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Original Paper

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Induction of inducible nitric oxide synthase increases the production of reactive oxygen species in RAW264.7 macrophages

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Synopsis

Macrophages produce a large volume of ROS (reactive oxygen species) through respiratory burst. However, the influence of iNOS [inducible NOS (nitric oxide synthase)] activation on ROS production remains unclear. In the present study, the kinetic generation of ROS in RAW264.7 murine macrophages was monitored by chemiluminescence. PMA induces a robust chemiluminescence in RAW264.7 cells, suggesting PKC (protein kinase C)-related assembly and activation of NOX (NADPH oxidase). The effects of iNOS induction on ROS production were examined. Induction of iNOS expression in RAW264.7 cells with LPS (lipopolysaccharide; 1 μg/ml) causes a significant increase in PMA-induced chemiluminescence, which could be enhanced by the NOS substrate, L-arginine, and could be abolished by the NOS inhibitor, L-NNA (N^G-nitro-L-arginine). Further experiments reveal that induction of iNOS expression enhances the PMA-stimulated phosphorylation of the $p47^{phox}$ subunit of NOX, and promotes the relocalization of cytosolic $p47^{phox}$ and $p67^{phox}$ subunits to the membrane. Inhibition of PKCζ by its myristoylated pseudosubstrate significantly decreased the PMA-stimulated phosphorylation of the $p47^{phox}$ in LPS-pretreated cells, suggesting that PKCζ is involved in the iNOS-dependent assembly and activation of NOX. Taken together, the present study suggests that the induction of iNOS up-regulates the PMA-induced assembly of NOX and leads to the enhanced production of ROS via a PKCζ-dependent mechanism.

Key words: inducible nitric oxide synthase (iNOS), macrophage, NADPH oxidase (NOX), RAW264.7 macrophage, reactive oxygen species (ROS), respiratory burst

INTRODUCTION

Macrophages are pivotal effector cells in the innate immune system [1,2]. When microbial products bind to pathogen recognition receptors, macrophages are activated and release a broad array of mediators that orchestrate the inflammatory responses of the host. Extensive studies have led to the recognition that a lot of oxidative reactions contribute to the microbicidal capability of macrophages during the respiratory burst. 'Respiratory burst' refers to the early observation that when phagocytes are exposed to certain stimuli, they consume large amounts of oxygen. This observation led to a more than 25-year-long search for the enzymatic origin of the respiratory burst and to the discovery and characterization of the phagocytic NOX (NADPH oxidase). NOX generates superoxide radicals ($O_2^{\bullet-}$) via a one-electron reduction of O_2 by NADPH [3,4], with secondary production of H_2O_2 and other activated forms of oxygen. NOX is a multicomponent enzyme consisting of at least two membrane-bound components ($gp91^{phox}$ and $p22^{phox}$ that together form the flavocytochrome b_{558}), three cytosolic components ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and a small GTPase Rac [5,6]. In resting phagocytic cells, NOX is dormant and its components exist separately in the membrane and in the cytosol. When cells are exposed to appropriate stimuli, NOX is activated to produce $O_2^{\bullet-}$ by association of these cytosolic components with the plasma or phagosome membrane components. Relocalization of $p47^{phox}$/$p67^{phox}$ to the membrane is generally driven by phosphorylation of several serine residues

Abbreviations used: aPKC, atypical PKC; cPKC, conventional PKC; DAG, diacylglycerol; DETC, diethyldithiocarbamate sodium salt; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleneiodonium; HBSS, Hanks balanced salt solution; NOS, nitric oxide synthase; iNOS, inducible NOS; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; MnTMPyP, manganese(III) tetrakis(1-methyl-pyridy)porphyrin pentachloride; NF-κB, nuclear factor κB; NOX, NADPH oxidase; nPKC, novel PKC; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; ROS, reactive oxygen species.

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in p47^{phox} [6,7]. Invalidation of the *NCF1* (neutrophil cytosolic factor 1) gene, which encodes p47^{phox}, or inhibition of serine p47^{phox} phosphorylation correlates with reduced $O_2^{\bullet -}$ production [8,9]. Several kinases have been shown to increase phosphorylation of p47^{phox} serine residues, including PKC (protein kinase C), ERK1/2 (extracellular-signal-regulated kinase 1/2) and p38 kinase [7,10]. Once produced, $O_2^{\bullet -}$ is released either inside or outside the cells and then rapidly converted into H_2O_2 and other oxidants [4].

The discovery that activated macrophages express iNOS [inducible NOS (nitric oxide synthase)], which produces copious amounts of NO from L-arginine [11,12], has provided new insights for understanding the mechanism of macrophage-mediated pathogen killing. iNOS-deficient macrophages are unable to kill intracellular *Leishmania major* parasites after activation with IFN-γ (interferon-γ) and TNFα (tumour necrosis factor-α) or LPSs (lipopolysaccharides) [13], suggesting that iNOS-derived NO is critical for the control of microbial pathogens. On stimulation by certain cytokines or pathogens, the macrophage iNOS gene is induced via a complex signalling network, and the transcriptional factor NF-κB (nuclear factor κB) plays a central role in the induction of iNOS. The NF-κB transcription factor family consists of five proteins: p65/Rel A, p50, c-Rel, Rel B and p52. Each protein can dimerize with other family members to form homo- and hetero-dimers through the N-terminal Rel homology domain, which is also involved in translocation to the nucleus and DNA binding. Once iNOS gene expression has been induced, macrophages can generate large volumes of NO continuously.

