar results.

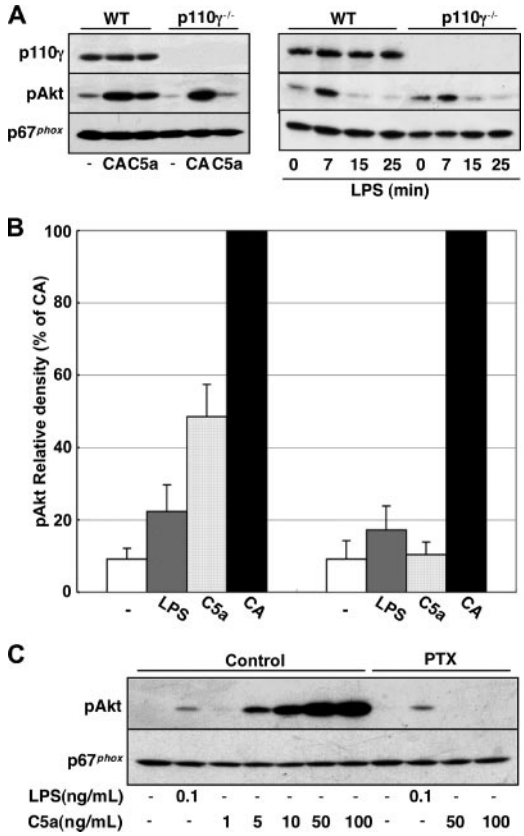

FIGURE 2. Irrelevance of p110γ in LPS-induced phosphorylation of Akt. *A*, Peritoneal macrophages from wild-type or p110γ$^{-/-}$ mice were stimulated with 0.1 μM calyculin A (CA) for 15 min, 50 ng/ml C5a for 2 min, or 100 ng/ml LPS for the indicated times. Whole cell lysates were analyzed by Western blotting with anti-p110γ, anti-phospho-Akt, and anti-p67^{phox}. The experiment was repeated three times with similar results. *B*, The results of Akt phosphorylation in *A* were quantified by NIH image and calculated as a percentage of relative intensity to that of calyculin A (CA). Results from three separate experiments are shown as mean ± SD. *C*, RAW 264.7 cells were incubated in the absence (−) or presence (+) of 100 ng/ml PTX for 18 h. The cells were then stimulated with 100 ng/ml LPS for 15 min or the indicated concentrations of C5a for 2 min. Whole cell lysates were analyzed by Western blotting with anti-phospho-Akt and anti-p67^{phox}. The experiment was repeated three times with similar results.

Irrelevance of p110γ in TLR-induced Akt activation

An anaphylatoxin, C5a, increased Akt phosphorylation in mouse macrophages. This effect was markedly attenuated in the macrophages from the p110γ$^{-/-}$ mouse (Fig. 2*A*). In contrast, the actions of LPS and a phosphatase inhibitor, calyculin A, were not impaired in the p110γ$^{-/-}$ cells (Fig. 2, *A* and *B*). The effects of other TLR ligands, such as Malp2 and CpG, were again not impaired in p110γ$^{-/-}$ cells (data not shown), indicating that p110γ is not involved in TLR-mediated Akt activation. When Akt phosphorylation was examined in cells other than thioglycolate-elicited macrophages (GM-CSF-derived bone marrow cells, untreated bone marrow cells, or spleen cells), the effect of C5a was completely abolished in the cells from p110γ$^{-/-}$ mice (data not shown). These results confirmed that the p110γ subtype is essential for C5a-induced Akt phosphorylation. Of these cells, GM-CSF-derived bone marrow macrophages were found to respond to LPS, though very weakly, and this response was not impaired in the p110γ-deficient cells (data not shown).

Pertussis toxin (PTX) is known to impair the function of G_i by ADP-ribosylating the α-subunit of the heterotrimeric GTP-binding protein (26). As expected, the effect of C5a, an agonist of GTP-binding protein-coupled receptors, was attenuated by PTX treatment (Fig. 2*C*). In sharp contrast, the LPS action on Akt phosphorylation was unaffected. Because p110γ is activated by the βγ subunits of GTP-binding proteins, the result further argued against the involvement of p110γ in the action of LPS. Although the B oligomer of PTX has a lectin-like effect at high concentrations (27), PTX by itself did not cause Akt activation or nonspecific augmentation of protein expressions at the conditions we used (Fig. 2*C*).

