Phosphoinositide 3-Kinase in Nitric Oxide Synthesis in Macrophage

CRITICAL DIMERIZATION OF INDUCIBLE NITRIC-OXIDE SYNTHASE*

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Phosphoinositide 3-kinase (PI3K) has important functions in various biological systems, including immune response. Although the role of PI3K in signaling by antigen-specific receptors of the adaptive immune system has been extensively studied, less is known about the function of PI3K in innate immunity. In the present study, we demonstrate that macrophages deficient for PI3K (p85α regulatory subunit) are impaired in nitric oxide (NO) production upon lipopolysaccharide and interferon-γ stimulation and thus vulnerable for intracellular bacterial infection such as Chlamydia pneumoniae. Although expression of inducible nitric-oxide synthase (iNOS) is induced normally in PI3K-deficient macrophages, dimer formation of iNOS protein is significantly impaired. The amount of intracellular tetrahydrobiopterin, a critical stabilizing cofactor for iNOS dimerization, is decreased in the absence of PI3K. In addition, induction of GTP cyclohydrolase 1, a rate-limiting enzyme for biosynthesis of tetrahydrobiopterin, is greatly reduced. Our current results demonstrate a critical role of class IA type PI3K in the bactericidal activity of macrophages by regulating their NO production through GTP cyclohydrolase 1 induction.

Phosphoinositide 3-kinase (PI3K)2 is a key enzyme in various signal transduction pathways, such as cytoskeletal rearrangement, survival, and cell cycle progression (1). PI3Ks are classified into three groups according to their structure and substrate specificity. Of those groups, class IA heterodimeric PI3Ks, with each enzyme consisting of a catalytic subunit and a regulatory subunit, are involved in receptor-mediated signaling in the immune system. We and others (2, 3) have developed mice lacking the gene for p85α, the most abundant and ubiquitously expressed regulatory subunit of the class IA PI3Ks, and reported the significance of these enzymes in B cell differentiation and activation (4–6). We found that class IA PI3Ks are essential for development and activation of B lymphocytes (5, 6), granulocytes (7), platelets (8), and mast cells (9). Little is known, however, about the function of PI3K in the innate immune response (10–12).

Innate immunity is critical in self-defense for the host against microbial infection, in which macrophages play central roles in the initiation of inflammation by producing proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6, and bactericidal radical effector molecules such as nitric oxide (NO) and reactive oxygen species. The considerable susceptibility to intracellular bacteria and protozoa in mice deficient in inducible nitric-oxide synthase (iNOS) (13–15), indicated that production of NO is important in host self-defense against intracellular microbes (16). NO is generated from arginine by three isotypes of NOS family enzymes, which are controlled by three independent genes: NOS1, NOS2, and NOS3 (17). Among them, iNOS (NOS2) is responsible for NO production in macrophages. Ligands for Toll-like receptors as well as cytokines can stimulate macrophages to induce iNOS, whose expression depends on transcription factors such as nuclear factor-κB (NF-κB), AP-1, STAT1, IRF1, and NF-IL6 (18–22). NO generation by iNOS is subject to multiple levels of regulation, including transcriptional, translational, and post-translational regulation. Homodimerization is essential for iNOS activity, and dimerization requires cofactors such as calmodulin, FAD, FMN, NADPH, and heme (23). The dimers are further stabilized by binding of tetrahydrobiopterin (BH4) and the substrate arginine (23). In contrast, NAP110 and kalirin associate with iNOS and thereby inhibit iNOS dimerization (24, 25). Cytosolic arginine content is affected by many factors including arginase and cationic amino acid transporter 2 (CAT2) membrane amino acid transporter proteins (26–29). Furthermore, iNOS protein itself is actively degraded in a proteasome-dependent pathway (30). Production of NO is thus regulated by many factors (31).