Although both NOX-derived $O_2^{\bullet -}$ and iNOS-derived NO play important roles in the killing of microbes, it remains unclear whether there is cross-talk between NOX and iNOS signalling in macrophages. In the present study, we have utilized a sensitive chemiluminescence assay to clearly define the kinetics of oxidants generated during the PMA-induced respiratory burst of murine RAW264.7 cells, cells that are often used as models for natural macrophages. We also investigated the modulation of NOX assembly by iNOS-derived NO. We provide direct evidence that iNOS activation up-regulates NOX assembly and promotes ROS (reactive oxygen species) generation via a PKCζ-dependent mechanism.

MATERIALS AND METHODS

Materials

MnTMPyP [manganese(III) tetrakis(1-methyl-pyridy)porphyrin pentachloride] and myristoylated pseudosubstrate of PKCζ (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH) were purchased from Calbiochem (La Jolla, CA, U.S.A.). LPS (from *Escherichia coli* 026:B6), PMA, L-arginine, L-NNA (N^G-nitro-L-arginine), DPI (diphenyleneiodonium) chloride and luminol were purchased from Sigma (St. Louis, MO, U.S.A.). Rabbit anti-phosphoserine antibody, DMEM (Dulbecco's modified Eagle's medium), fetal bovine serum and cell culture supplements were obtained from Invitrogen (Beijing, China). Primary antibodies against iNOS, β-actin, PKCα, PKCδ, phospho-PKCα, phospho-PKCδ, NOX subunits (p47^{phox}, phospho-p47^{phox} and p67^{phox}) and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Primary antibodies against PKCζ and phospho-PKCζ were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Other chemicals were of analytical grade and were made in China.

Cell culture and LPS exposure

RAW264.7 murine macrophages were obtained from the Chinese Type Culture Collection (Shanghai, China) and were maintained in DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal bovine serum [14]. Cells were incubated at 37°C under a humidified atmosphere containing 5% CO_2/95% air. In some experiments, RAW264.7 macrophages at 50% confluence were stimulated with LPS (1 μg/ml) in serum-free DMEM for the durations indicated.

Assay of iNOS induction and NO production

iNOS expression was assayed by Western blotting, as previously reported [14]. Briefly, RAW264.7 cells were treated with or without LPS (1 μg/ml) for the durations indicated, and collected after trypsinization. Whole cell lysates were prepared by lysing cells in 400 μl of a buffer consisting of 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 20 mM PMSF. Proteins were separated by SDS/PAGE (10% gels) and then electroblotted on to a PVDF membrane. The membrane was probed with antibodies against iNOS and β-actin and then incubated with horseradish peroxidase-conjugated secondary antibodies. After four washes with TBST [10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.05% Tween 20], the target proteins were detected using an enhanced chemiluminescence assay.

NO produced by activated macrophages was detected by EPR spin trapping [14]. Briefly, macrophages cultured in 25 cm^2 cell culture flasks were treated with LPS (1 μg/ml) for 12 h. The spin-trapping agent containing 1 mM $FeSO_4$, 5 mM DETC (diethyldithiocarbamate sodium salt) and 5 mM $Na_2S_2O_3$ was then added to the activated macrophages, which were incubated at 37°C for an additional 3 h. The paramagnetic [ON-Fe^{2+}(DETC)$_2$] complex was enriched by extraction with 200 μl of ethyl acetate and detected with a Bruker ER-200 D-SRC EPR spectrometer under the following conditions: X-band; sweep width 400 G; microwave power 20 mW; 100 kHz modulation with amplitude 3.2 G; time constant 0.128 s.

Monitoring of ROS production

The kinetic generation of ROS during the PMA-stimulated respiratory burst of RAW264.7 macrophages was monitored by luminol-enhanced chemiluminescence with a computerized BPCL-IV illuminator, as reported previously [15]. After treatment with or without LPS (1 μg/ml) for 12 h, macrophages were

detached from the culture flasks with a rubber policeman, washed twice with HBSS (Hanks balanced salt solution) and harvested by centrifugation. Ten million RAW264.7 macrophages were suspended in 1 ml of HBSS containing 10 μM luminol, transferred into quartz test tubes, fitted into the detection cavity of a BPCL-IV Ultra-weak Chemiluminescence Analyzer, and equilibrated at 37°C. After the addition of PMA (10 ng/ml), the kinetic generation of luminol-dependent chemiluminescence was recorded immediately. In some experiments, the cells were pre-incubated with L-NNA (0.5 mM, 1 mM and 2 mM) for 30 min before the addition of PMA. The chemiluminescence was assayed in triplicate.

Mobilization of p47^{phox} and p67^{phox} subunits to the plasma membrane

Translocation of p47^{phox} and p67^{phox} NOX subunits to the membrane was analysed by Western blotting, as previously reported [16], with minor modifications. Briefly, RAW264.7 cells were pretreated with or without LPS (1 μg/ml) for 12 h, stimulated with PMA (10 ng/ml) for 3 min, collected in 400 μl of ice-cold buffer consisting of 10 mM Tris/HCl (pH 7.3), 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM ATP, 2 mM Na_3VO_4, 100 μM phenylarsine oxide, 3 mM di-isopropyl fluorophosphate, 10 μg/ml leupeptin and 10 μg/ml aprotinin, and disrupted by sonication on ice. After ultracentrifugation at 48537 rev./min (TLA100.3 rotor, Beckman) for 30 min at 4°C, the membrane pellet obtained was resuspended in solubilization buffer consisting of 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P40, and 0.1% SDS and then re-centrifuged at 20000 ***g*** for 40 min at 4°C. The solubilized membrane proteins in the supernatant were separated by SDS/PAGE (10% gels), and the target proteins (p67^{phox} and p47^{phox} subunits of NOX) were detected by Western blotting.