Preparation of Δp110α and Δp110β cells

To identify the PI3K subtypes responsible for TLR-mediated Akt activation, we prepared RAW 264.7 cells deficient in p110α or p110β (Δp110α or Δp110β, respectively). The pH1 vectors expressing shRNA targeting either of these PI3K subtypes were transfected to produce these cells. The control cells that were transfected with a control pH1 vector showed the presence of p110α, p110β, p110γ, and p85 (Fig. 3*A*). In Δp110α and Δp110β cells, either p110α or p110β was completely abolished, respectively (Fig. 3*A*). The amount of p85 or p110γ was unchanged among these cells (Fig. 3*A*). The whole cell lysates were next subjected to immunoprecipitation with the Ab against the p85 regulatory subunit of class Ia PI3K, and the immune complex was analyzed for the catalytic subunits (Fig. 3*B*). The result indicated that both the p85-p110α and the p85-p110β complex exist in RAW 264.7 cells. In Δp110α cells, the regulatory p85 subunit bound only p110β as the catalytic subunit. In contrast, p85 in Δp110β cells bound only p110α as the catalytic subunit.

Involvement of p110β in TLR-induced Akt activation

In Δp110α cells, LPS-induced Akt phosphorylation was enhanced, even though the total amount of Akt was unchanged (Fig. 4*A*). Inhibition of PI3K by wortmannin completely abolished LPS action (Fig. 4, *A–C*). In sharp contrast, the LPS-induced Akt phosphorylation was impaired markedly in Δp110β cells (Fig. 4, *B* and

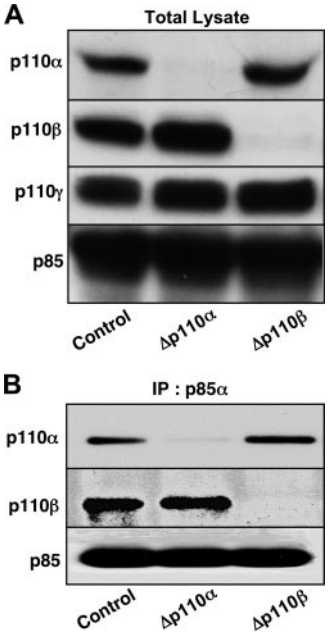

FIGURE 3. Preparation of RAW 264.7 cells deficient in p110α or p110β. RAW 264.7 cells were transfected with 20 μg of an shRNA vector targeting p110α (Δp110α), 10 μg each of two shRNA vectors targeting p110β (Δp110β), or 20 μg of control vector (control) by electroporation. The cells resistant to puromycin were selected and cloned as in *Materials and Methods*. *A*, Whole cell lysates were analyzed by Western blotting with anti-p110α, anti-p110β, anti-p110γ, and anti-p85. *B*, Cell lysates were mixed with anti-p85α and the immune complexes were analyzed by Western blotting with anti-p110α, anti-p110β, and anti-p85.

C). The machinery that directly phosphorylates Akt may not be affected in both cells, because a serine/threonine phosphatase inhibitor, calyculin A, induced Akt phosphorylation normally (Fig. 4, *A* and *B*). Although the extent of the LPS-induced Akt activation was quite different among these cell lines, the time course of the activation were essentially identical over the 5–45 min incubation period (Fig. 4*D*). The effects of other TLR ligands, Malp2, CpG, and poly(I:C), were all impaired in Δp110β cells and enhanced in Δp110α cells (data not shown).

Con A-induced Akt phosphorylation was unaffected in Δp110α cells (Fig. 4*A*). In contrast, its action was observable, but severely attenuated, in Δp110β cells (Fig. 4*B*). Thus, the effect of Con A is, like TLR ligands, mediated primarily by p110β. The weak Akt phosphorylation was still observable in Δp110β cells and may be mediated by p110γ because it has been reported that Con A-induced phosphatidylinositol (3,4,5)-trisphosphate generation is through both phosphotyrosine- and G$\beta\gamma$-mediated mechanisms (28).