PI3K inhibitors have been useful for the investigation of the effect of PI3K on NO production in macrophage cell lines (32–34). However, results from different reports have not been consistent. To clarify the precise function of PI3K in the regulation of NO production and mechanisms of host defense against microbes, we utilized our p85α-deficient mice as PI3K knock-out mice. In the present study, we found that PI3K-deficient macrophages produced less NO than wild-type macrophages after stimulation of with interferon-γ (IFN-γ) and lipopolysaccharide (LPS), and that these macrophages were further susceptible to bacterial infection.
Although production of NO decreased in PI3K-deficient macrophages, the induction of iNOS protein was unaffected. We found that the formation of dimers of iNOS protein, which are required for enzymatic activity, was remarkably impaired in PI3K-deficient macrophages. We also observed a decreased amount of BH4, which stabilizes iNOS dimers. The expression of GTP cyclohydrolase 1 (GCH1), a rate-limiting enzyme for BH4 synthesis was significantly decreased in the absence of PI3K as well. Collectively, our results indicate that class IA PI3K is important in dimerization of iNOS protein and thus critical in NO production and the innate immune response.

MATERIALS AND METHODS

Chemicals—LPS, LY294002, wortmannin, Nω-nitro-l-arginine methyl ester (L-NAME), and Nε-iminoethyl-l-lysine (l-NIL) were purchased from Sigma. IFN-γ was purchased from Peprotec (London, UK).

Animals—PI3K-deficient mice (6) on a C57BL/6 background and control C57BL/6 mice (Chiyoda, Tokyo, Japan) were housed under the specific pathogen-free condition in our mice facility.

Cell Cultures—The mice were sacrificed, and the cells were harvested from peritoneal cavities by washing with RPMI 1640 containing 10% heat-inactivated fetal calf serum, 1.0 mM sodium pyruvate, 10 mM HEPES buffer, 0.1 mM nonessential amino acid solution, 1000 units/ml penicillin, 1000 mg/ml streptomycin, and 55 μM 2-mercaptoethanol (all purchased from Invitrogen). After an overnight incubation at 37 °C in a 5% CO2 atmosphere, cells adhering to plastic dishes were used as macrophages. Mouse embryonic fibroblasts were obtained from day 14 fetuses. After the livers were removed, the fetuses were homogenized and incubated with trypsin for 30 min, then with collagenase for 30 min and finally with DNase for 30 min at 37 °C.

Chlamydial Infection—Chlamydia phila pneumoniae J138 (35) was passaged, and the samples were stored at −80 °C until use. The infection of macrophages was established by centrifugation (700 × g) at 22 °C for 1 h, followed by incubation at 37 °C in a 5% CO2 atmosphere for 1 h (36) with or without 10 μM LY294002, 50 mM wortmannin, and indicated concentrations of NOS inhibitors. Then the inoculum was aspirated and replaced with RPMI 1640 containing no penicillin or streptomycin.

Infected cells were incubated for up to 72 h.

Assessment of C. pneumoniae Infection—Infected macrophages were fixed for 15 min in 95% ethanol and stained with the Chlamydia FA test reagent (Denka Seiken Co., Tokyo, Japan) or RR402 (Washington Research Foundation, Seattle, WA) (36). A fluorescence microscopy was used to determine the number of inclusion body-forming units. At the same time, DNA from cultures was purified, and the amount of chlamydial DNA was determined by using real time PCR method (QuantiTec real time PCR kit, Qiaegen, Venio, The Netherlands). Primers for specific sequences of the chlamydial genome were 5′-CTCTGTGT-GTAGATTGCCT-3′ and 5′-GGACATTTCTACAACCTCG-3′; primers for mouse GAPDH for controls were 5′-TTTGGGTGTGTA-CATCAGACATCC-3′ and 5′-AGGTCAACAGCTTATGCAG-A3′.