Detection of p47^{phox} phosphorylation

RAW264.7 cells grown in 60 mm dishes were pretreated with or without LPS (1 μg/ml) for 12 h, stimulated with PMA (10 ng/ml) for 1.5 min and lysed in 1 ml of a buffer consisting of 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 20 mM PMSF. Aliquots of the cellular lysates (containing 500 μg of protein) were incubated with rocking overnight at 4°C with anti-p47^{phox} antibody. Protein was then immunoprecipitated using gamma-bind G–Sepharose beads (Amersham Biosciences). The proteins immunoprecipitated with the anti-p47^{phox} antibody were separated on an SDS/10% PAGE gel and then electroblotted on to a PVDF membrane. The phosphorylation of p47^{phox} was detected by Western blotting with an anti-phosphoserine antibody.

Detection of phosphorylated PKC isoforms

The activation of PKCs in PMA-stimulated macrophages was detected by Western blotting of phosphorylated PKC isoforms. RAW264.7 cells grown in 60 mm culture dishes were pretreated with or without LPS (1 μg/ml) for 12 h, stimulated with PMA (10 ng/ml) for 1.5 min, and whole cell lysates were prepared. Proteins were separated on an SDS/10% PAGE gel, and the levels of both total and phosphorylated cPKC (conventional PKC) isoform PKCα, nPKC (novel PKC) isoform PKCδ and aPKC (atypical PKC) isoform PKCζ were detected by Western blotting.

RESULTS

LPS induces the expression of iNOS and stimulates the production of NO

The induction of iNOS expression in RAW264.7 macrophages by LPS exposure was examined by Western blotting. iNOS was not detected in quiescent RAW264.7 macrophages; when RAW264.7 macrophages were incubated with LPS, iNOS expression was induced in a time-dependent manner, as shown in Figure 1(A). The generation of NO was measured by EPR spin-trapping, and a three-line EPR spectrum corresponding to the [ON-$Fe^{2+}$$(DETC)_2$] complex at $g = 2.035$ (where g is the g-factor of the electron paramagnetic resonance) was observed in RAW264.7 macrophages treated with LPS for 12 h (Figure 1B), suggesting that iNOS-expressing macrophages do generate NO. No EPR signal was observed in quiescent RAW264.7 macrophages without LPS treatment.

Kinetic generation of ROS in PMA-stimulated macrophages

After being stimulated with PMA, which activates the PKC pathway in RAW264.7 macrophages, respiratory burst occurred. The kinetic generation of ROS during the respiratory burst was observed by luminol-enhanced chemiluminescence. As shown in Figure 2(A), the generation of ROS reached its maximum (~700 c.p.s.) 200 s after the addition of PMA, and then decayed gradually. To ascertain if the chemiluminescence was due to the assembly and activation of NOX, RAW264.7 macrophages were pretreated with the NOX inhibitor DPI, or the superoxide anion scavenger MnTMPyP. PMA-stimulated chemiluminescence was markedly inhibited in macrophages pretreated with DPI (5 μM) or MnTMPyP (25 μM) (Figure 2A).

Incubation of RAW264.7 macrophages with LPS did not induce the production of ROS. However, induction of iNOS by LPS significantly increased the PMA-stimulated production of ROS in RAW264.7 macrophages. In cells pretreated with LPS for 12 h, the PMA-stimulated chemiluminescence intensity increased to ~1100 c.p.s. (Figure 2B), significantly higher than that of cells without LPS pretreatment (~700 c.p.s.).

To explore the contribution of endogenous NO to the increase of ROS production during PMA-stimulated respiratory burst, L-arginine (the substrate of iNOS) was added to the cells, and the kinetic production of ROS was monitored. In RAW264.7 macrophages that were pretreated with LPS and expressed high levels of iNOS, the addition of L-arginine enhanced the intensity of respiratory burst in a dose-dependent manner. The addition of 0.5 mM L-arginine increased the PMA-stimulated chemiluminescence intensity to ~2400 c.p.s., while the addition of 2 mM L-arginine

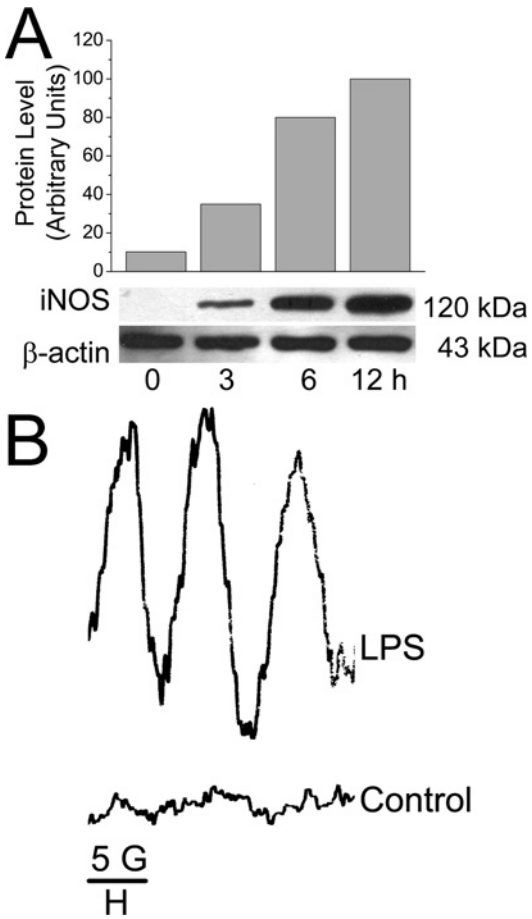

Figure 1 Induction of iNOS expression and NO production in RAW264.7 murine macrophages