Irrelevance of PI3K to LPS-induced activation of MAPK cascades

Stimulation of RAW 264.7 cells with LPS induced the phosphorylation of p38MAPK, Erk1/2, and SAPK/JNK (Fig. 5). These effects of LPS may not be dependent on PI3K because wortmannin did not inhibit the activation (Fig. 5). The activation of MAPKs was almost identical among wild-type, Δp110α (Fig. 5, *A* and *C*), and Δp110β cells (Fig. 5, *B* and *C*). The results indicated that signal transduction systems, other than PI3K-dependent systems, were not influenced by the transfection of the shRNA probes used. These results were reproducible with multiple clones of Δp110α and Δp110β cells (data not shown).

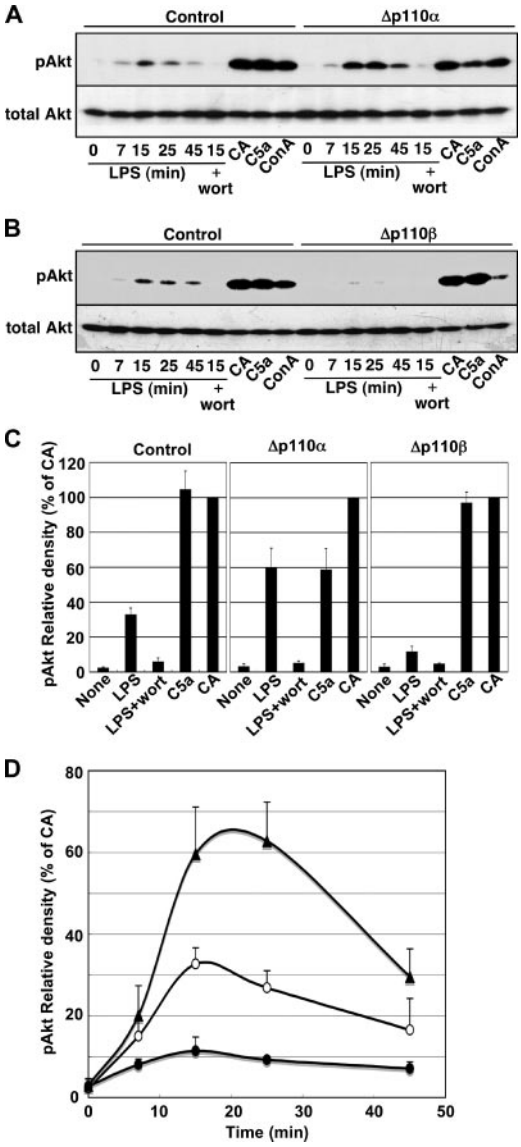

FIGURE 4. Failure of LPS to activate Akt in Δp110β cells. *A* and *B*, Control RAW 264.7 cells, Δp110α cells (*A*), or Δp110β cells (*B*) were untreated or treated with 1 μM wortmannin (+wort) for 15 min, followed by stimulation with 100 ng/ml LPS for the indicated times, 0.1 μM calyculin A (CA) for 15 min, 50 ng/ml C5a for 2 min, or 1 mg/ml Con A for 10 min. Whole cell lysates were analyzed by Western blotting with the Ab against phosphorylated Akt (pAkt). The membrane used for the blotting was reprobed with anti-Akt (total Akt). The experiment was repeated three times with similar results. Experiments using other colonies of Δp110α and Δp110β cells (two colonies each) showed similar results. *C* and *D*, The results of Akt phosphorylation in *A* and *B* were quantified by NIH image and calculated as a percentage of the relative intensity to that of calyculin A (CA). Results from three separate experiments are shown as mean $\pm$ SD. In *D*, control vector (○), Δp110α (▲), and Δp110β (●) are shown.