Determination of Nitrite and Nitrate—Macrophages (1 × 106 cells) were treated with Dulbecco’s modified Eagle’s medium containing 1 μg/ml LPS and 100 units/ml IFN-γ with or without 10 μM LY294002, and supernatants were collected after centrifugation at 500 × g for 5 min at 4 °C. The concentration of nitrite (NO2−) + nitrate (NO3−) in cultured supernatant was determined via an NO2−/NO3− Assay Kit (Assay Design, Ann Arbor, MI). Briefly, 25-μl aliquots of supernatant were incubated with 25 μl of NADH solution and 50 μl of nitrate reductase solution for 30 min at 37 °C. An equal amount of Griess reagent was then added, and after 10 min in the dark, the color intensity of the samples was measured at 550 nm. Sodium nitrate was used to derive standard curve. The nitrite or nitrate concentration were also determined directly by high performance liquid chromatography (HPLC) coupled with a Griess reagent-flow reactor (37).

arginase Assay—Arginase activity was assessed as described previously (38). In brief, 1 × 106 cells were lysed with 50 μl of lysing solution containing 0.1% Triton X-100, 0.1% peptatin, aprotinin, and antipain for 30 min at room temperature. After 50 μl of buffer containing 10 mM MnCl and 50 mM Tris-HCl (pH 7.5) was added, arginase was activated by incubation for 10 min at 55 °C. The samples were mixed well with equal amounts of 0.5 mM l-arginine for 60 min at 37 °C, and the enzyme reaction was stopped by addition of 400 μl of a mixture of acid (H2SO4; H3PO4:H2O = 1:3:7). The urea produced was visualized by use of 25 μl of 9% of 1-phenyl-1,2-propanedione-2-oxime solved in ethanol at 100 °C for 45 min. The color intensity of the samples was measured at 550 nm. Urea (Katayama chemical, Osaka, Japan) was used to make standard curve.

Real Time RT-PCR Analysis—Real time RT-PCR was performed with QuantiTec Real-Time RT-PCR kit (Qiagen) using Light Cycler (Roche Diagnostics) to quantify mRNA. The primers (sense/antisense) were iNOS, 5′-CTGCTTTTGCAAGTGCAGT-3′ and 5′-GGACACCA-AACACCAAGCTC-3′; GCH1, 5′-GTTGTAAGACTACCTTGTCTC-3′ and 5′-CAATTCTGGCGAGTTACTGAGAC-3′; GAPDH, 5′-CAA-CTTTGTGAAACTCATTTTCTCG-3′ and 5′-CTCTCTTGTGCATGGTCTCCC-3′; AT2, 5′-GCTAGAGAACACCTTCC-3′ and 5′-AGGCA-ATAAGACAGCTT-3. Thermal cycling parameters were 15 s at 94 °C, 20 s at 55 °C, and 10 s at 72 °C, followed by fluorescence acquisition. Data were calculated as a ratio, via the reference mRNA (GAPDH).

Western Blotting—Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitor mixture (Nacalai tesque, Kyoto, Japan). Proteins were resolved by using SDS-PAGE with 7.5% gel, transferred onto a nitrocellulose filter, and probed with specific antibodies. For fully denaturing SDS-PAGE, samples were boiled. For measurement of iNOS dimer formation, samples were dissolved with sample buffer without boiling; electrophoresis was performed at 4 °C for low temperature SDS-PAGE (39 – 43). Anti-iNOS polyclonal antibody (Affinity Bioreagents, Golden, CO), anti-GAPDH antibody (Chemicon International, Temecula, CA), and anti-GCH1 antiserum (44) were used for Western blotting, and specific proteins were detected with LumiGLO Reagent and Peroxide (Cell Signaling, Beverly, MA).

Determination of the Amount of BH4—BH4 content in the cell lysate was determined as the bioperin amount produced after iodine oxidation. The samples of cell lysate were sonicated, oxidized with 1% I2 and 2% KI in 1 N HCl for 60 min in the dark at room temperature, and analyzed by HPLC with fluorescence detection (45).