(A) Time-dependent induction of iNOS expression in RAW264.7 murine macrophages treated with LPS. RAW264.7 macrophages were cultured in 60 mm dishes, treated with 1 μg/ml LPS for the times indicated, and the expression of iNOS was assayed by Western blotting. (B) Production of NO in LPS-treated RAW264.7 macrophages. RAW264.7 macrophages cultured in 25 cm^2 cell-culture flasks were treated with or without 1 μg/ml LPS for 12 h. The spin-trapping agent containing 1 mM $FeSO_4$, 5 mM DETC and 5 mM $Na_2S_2O_3$ was then added to the activated macrophages, which were incubated at 37°C for an additional 3 h. The paramagnetic [ON-$Fe^{2+}$$(DETC)_2$] complex was enriched by extraction with 200 μl of ethyl acetate and detected with a Bruker ER-200 D-SRC EPR spectrometer.

increased the PMA-stimulated chemiluminescence intensity to ~5600 c.p.s. (Figure 2C). No apparent increase in ROS production could be observed in RAW264.7 macrophages without LPS pretreatment (Figure 2D), suggesting that L-arginine increases ROS generation in a manner that is dependent on the expression of iNOS.

The involvement of endogenous NO in ROS production was further confirmed by incubating cells with L-NNA, the inhibitor of iNOS. The addition of L-NNA inhibited the L-arginine-enhanced chemiluminescence intensity in LPS-pretreated RAW264.7 macrophages in a dose-dependent manner (Figure 2E), suggesting the involvement of iNOS-derived NO in the generation of ROS.

To ascertain whether the addition of L-arginine increases the PMA-stimulated chemiluminescence via generation of peroxynitrite, RAW264.7 macrophages were incubated with 1 mM L-arginine for 30 min and then stimulated with PMA. At 4 min after the addition of PMA, when the generation of chemiluminescence reaches its maximal rates, macrophages were incubated with 1 mM L-NNA. Interestingly, the addition of L-NNA at this time point shows no apparent influence on chemiluminescence (Figure 2F). If the L-arginine increases the PMA-stimulated chemiluminescence via the formation of peroxynitrite, the addition of L-NNA, which inhibits the activity of iNOS immediately and thus prevents the generation of NO and its downstream products peroxynitrite, should decrease the chemiluminescence immediately. The results of Figure 2(F) suggest that the L-arginine increases the PMA-stimulated chemiluminescence via a mechanism unrelated to the formation of peroxynitrite. The addition of L-arginine might increase the PMA-stimulated production of superoxide anion.

Induction of iNOS potentiates the phosphorylation of $p47^{phox}$ and the translocation of $p47^{phox}$ and $p67^{phox}$ to the plasma membrane

The translocation of the $p47^{phox}$ and $p67^{phox}$ subunits of NOX from the cytosol to the plasma membrane is an essential step for the activation of NOX. In order to elucidate the effect of induction of iNOS on NOX activation, the membrane levels of $p47^{phox}$ and $p67^{phox}$ were evaluated in RAW264.7 cells stimulated with PMA. Figure 3(A) shows that after incubation with PMA for 3 min, the $p47^{phox}$ and $p67^{phox}$ levels in the plasma membrane increased significantly, suggesting that PMA stimulation induces rapid translocation of $p47^{phox}$ and $p67^{phox}$ subunits to the plasma membrane.

Pretreatment of RAW264.7 macrophages with LPS significantly promoted PMA-induced mobilization of $p47^{phox}$ and $p67^{phox}$ subunits to the plasma membrane. This effect could be partially abolished by the iNOS inhibitor, namely L-NNA, suggesting that the induction of iNOS and the production of endogenous NO accelerated PMA-stimulated assembly of NOX.

Translocation of $p47^{phox}$ is triggered by its phosphorylation, which induces a conformational change that mediated its interaction with both $gp91^{phox}$ and $p67^{phox}$. To ascertain whether the expression of iNOS accelerates the mobilization of $p47^{phox}$ to the plasma membrane by modulating its phosphorylation, the effects of LPS pretreatment on PMA-stimulated $p47^{phox}$ phosphorylation were studied by immunoprecipitation. As shown in Figure 3(B), induction of iNOS by LPS pretreatment significantly increased the PMA-stimulated phosphorylation of the serine residues of $p47^{phox}$. L-NNA, the inhibitor of iNOS, inhibited the phosphorylation of $p47^{phox}$ significantly. This result indicates that the induction of iNOS potentiates PMA-stimulated NOX assembly by NO-mediated up-regulation of $p47^{phox}$ phosphorylation.