Augmentation of iNOS expression and nitrite production in Δp110β cells and their inhibition in Δp110α cells

Treatment of control RAW 264.7 cells with LPS caused a marked expression of iNOS (Fig. 6*A*). Interestingly, the iNOS expression was augmented in Δp110β cells, where the TLR-induced activation of Akt was severely impaired (see Fig. 4*B*). In contrast, Δp110α cells with the enhanced Akt activation (see Fig. 4*A*) showed impaired expression of iNOS. These results confirmed the previous reports suggesting the negative regulatory role of Akt in

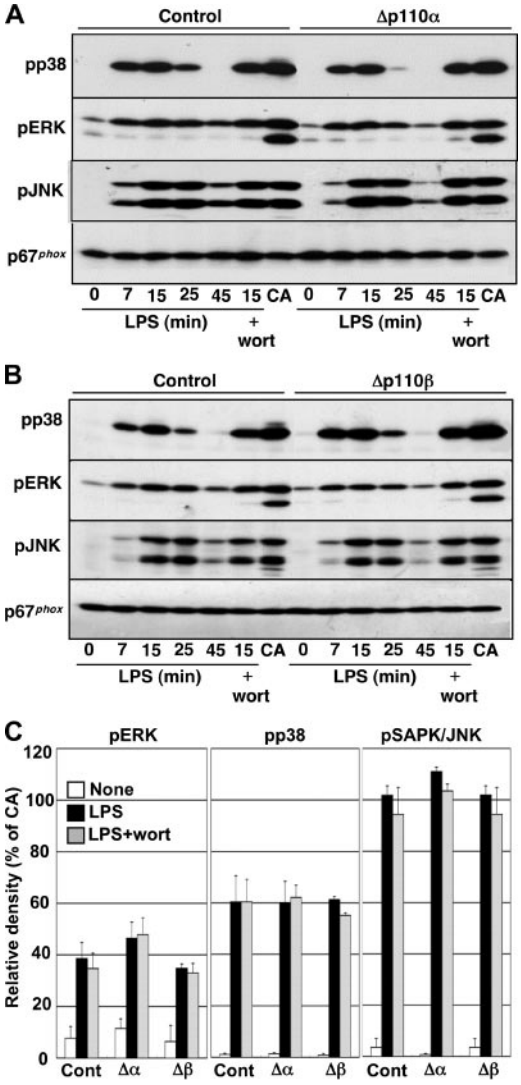

FIGURE 5. Irrelevance of PI3K to LPS-induced activation of MAPK cascades. *A* and *B*, Control RAW 264.7 cells, Δp110α cells (*A*), and Δp110β cells (*B*) were untreated or treated with 1 μM wortmannin (+wort) for 15 min, followed by stimulation with 100 ng/ml LPS or 0.1 μM calyculin A (CA) for 15 min. Whole cell lysates were analyzed by Western blotting with Ab against the phosphorylated form of p38MAPK (pp38). The membrane used for the blotting was reprobed with anti-phospho-Erk1/2 (pERK), anti-phospho-SAPK/JNK (pJNK), and anti-p67^{phox}. The experiments were repeated three times with similar results. *C*, The results in *A* and *B* were quantified by NIH image and calculated as a percentage of the relative intensity to that of calyculin A (CA). Results from three separate experiments are shown as mean ± SD.