RESULTS

Production of NO Induced by LPS and IFN-γ in Macrophages Depended on PI3K—Previous reports have described the effect of PI3K inhibitors on NO synthesis, but these reports have provided controversial results. We therefore investigated the effect of the PI3K inhibitor LY294002 on murine peritoneal macrophages. Although macrophages produced a decreased amount of NO, as assessed by measuring total amount of nitrite plus nitrate in the culture supernatant, simultaneous stimulation with LPS (1 μg/ml) and IFN-γ (100 units/ml) induced a large amount of NO synthesis in 2 days (Fig. 1A).

We found that LY294002 inhibited LPS and IFN-γ induced NO synthesis in murine peritoneal macrophages (Fig. 1A). An earlier study reported that wortmannin, another PI3K inhibitor, enhanced LPS-induced NO production in peritoneal macrophages (46). Because wort-
mannin has been known to inhibit many other ATP-dependent kinases, we decided to utilize our PI3K-deficient mice to investigate the effect of PI3K on NO synthesis. Although these PI3K-deficient mice still expressed p55\(^{H9251}\) and p50\(^{H9251}\), two alternative splicing products from the p85\(^{H9251}\) gene, we could detect little p50\(^{H9251}\) and p55\(^{H9251}\) protein in both wild-type and PI3K-deficient macrophages by Western blotting analysis (data not shown). Therefore, class IA-type PI3K was likely absent in the p85\(^{H9251}\)-deficient macrophages. In wild-type macrophages, production of NO increased dramatically after treatment with LPS plus IFN-\(\gamma\) for 48 h, but this increase was reduced by 10\(^{\mu\text{M}}\) LY294002 (Fig. 1A). In contrast, peritoneal macrophages from PI3K-deficient mice produced about half the amount of NO after stimulation with LPS/IFN-\(\gamma\) compared with wild-type macrophages (Fig. 1B). At the same time, production of nitrate and nitrite were measured separately by HPLC coupled with a Griess reagent-flow reactor. Stimulation-dependent production of both nitrate and nitrite were reduced in PI3K-deficient macrophages (Fig. 1C).

**Induction of iNOS Was Independent of PI3K**—Inasmuch as NO is produced by iNOS (NOS2) in macrophages, we investigated the expression of iNOS in PI3K-deficient macrophages. PI3K was reported to be involved in activation of NF-\(\kappa\)B upon LPS stimulation (47), and NF-\(\kappa\)B was reportedly one of the major transcription factors associated with transcriptional regulation of the iNOS gene. Contrary to our expectation, iNOS mRNA expression induced by LPS and IFN-\(\gamma\) was unaltered in PI3K-deficient macrophages (Fig. 2A) even though production of NO was significantly decreased (Fig. 1). Western blotting analysis also showed that induction of iNOS protein by LPS/IFN-\(\gamma\) was unaltered in PI3K-deficient macrophages (Fig. 2B). Consistent with the finding that induction of iNOS was unaffected, LPS/IFN-\(\gamma\)-induced interleukin-6 production, which also depends on NF-\(\kappa\)B activation (48) was unchanged in PI3K-deficient macrophages (data not shown).

**Substrate Availability Was Not Altered in PI3K-deficient Macrophages**—Despite the normal amount of iNOS protein, production of NO was...
is the availability of its substrate L-arginine. L-Arginine is utilized by iNOS in addition to the induction of the iNOS gene. One of these factors is activated in type macrophages. NO production by iNOS is regulated by many factors including PI3K deficiency. Dimerization of iNOS was greatly reduced in PI3K-deficient macrophages.