Induction of iNOS potentiates the phosphorylation of $p47^{phox}$ via PKCζ

In PMA-stimulated macrophages, DAG (diacylglycerol)-dependent PKC isoforms were activated, leading to the

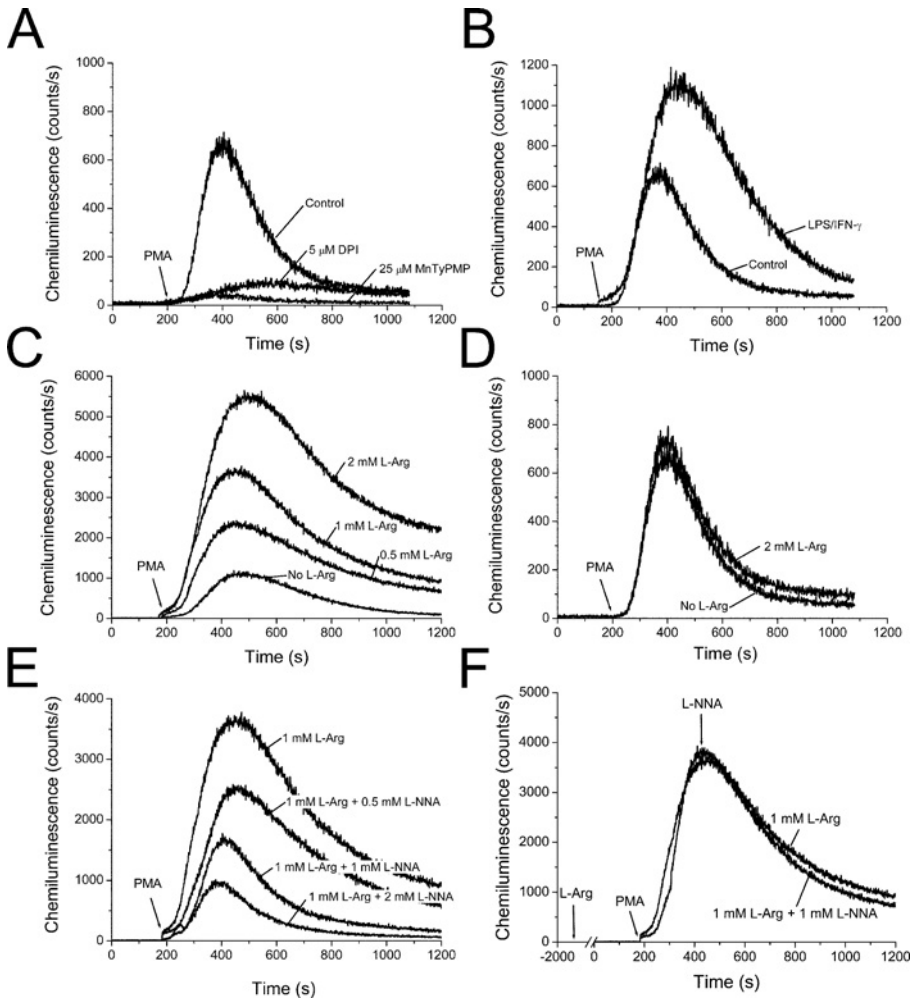

Figure 2 **Kinetic generation of ROS in PMA-stimulated RAW264.7 macrophages**

(A) Inhibition of NOX activity by DPI, or scavenging of superoxide by MnTMPyP, significantly decreased PMA-stimulated chemiluminescence in RAW264.7 macrophages. RAW264.7 macrophages (1×10^7 cells) were suspended in 1 ml of HBSS containing 10 μM luminol and pretreated with the NOX inhibitor DPI (5 μM) or the superoxide anion scavenger MnTMPyP (25 μM) for 30 min. Immediately after the addition of PMA (10 ng/ml), the kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. (B) Induction of iNOS increases ROS production in RAW264.7 macrophages. RAW264.7 macrophages were treated with or without LPS for 12 h. RAW264.7 macrophages (1×10^7 cells) suspended in 1 ml of HBSS containing 10 μM luminol were stimulated with PMA (10 ng/ml) and the kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. (C) L-Arginine potentiates ROS production in LPS-pretreated RAW264.7 macrophages. RAW264.7 macrophages were treated with LPS for 12 h. RAW264.7 macrophages (1×10^7 cells) were suspended in 1 ml of HBSS containing 10 μM luminol and pretreated with the iNOS substrate L-arginine (L-Arg) (0.5, 1 or 2 mM) for 30 min. The PMA-stimulated kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. (D) L-Arginine shows no effect on ROS production in RAW264.7 macrophages without LPS pretreatment. RAW264.7 macrophages (1×10^7 cells) were suspended in 1 ml of HBSS containing 10 μM luminol and pretreated with 2 mM L-arginine for 30 min. The PMA-stimulated kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. (E) Inhibition of iNOS decreases L-arginine-enhanced ROS production in LPS-pretreated RAW264.7 macrophages. RAW264.7 macrophages were treated with LPS for 12 h. RAW264.7 macrophages (1×10^7 cells) were suspended in 1 ml of HBSS containing 10 μM luminol and pretreated with the iNOS substrate (L-arginine; 1 mM) and the iNOS inhibitor (L-NNA; 0.5, 1 or 2 mM) for 30 min. The PMA-stimulated kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. (F) Inhibition of iNOS shows no effect on L-arginine-enhanced ROS production in LPS-pretreated RAW264.7 macrophages. RAW264.7 macrophages were treated with LPS for 12 h. RAW264.7 macrophages (1×10^7 cells) were suspended in 1 ml of HBSS containing 10 μM luminol and pretreated with the iNOS substrate (L-arginine; 1 mM) for 30 min, stimulated with PMA (10 ng/ml), and the kinetic generation of ROS-dependent chemiluminescence was recorded by a BPCL-IV Ultra-weak Chemiluminescence Analyzer. At 4 min after the addition of PMA, when the generation of ROS reaches its maximum rate, the iNOS inhibitor (L-NNA; 1 mM) was added to cells. For (A–F), the chemiluminescence was assayed in triplicate.