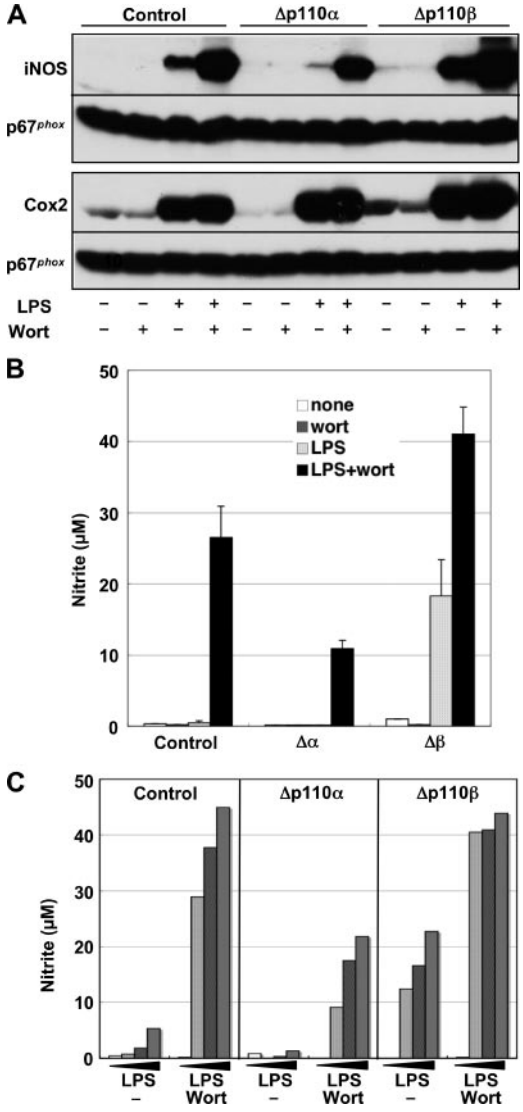

FIGURE 6. Enhancement of LPS-induced nitrite production in Δp110β cells. *A*, RAW 264.7 cells were incubated in the absence (−) or presence (+) of 1 μM wortmannin (wort) for 15 min, and then with (+) or without (−) the addition of 10 ng/ml LPS for 6 h (COX2) or for 15 h (iNOS). Whole cell lysates were analyzed by Western blotting with anti-iNOS or anti-COX-2. The membranes used for the blotting were reprobed with anti-p67^{phox}. The experiments were repeated three times with similar results. *B*, RAW 264.7 cells were incubated in the absence or presence of 1 μM wortmannin (wort) for 15 min, and then with the addition of 1 ng/ml LPS for 15 h. Accumulation of nitrite in the supernatants was determined using Griess reagent. Results from three separate experiments each performed in duplicate are shown as mean ± SD. *C*, The cells were treated as in *B* except that increasing concentrations (0, 1, 3, and 10 ng/ml) of LPS were used. Data shown indicate the mean value of duplicate determinations. A repeated experiment showed a similar result.

TLR-induced iNOS expression (22). Akt may play only a minor role in the regulation of COX-2 because LPS-induced expression of COX-2 was not significantly changed among these cells (Fig. 6*A*). Treatment of these cells with wortmannin effectively augmented the LPS-induced iNOS expression without a marked effect on COX-2 expression, regardless of the cell type. Fig. 6*B* shows the results obtained when the nitrite production of these cells was determined. As expected, nitrite production in response to LPS was enhanced in Δp110β cells (Fig. 6*B*). We have previously reported that the enhancing effect on nitrite production by the inhibition of PI3K is prominent when lower concentrations of LPS are used (22). Actually, the effect of wortmannin or that of p110β deficiency was to a lesser extent at optimal stimulation, but it was still observed at 1–10 ng/ml LPS (Fig. 6*C*). The p110α-deficient cells hardly produced nitrite in response to LPS in the absence of wortmannin (Fig. 6*C*).

Augmentation of IL-12 production in Δp110β cells and its inhibition in Δp110α cells

We have reported that the inhibition of PI3K results in an augmentation of IL-12 and IL-6 production in human monocyte-derived macrophages (22). Therefore, it is intriguing to find IL-12 production in the PI3K-deficient cells. Control RAW 264.7 cells showed a detectable increase of IL-12p40 production in response

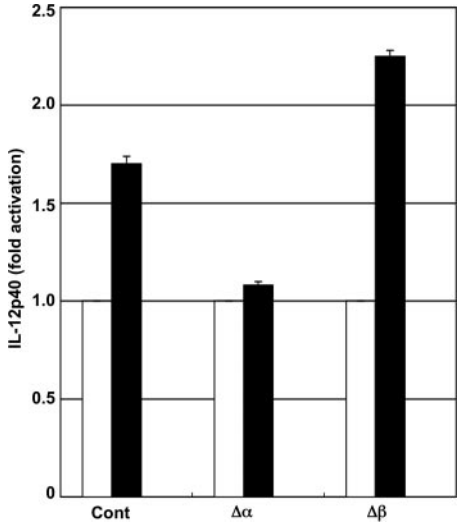

FIGURE 7. Enhancement of LPS-induced IL-12p40 production in Δp110β cells. RAW 264.7 cells were incubated in the presence (■) or absence (□) of 100 ng/ml LPS plus 50 ng/ml mouse recombinant IFN-γ for 20 h. IL-12p40 in the culture supernatants were determined by ELISA kit. Data are the fold activation over those observed in the absence of LPS. Results from three separate experiments, each of which were performed in duplicate, are shown as mean ± SD.

to LPS (Fig. 7), although the level of cytokine production was very low compared with that of primary mouse macrophages. LPS-induced IL-12p40 production was increased in Δp110β cells. In contrast, LPS hardly increased the cytokine production in Δp110α cells (Fig. 7).