**FIGURE 3. Dimerization of iNOS protein was impaired in PI3K-deficient macrophages.** A, cell lysate from wild-type (WT) or PI3K-deficient macrophages after 2 days of stimulation with LPS + IFN-γ (stim) was subjected to 7.5% SDS-PAGE with or without sample boiling and followed by Western blotting with anti-iNOS antibody. Dimers and monomers of iNOS were separated by low temperature SDS-PAGE (39–43), and the ratio of dimer to monomer was calculated by densitometric analysis. B, macrophages from iNOS-deficient, PI3K-deficient, and wild-type mice were cultured with or without sample boiling and followed by Western blotting with anti-iNOS antibody. Dimers and monomers of iNOS were separated by low temperature SDS-PAGE (39–43), and the ratio of dimer to monomer was calculated by densitometric analysis. Upper band was specifically disappeared upon sample boiling, which corresponded to the monomer and the dimer of iNOS protein, respectively. Upper band was specifically disappeared upon sample boiling, which was known to induce dissociation of iNOS dimer (39, 43) (Fig. 3B), and complete absence of both of the bands in iNOS-deficient macrophages confirmed the identity of the bands (Fig. 3B). As expected, we could not detect any iNOS proteins without LPS/IFN-γ-stimulation (Fig. 3A). We found that the formation of iNOS dimer was reduced in PI3K-deficient macrophages compared with that in wild-type macrophages (Fig. 3). Densitometric analysis revealed that ratio of dimers to monomers was slightly lower than that in wild-type macrophages after stimulation. There-fore, decreased production of BH4 seems to be responsible for reduced formation of iNOS dimers. If the lower BH4 content is the cause of reduced iNOS activity, the addition of exogenous BH4 should reestablish NO synthesis in PI3K-deficient macrophages. To test this hypothesis, we added sepiapterin, an immediate precursor of BH4, to culture medium during LPS/IFN-γ stimulation. As shown in Fig. 4B, addition of sepiapterin suc-

**FIGURE 4.** The intracellular biopterin content was reduced in PI3K-deficient macrophages. A, the intracellular amount of biopterin was measured by the iodine oxidation method followed by HPLC (45) of cell lysates of LPS + IFN-γ-stimulated macrophages. The addition of sepiapterin (10 μM) to the culture medium restored stimulation-dependent NO production (B) and iNOS protein dimerization (C) in PI3K-deficient macrophages. Error bars represent S.D., and shown are the representatives of more than three experiments.
cessfully restored NO production in PI3K-deficient macrophages. At the same time, the addition of sepiapterin restored impaired iNOS dimer formation found in PI3K-deficient macrophages (Figs. 3B and 4C). All of these results indicate that lower production of BH4 is responsible for reduced NO synthesis in PI3K-deficient macrophages.

Induction of GCH1 Was Impaired in PI3K-deficient Macrophages—BH4 is synthesized from GTP by a three-step enzymatic reaction pathway, and GCH1 is a rate-determining enzyme among the three (55). Therefore, we investigated the expression of GCH1 in PI3K-deficient macrophages. Real-time RT-PCR analysis showed that transcription of the GCH1 gene was induced after LPS/IFN-γ-stimulation, and this induction was significantly lower in PI3K-deficient macrophages (Fig. 5A). Western blot analysis also showed that the amount of GCH1 protein in stimulated macrophages was significantly reduced in the absence of PI3K (Fig. 5B). These results indicate that insufficient induction of GCH1 could be responsible for reduced NO production in PI3K-deficient macrophages.

![Image](https://example.com/image.png)

**FIGURE 5.** Induction of GCH1 was impaired in PI3K-deficient macrophages. A, total RNA was extracted from LPS + IFN-γ-stimulated macrophages and analyzed for expression of GCH1 mRNA by real-time RT-PCR. The amount relative to GAPDH was calculated. B, protein expression of GCH1 in stimulated macrophages was determined by Western blotting analysis with anti-GCH1 antisera (44). Error bars represent S.D., and shown are the representative of more than three experiments. *, significant difference between samples by t test (p < 0.05).