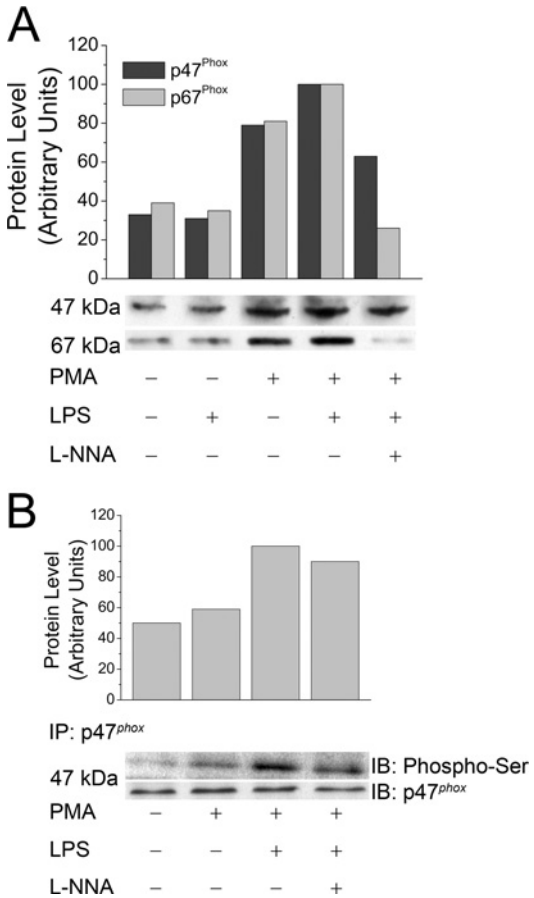

Figure 3 Induction of iNOS potentiates PMA-stimulated assembly of NOX

(A) Induction of iNOS potentiates the PMA-induced translocation of p47^{phox} and p67^{phox} NOX subunits to the plasma membrane. RAW264.7 macrophages were cultured in 60 mm dishes, treated with or without 1 μg/ml LPS for 12 h and stimulated with 10 ng/ml PMA for 3 min. Plasma membrane fractions were isolated from RAW264.7 macrophages and the levels of p47^{phox} and p67^{phox} NOX subunits were assayed by Western blotting. (B) Induction of iNOS increases the phosphorylation of p47^{phox}. RAW264.7 macrophages were cultured in 100 mm dishes, treated with or without 1 μg/ml LPS for 12 h and stimulated with 10 ng/ml PMA for 1.5 min. p47^{phox} was immunoprecipitated (IP) from the cell lysate of RAW264.7 macrophages, and phosphorylation of p47^{phox} was assayed by Western blotting (IB). L-NNA, 1 mM.

phosphorylation of p47^{phox}. We assayed the influence of iNOS induction on the phosphorylation (activation) of two DAG-dependent PKC isoforms (PKCα and PKCδ) in PMA-stimulated RAW264.7 macrophages. As shown in Figure 4(A), PMA stimulated the phosphorylation of the cPKC isoform PKCα and the nPKC isoform PKCδ in RAW264.7 macrophages, and LPS pretreatment showed no effect on the PMA-stimulated phosphorylation of PKCα and PKCδ.

The activation of the aPKC isoform PKCζ, which lacks PMA-binding sites, is DAG independent. Stimulation of RAW264.7 macrophages by PMA showed no effect on the phosphorylation of PKCζ in RAW264.7 macrophages (Figure 4B). Interestingly, pretreatment of RAW264.7 macrophages with LPS induced significant phosphorylation of PKCζ in RAW264.7 macrophages. When LPS-pretreated RAW264.7 macrophages were stimulated with PMA, the phosphorylation of PKCζ increased significantly. This effect was abolished by the iNOS inhibitor, namely L-NNA. These results suggest that the induction of iNOS and the generation of endogenous NO might potentiate the activation of PKCζ.

To further confirm the possible involvement of PKCζ in iNOS-potentiated p47^{phox} phosphorylation, LPS-pretreated RAW264.7 macrophages were incubated with a myristoylated pseudosubstrate of PKCζ (a proven selective inhibitor of PKCζ) before PMA stimulation, and the phosphorylation of p47^{phox} was assayed. As shown in Figure 4(C), the phosphorylation of p47^{phox} decreased drastically after treatment with myristoylated pseudosubstrate of PKCζ. These results suggest that PKCζ is the key molecule that mediates the up-regulation of p47^{phox} phosphorylation in LPS-pretreated macrophages.

DISCUSSION

The early observation of respiratory burst in macrophages has led to a greater than 25-year-long search for the enzymatic origin of oxygen consumption and to the discovery and characterization of NOX [6]. The existence of iNOS within macrophages was suggested over 20 years ago [17,18]. Although it is well accepted that NOX, NOS-derived ROS and NO participate in host defence by killing invading microbes, the possible interactions and cross-talk between ROS and NO has remained a subject of debate. Like $O_2^{\bullet -}$, NO is relatively unreactive toward biomolecules. As free radicals, $O_2^{\bullet -}$ formed in the NOX-catalysed respiratory burst, as well as NO formed from iNOS-catalysed reactions, react at diffusion-controlled rates to generate strongly oxidizing, but unstable peroxynitrite compounds ($ONOO^-$ and $ONOOCO_2^-$) and other related compounds [19,20]. Since peroxynitrite oxidizes and nitrates a variety of biological targets, this led to proposals that peroxynitrite and peroxynitrite-derived species are the potential mediators of NO microbicidal and cytotoxic effects [21–23]. In a previous study, Li et al. [24] reported that L-arginine enhances chemiluminescence during PMA-stimulated respiration of rat peritoneal macrophages. They hypothesized that the L-arginine-dependent increase in chemiluminescence was due to the generation of peroxynitrite [24]. However, this viewpoint has been disputed on the basis of the results of several reports. By analysing the protein tyrosine nitration in activated macrophages, Pfeiffer et al. [25,26] demonstrated that a peroxidase/nitrite pathway, rather than the peroxynitrite pathway, contributes to the protein nitration. A study by Palazzolo-Ballance et al. [27] also suggested that iNOS-generated NO_2^- is used to produce NO_2 as a terminal microbicidal oxidant and nitrating agent in murine RAW264.7 macrophages. They have suggested that the expression of iNOS and NOX is temporally well-separated after macrophage activation. Our results also suggest that L-arginine enhances chemiluminescence during PMA-stimulated respiration via a mechanism unrelated to the formation of peroxynitrite (Figure 2F). However, potential