Discussion

There are a few reports concerning the specific functions of PI3K subtypes in TLR signaling. The class III PI3K is known to regulate CpG-induced (TLR9 ligand) cytokine production and NF-κB activation (29). TLR9 exists in cytosolic compartments in the vicinity of the plasma membrane in dendritic cells (30). Therefore, to stimulate their receptors, ligands of TLR9 should be incorporated into the cells by endocytosis. Because class III PI3K has been implicated in vesicle trafficking (31), it is expected that TLR9-mediated events are impaired by PI3K inhibitors at an initial step before receptor ligation. Actually, class III PI3K acts upstream of MyD88 and regulates CpG uptake; this is in sharp contrast to class I PI3Ks that act downstream of MyD88 (29). It has been shown that LPS-induced activation of Akt and NF-κB is abrogated in neutrophils obtained from p110$\gamma^{-/-}$ mice (32). The authors have also shown that the in vivo administration of endotoxin activates Akt in lung edema neutrophils and that this activation is abrogated in p110$\gamma^{-/-}$ mice (32). These results suggest the specific role of p110γ in TLR signaling. In the present study, however, we observed that Akt in macrophages from p110$\gamma^{-/-}$ mice respond normally to LPS (Fig. 2, *A* and *B*). The irrelevance of p110γ in LPS-mediated Akt activation was further supported by the fact that the effect of C5a, an agonist of G protein-coupled receptors, was attenuated by PTX treatment, whereas LPS induction of Akt phosphorylation was unaffected (Fig. 2*C*). Although the reason for this apparent contradiction between neutrophils and peritoneal macrophages is currently unknown, it is of interest to note that a previous report also indicates that LPS increases PI3K activity that associates with p85 (the regulatory subunit of class Ia PI3K) in the neutrophils (32). This fact seems to suggest that the class Ia subtype plays some role in the LPS-signaling of neutrophils.

Involvement of PI3K subtypes, besides p110γ, in TLR-mediated activation of Akt has been suggested in previous studies. A dominant negative mutant of the regulatory subunit of class Ia PI3K (Δp85) has suggested a role for this subclass (18). Involvement of this subclass has also been shown in macrophages obtained from p85$\alpha^{-/-}$ mice (33). However, the isoform of class Ia PI3K responsible for the regulation has not been identified because the targeted disruption of either p110α or p110β in animals causes death at an early embryonic stage (34). In the present study, RAW 264.7 cells were transfected with the pH1 vector that produces p110α or p110β siRNA. Cells showing complete depletion of either subtype were cloned and finally established as Δp110α and Δp110β cells (Fig. 3). Cells with minor defects in the subtype expression did not show any apparent changes in TLR-mediated phosphorylation of Akt (data not shown). The Akt activation was impaired in Δp110β cells, suggesting the role of p110β in transmitting the TLR signal to Akt (Fig. 4, *B–D*). The result also indicated that the p85-p110α complex was unable to activate Akt in response to TLR ligands. Unexpectedly, Akt phosphorylation was significantly enhanced in Δp110α cells (Fig. 4, *A*, *C*, and *D*). Although the reason is not known, this enhancement has been observed reproducibly in multiple clones of Δp110α cells (data not shown). A similar enhancement of p110β-mediated response in Δp110α cells has also been reported in Chinese hamster ovary cells (16).