Intracellular Bacterial Growth in PI3K-deficient Macrophages Was Increased—NO is one of the most important bactericidal effector molecules against intracellular bacteria in macrophages, so PI3K-deficient macrophages would be expected to be more susceptible to bacterial infections. To confirm this expectation, we investigated the growth and inclusion formation of *C. pneumoniae* in PI3K-deficient macrophages, as *Chlamydophila* is an obligate intracellular Gram-negative bacterium that is sensitive to NO (13, 14). As was reported earlier (56), the augmented growth of *C. pneumoniae* organisms in the presence of iNOS inhibitors in peritoneal macrophages was observed (Fig. 6A). As expected, growth of *C. pneumoniae* organisms also increased in PI3K-deficient macrophages, as determined by both the number of inclusion bodies formed (Fig. 6B) and the amount of chlamydial DNA (Fig. 6C). Treatment with PI3K inhibitors also increased chlamydial growth in wild-type macrophages (Fig. 6C). Such increases were observed only in macrophages, however, chlamydial infection was reduced in PI3K-deficient mouse embryonic fibroblasts and LY294002-treated wild-type mouse embryonic fibroblasts (Fig. 6D).

**FIGURE 6.** Chlamydial growth increased in PI3K-deficient macrophages. A, Macrophages were infected with *C. pneumoniae* in the presence or absence of an NOS inhibitor, L-NIL (75 or 100 nM) or L-NAME (0.3 μM). The amount of chlamydial DNA was measured by real-time PCR. B, peritoneal macrophages from wild-type and PI3K-deficient mice were infected with *C. pneumoniae* and incubated for 3 days. Chlamydial inclusions were stained with fluorescein isothiocyanate-conjugated anti-Chlamydia antibody and counted. Numbers below the photographs were obtained by counting the number of inclusions in several fields. WT, wild-type. C and D, peritoneal macrophages and mouse embryonic fibroblasts (MEF) were infected with *C. pneumoniae* and were cultured with or without PI3K inhibitor wortmannin (wm: 50 nM) or Ly294002 (Ly: 10 μM). The amount of chlamydial DNA was measured by real-time PCR with primers for the *Chlamydophila*-specific sequences. Results were given as a ratio relative to GAPDH of the host cell. Error bars represent S.D., and shown are the representative of more than three experiments.
in NO synthesis. To our surprise, induction of the iNOS protein in PI3K-deficient macrophages was not affected, whereas iNOS activity was significantly decreased. Although NO synthesis is mainly regulated by the induction of iNOS gene expression, enzymatic activity of iNOS is post-translationally regulated by many complex factors including dimerization, degradation, phosphorylation, and association with inhibitory proteins, as well as substrate availability (60).

The production of NO is influenced by the amount of substrate for iNOS, L-arginine, which is also a substrate for arginase, an enzyme in the urea metabolic pathway. The production of NO is thus affected by arginase activity. Increased arginase activity is known to be responsible for reduced NO production in BALB/c mice (27). The amount of L-arginine in cytosol is also affected by its transport from outside the cell, which is mediated by CAT2 (28). In fact, the CAT2-deficient macrophage failed to produce detectable NO (29). As shown here, neither arginase activity nor CAT2 expression was affected by the absence of PI3K (Fig. 2, C and D). We therefore concluded that a lack of the substrate supply was not the cause of impaired NO production in PI3K-deficient macrophages.

Dimerization of NOs is essential for enzymatic activity, and it requires multiple cofactors including calmodulin, heme, FAD, NADPH, FMN, and BH4 (23, 52, 53). The availability of BH4, of all these cofactors, is critical for the dimerization of the iNOS (17). We observed a decrease in cytosolic biopterin concentration (Fig. 4A) and recovery of both iNOS dimerization and NO production after the addition of sepiapterin (Figs. 3B and 4, B and C) in PI3K-deficient macrophages. The addition of sepiapterin was reported to increase intracellular BH4 (61) and augment NO production in mast cells (62). These data suggested that the smaller amount of BH4 was the major reason for reduced NO synthesis in PI3K-deficient macrophages. We measured biopterin because concentration of total biopterin after sample oxidation is widely accepted to represent the amount of intracellular BH4 (45). Although total biopterins contain trace amounts of oxidized BH2 and endogenous biopterin other than oxidized BH4, concentration of BH2 and endogenous biopterin in macrophages was below the detection limit.