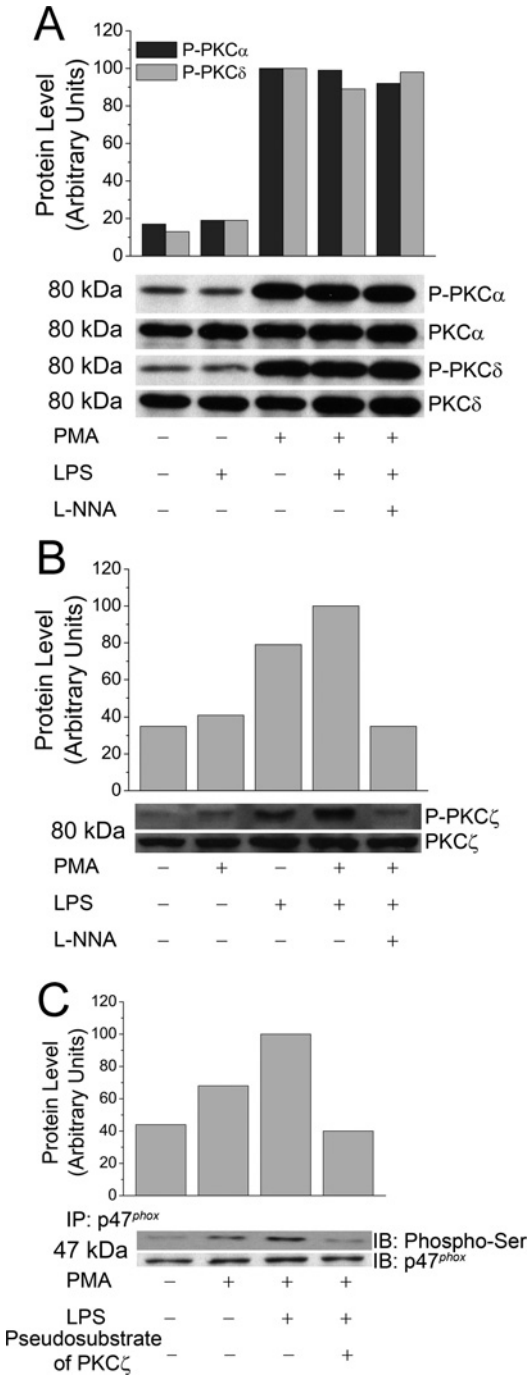

Figure 4 Induction of iNOS increases the phosphorylation of p47^{phox} via PKCζ

(A) Activation of PKCα and PKCδ in PMA-stimulated RAW264.7 macrophages. RAW264.7 macrophages were cultured in 60 mm dishes, treated with or without 1 μg/ml LPS for 12 h and stimulated with 10 ng/ml PMA for 1.5 min. The levels of phosphorylated PKCα (P-PKCα), total PKCα, phosphorylated PKCδ (P-PKCδ) and total PKCδ were analysed by Western blotting. (B) LPS activates PKCζ in RAW264.7 macrophages. RAW264.7 macrophages were cultured in 60 mm dishes, treated with or without 1 μg/ml LPS for 12 h and stimulated with 10 ng/ml PMA for 1.5 min. The levels of phosphorylated PKCζ and total PKCζ were analysed by Western blotting.

interactions between NOX activation and NOS induction need to be elucidated.

The present study has investigated the influence of iNOS-generated NO on NOX assembly and activation, and the results presented here reveal that NO plays a significant role in the up-regulation of NOX activation and ROS production during the respiratory burst of murine RAW264.7 macrophages. Using a luminol-enhanced chemiluminescence assay, we have confirmed that induction of iNOS expression by LPS can evoke an L-arginine-dependent increase in ROS production in RAW264.7 cells, which can effectively be inhibited by L-NNA, an inhibitor of NOS. Further investigation demonstrated that iNOS-derived NO contributes to the activation of NOX.

Next, we searched for the molecule that may link iNOS induction with NOX activation. Key to the assembly and activation of NOX is phosphorylation of the p47^{phox} C-terminal autoinhibitory region by multiple serine/threonine kinases, leading to the translocation of the cytosolic complex p47^{phox}/p67^{phox} to the membrane. Some of these phosphorylations are also implicated in the acquisition of full catalytic activity. PKC is a family of serine/threonine kinases and at least 12 isoforms of PKC are currently known to exist [28]. In macrophages PKC plays a critical role in the phosphorylation of p47^{phox} and the subsequent activation of NOX. Based on homology and cofactor requirements, the isoforms have been divided into three families. The cPKCs (α, βI, βII and γ) are Ca^{2+}- and DAG-dependent; the nPKCs (δ, ε, η and θ) are Ca^{2+}-independent, but DAG-dependent; and the aPKCs (ι, λ, μ and ζ) are both Ca^{2+}- and DAG-independent and lack PMA-binding sites. cPKCs α, β1 and β2, nPKCs δ, ε and θ and aPKCs ι, λ, μ and ζ are all expressed in RAW264.7 murine macrophages [29,30].