There are many reports showing that TLR ligation activates PI3K. However, there have been conflicts over the physiologic consequences of this phenomenon (18). The inhibition of PI3K has been reported to result in the inhibition of TLR2-mediated activation of NF-κB (35) or TLR3-mediated activation of IFN regulatory factor-3 (36). Activation of PI3K is also needed for LPS-induced IL-1β expression (37). PI3K activation is indispensable for LPS-induced (38) and CpG-induced (39) activation of NF-κB. These reports indicate a positive regulatory role of PI3K in TLR signaling. In contrast, many lines of evidence have attributed a negative regulatory role to PI3K. LPS-induced nitrite production is increased in the presence of PI3K inhibitors (40). Gene targeting of the regulatory subunit of PI3K (p85α), as well as treatment with PI3K inhibitors, results in augmentation of LPS-induced IL-12 production (33). TRIF-dependent NF-κB activation and IFN-β synthesis are enhanced by PI3K inhibition in dendritic cells (41). We have also reported that TLR2- or TLR4-mediated production of IL-12 and IL-6 in human monocyte-derived macrophages, as well as TLR2-, TLR3-, TLR4-, and TLR9-mediated production of nitrite and several cytokines, including IFN-β in mouse macrophages, are all enhanced by inhibition of PI3K (22). In the present study, we observed that Δp110β cells with impaired Akt activation (Fig. 4*B*) exhibited an augmented expression of iNOS (Fig. 6*A*), whereas Δp110α cells with enhanced Akt activation (Fig. 4*A*) had impaired expression of iNOS (Fig. 6*A*). These results argue for a negative regulatory role of the PI3K-Akt system in TLR-induced events. Another intriguing finding in the present study is that a PI3K inhibitor, wortmannin, markedly augmented LPS-induced iNOS expression and nitrite production in both Δp110α and Δp110β cells. Because Akt activation is minimal in Δp110β cells, this effect of wortmannin may not have been due to the inhibition of the PI3K-Akt system. Thus the result suggests that inhibition of the Akt-mediated negative feedback system (22) is not the sole mechanism by which wortmannin causes the dramatic augmentation of nitrite production. Wortmannin has been shown to inhibit class II and III subtypes of

PI3K (6). The compound is also known to inhibit myosin L chain kinase at higher concentrations (42). Such actions may be contributing to the enhanced cell response to LPS.

PI3Ks of the class Ia subtype are activated when the Src homology 2 domains of p85 bind the tyrosine-phosphorylated proteins that possess a pYXXM motif (6). Several reports indicate that p85 binds the receptor itself to activate PI3K. For example, TLR2 is tyrosine-phosphorylated within the YXXM motif of its TIR domain and directly binds p85 (35). Likewise, the TIR domain of TLR3 is phosphorylated on the tyrosine residue. Although the tyrosine residue is not within the optimal consensus sequence for p85 binding, this phosphorylation has been reported to be able to recruit p85 (36). Most TLRs, at least TLR2, TLR3, TLR4, TLR5, and TLR9, activate PI3K. This fact suggests that adaptor proteins shared by TLRs may play a role in activating PI3K. One candidate protein is MyD88, an adaptor shared by all TLRs except TLR3. In fact, MyD88 associates with p85 in response to flagellin and LPS (43). MyD88, with a point mutation in its YXXM motif (Y257F), fails to interact with p85 (44). It is reported that IL-1β-, IL-18-, and LPS-induced activation of PI3K is diminished in IL-1R-associated kinase 1-deficient cells (45). Because IL-1R-associated kinase 1 is known to directly bind MyD88, the protein kinase may play a role in MyD88-dependent PI3K recruitment. In the present study, we showed that p110β is specifically used in TLR signaling; however, the basis for this specificity is not clear based on the mechanisms described, especially because p110α and p110β use p85 as a common regulatory subunit. Although little is known concerning the difference in the biochemical properties among class Ia PI3K subtypes, recent studies have reported the different physiological functions of the subtypes, including the specific function of p110α in insulin receptor signaling (46). We have also observed that p110α, but not p110β, is indispensable in the engulfment of macrophages (our unpublished data). Although the domain structures of class Ia PI3K subtypes are similar, there are several regions with sequence heterogeneity. Future studies using chimeric enzymes or determining subtype-specific localization may help elucidate the specific physiological functions of each subtype.

Disclosures

The authors have no financial conflict of interest.

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