Recent results indicate that a reduction of either BH4 or arginine in the NOS complex leads to production of superoxide ($O_2^-$) instead of NO, the process known as “uncoupled catalysis” (63). Because we observed decreased BH4 synthesis in the absence of PI3K, uncoupled production of active oxygen may have resulted in substantially reduced nitrite production. If that is the case, increased production of nitrate and a concomitant decreased generation of nitrite would be observed in PI3K-deficient macrophages, because NOS-derived superoxide is known to react rapidly with NO to form nitrate (64). We thus measured both nitrite and nitrate formed in the supernatant of cultured macrophages after stimulation with LPS/IFN-$\gamma$ by HPLC coupled with the Griess reagent-flow reactor analysis (37). Both nitrite and nitrate levels decreased to the same degree in cultures of PI3K-deficient macrophages, which indicated that nitrate production did not increase (Fig. 1C). We therefore concluded that there was little uncoupled catalysis of iNOS in PI3K-deficient macrophages, despite the substantial decrease in intracellular BH4 concentration.

We found that the amount of GCH1, a rate-limiting enzyme in the BH4 biosynthesis pathway (55) was reduced in PI3K-deficient macrophages (Fig. 5). It is interesting that insulin-dependent GCH1 induction in endothelial cells also depends on PI3K, as wortmannin inhibited GCH1 induction in endothelial cells (65). Reduced induction of GCH1 after stimulation with LPS/IFN-$\gamma$ could thus be responsible for reduced production of BH4, which results in impaired formation of iNOS dimers in PI3K-deficient macrophages.

It is possible that phosphorylation could regulate iNOS activity, because phosphorylation of endothelial NOS by Akt is known to increase its activity (66, 67). In contrast, phosphorylation of neuronal NOS by Ca$^{2+}$/calmodulin-dependent protein kinases (68, 69) or protein kinase C reduces neuronal NO activity. In fact, there is one report that phosphorylation of iNOS in RAW264.7 cells was blocked by a PI3K inhibitor (57). We observed both serine and tyrosine phosphorylation of iNOS upon stimulation; however, the extent of phosphorylation seemed unaffected by the lack of PI3K (data not shown).

C. pneumoniae is an obligate intracellular Gram-negative bacterium and is a common cause of respiratory infection. Increased chlamydial growth in RAW264.7 cells with an iNOS inhibitor (Ref. 56 and Fig. 6A) as well as in iNOS-deficient macrophages (13, 14) clearly indicate that growth of the bacteria in macrophage is sensitive to NO. Consistent with reduced NO production in PI3K-deficient macrophages, chlamydial growth was increased in PI3K-deficient macrophages and in PI3K inhibitor-treated wild-type macrophages (Fig. 6B). Fibroblasts produce little NO upon LPS/IFN-$\gamma$-stimulation (70, 71) and unaltered chlamydial infection in iNOS-deficient fibroblasts (72) suggested that production of NO is not important in exclusion of the intracellular bacteria in fibroblasts. On the other hand, PI3K-mediated endocytosis was reported to be critical for entry of Chlamydia into cytosol of Hep2 cell line (73). Therefore, reduced chlamydial infection in PI3K-deficient mouse embryonic fibroblasts (Fig. 6D) could indicate the significance of PI3K in entry process of the bacteria in cells such as fibroblast.

In summary, we showed here that class IA PI3K is important in the dimerization of iNOS protein, by production of a cofactor BH4, possibly through induction of GCH1. Additional studies are required to elucidate how PI3K regulates expression of the gene GCH1.

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PI3K in NO Synthesis in Macrophage