Direct activation of cPKC isoform PKCα and nPKC isoform PKCδ by PMA triggered rapid activation of NOX and consequent generation of ROS in RAW264.7 cells. To explore whether iNOS-derived NO modulates NOX activation by PKCα and PKCδ, the influence of NO on the phosphorylation of PKCα and PKCδ was investigated. iNOS-derived NO showed no effects on PMA-stimulated PKCα and PKCδ activation in LPS-pretreated cells. However, aPKC isoform PKCζ, which lacks PMA-binding sites, was activated during the induction of iNOS by LPS. In RAW264.7 macrophages pretreated with LPS for 12 h, the phosphorylation of PKCζ increased significantly. However, when LPS-pretreated RAW264.7 macrophages were stimulated with PMA, the phosphorylation of PKCζ increased further. To ascertain whether PKCζ plays a role in the activation of NOX, RAW264.7 macrophages were pretreated with the PKCζ inhibitor and the level of phosphorylated p47^{phox} was detected. We found that the myristoylated pseudosubstrate of PKCζ, a proven specific inhibitor of PKCζ, blocked the NO-enhanced phosphorylation of p47^{phox}.

(C) Inhibition of PKCζ decreases the phosphorylation of p47^{phox}. RAW264.7 macrophages were cultured in 100 mm dishes, treated with or without 1 μg/ml LPS for 12 h and stimulated with 10 ng/ml PMA for 1.5 min. p47^{phox} was immunoprecipitated (IP) from the cell lysate of RAW264.7 macrophages and phosphorylation of p47^{phox} was assayed by Western blotting (IB). L-NNA, 1 mM; pseudosubstrate of PKCζ, 50 μM.

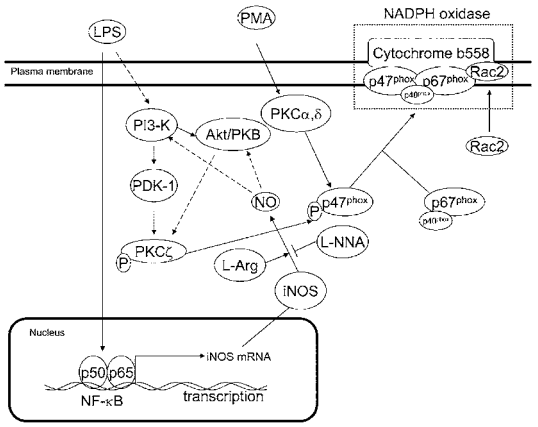

Figure 5 Interactions and cross-talk between iNOS induction and NOX activation in murine RAW264.7 macrophages

These results suggest that PKCζ activation plays an important role in the phosphorylation of p47^{phox} and consequent NOX activation in LPS-pretreated RAW264.7 macrophages. iNOS-generated NO potentiates PMA-stimulated NOX assembly via a PKCζ-related p47^{phox} phosphorylation mechanism.

The activation of PKCζ is necessary, but not sufficient, to enhance NOX activation and increase ROS production in RAW264.7 macrophages. In LPS-pretreated RAW264.7 macrophages in which PKCζ has been activated, no detectable ROS production was observed until PKCα and PKCδ were activated with PMA (Figures 2B and 2C). Our results indicate that there may be as yet undiscovered cross-talk between cPKC isoform PKCα, nPKC isoform PKCδ and aPKC isoform PKCζ during respiratory burst in RAW264.7 macrophages.

The molecular mechanism that governs PKCζ activation in LPS-pretreated macrophages still needs further elucidation. PKCζ is an aPKC isoform that does not require either calcium or DAG/phorbol esters for activation. It can be activated *in vitro* by PIP_3 (phosphatidylinositol 3,4,5-triphosphate), phosphatidic acid, ceramide and arachidonic acid and/or by direct interaction with binding proteins [31–33]. PI3K (phosphoinositide 3-kinase) is the key molecule that mediates LPS-induced PKCζ activation. The activation loop consensus threonine residue Thr^{410} of PKCζ is phosphorylated by PI3K-induced PDK1 (phosphoinositide-dependent kinase 1) and is sufficient to activate PKCζ [34]. However, PDK1 is not the only pathway downstream of PI3K that mediates the activation of PKCζ. Inhibiting PKB (protein kinase B; also called Akt), a signalling component immediately downstream of PI3K, decreased the phosphorylation of PKCζ to some extent (results not shown). PKB shares partially redundant functions with PDK1 during LPS-induced, PI3K-dependent activation of PKCζ. Results from the present study also demonstrate that iNOS-derived NO is essential for the phosphorylation (activation) of PKCζ, since treatment of RAW264.7 macrophages with L-NNA significantly decreased the phosphorylation of PKCζ. NO may activate PKCζ via modulating PI3K-dependent pathways (PDK1, PKB etc.) during LPS-induced macrophage activation.

The aPKC isoform PKCζ is essential for LPS-induced macrophage activation [35]. In the present study, we report for the first time that PKCζ is the key molecule that links iNOS induction and NOX activation. PKCζ activation is necessary, but not sufficient, for enhancing NOX activation and increasing ROS production in iNOS-expressing RAW264.7 macrophages (Figure 5). Our results suggest that iNOS and NOX show potentially synergistic effects in ROS generation during respiratory bursts in macrophages.

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