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Secretory Phospholipase A_2-Potentiated Inducible Nitric Oxide Synthase Expression by Macrophages Requires NF-κB Activation[1]

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The effect of secretory group II phospholipase A_2 ($sPLA_2$) on the expression of the inducible NO synthase (iNOS) and the production of NO by macrophages was investigated. $sPLA_2$ by itself barely stimulated nitrite production and iNOS expression in Raw264.7 cells. However, in combination with LPS, the effects were synergistic. This potentiation was shown for $sPLA_2$ enzymes from $sPLA_2$-transfected stable cells or for purified $sPLA_2$ from human synovial fluid. The effect of PLA_2 on iNOS induction appears to be specific for the secretory type of PLA_2. LPS-stimulated activation of iNOS was inhibited by the well-known selective inhibitors of $sPLA_2$ such as 12-epi-scalaradial and ρ-bromophenacyl bromide. In contrast, the cytosolic PLA_2-specific inhibitors methyl arachidonyl fluorophosphate and arachidonyltrifluoromethyl ketone did not affect LPS-induced nitrite production and iNOS expression. Moreover, when we transfected cDNA-encoding type II $sPLA_2$, we observed that the $sPLA_2$-transfected cells produced two times more nitrites than the empty vector or cytosolic PLA_2-transfected cells. The $sPLA_2$-potentiated iNOS expression was associated with the activation of NF-κB. We found that the NF-κB inhibitor pyrrolidinedithiocarbamate prevented nitrite production, iNOS induction, and mRNA accumulation by $sPLA_2$ plus LPS in Raw264.7 cells. Furthermore, EMSA analysis of the activation of the NF-κB involved in iNOS induction demonstrated that pyrrolidinedithiocarbamate prevented the NF-κB binding by $sPLA_2$ plus LPS. Our findings indicated that $sPLA_2$, in the presence of LPS, is a potent activator of macrophages. It stimulates iNOS expression and nitrite production by a mechanism that requires the activation of NF-κB. *The Journal of Immunology*, 2000, 164: 6359–6365.

Macrophage activation is a key component of the immune response. Several proinflammatory cytokines and bacterial products such as LPS participate actively in this process (1–3). Besides activating macrophages, LPS induces the synthesis of additional cytokines such as TNF-α, IL-1β, and IL-6, which leads to amplification of the original response (4, 5). Activated macrophages release NO, which is an important bactericidal and cytostatic gas (1–2, 6). However, massive production of this mediator can have detrimental effects on the host organism, such as occurs during septic shock or multiple organ failure (7, 8). For this reason, the study of the mechanism of the actions of the inflammatory cytokines and drugs has attracted strong interest (7–10). NO is the product of conversion of L-arginine to L-citrulline, which is catalyzed by the enzyme NO synthase (NOS)[3] (11). Three isoforms of NOS have been cloned and characterized: endothelial NOS, neuronal NOS, and inducible NOS (iNOS) (12, 13). NO, produced in low levels by the endothelial and neuronal NOS isoforms, functions as a signaling molecule in several biological processes including the regulation of vascular tone and neuronal signaling (13–15). NO, produced in large quantities following induction of iNOS by cytokines and LPS, can have cytotoxic or cytostatic effects on macrophage (2). iNOS is expressed in various cell types, which include vascular smooth muscle cells, hepatocytes, astrocytes, and macrophages and is induced in response to proinflammatory cytokines or bacterial LPS (16–19).

NF-κB appears to play a primary role in the transcriptional regulation of the iNOS gene in macrophages (20, 21). In unstimulated cells, NF-κB is present as an inactive heterodimer of p50/p65 subunits bound to the NF-κB inhibitor protein IκB. Upon stimulation, IκB becomes phosphorylated on specific serine residues. This targets IκB for degradation in an ubiquitin-dependent process (22). Antioxidant inhibitors of NF-κB activation, pyrrolidinedithiocarbamate (PDTC) and diethyldithiocarbamic acid, prevent the induction of iNOS expression and nitrite production by LPS in Raw264.7 cells, indicating that NF-κB participates in the LPS-induced iNOS expression (21–23).

The details of the signal transduction cascade involved in the induction of iNOS in response to LPS are an active area of investigation. Although LPS-induced iNOS induction in macrophages has been reported previously (20, 21, 23), the molecular events involved in this process are not yet fully understood. Many reports have suggested a potential role for phospholipase A_2 (PLA_2) in LPS-mediated iNOS induction. Secretory PLA_2 ($sPLA_2$) is a lipolytic enzyme that catalyzes the hydrolysis of the acyl ester bond at the *sn*-2 position of phospholipids. $sPLA_2$ is thought to be an important inflammatory agent because it is induced by inflammatory

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[3] Abbreviations used in this paper: NOS, NO synthase; PLA_2, phospholipase A_2; $sPLA_2$, secretory type II PLA_2; $cPLA_2$, cytosolic PLA_2; iNOS, inducible NOS; PDTC, pyrrolidinedithiocarbamate; MAFP, methyl arachidonyl fluorophosphate; AA-$COCF_3$, arachidonyltrifluoromethyl ketone; [^{3}H]AA, [5,6,89,11,12,14,15-^{3}H]arachidonic acid; PI 3-K, phosphatidylinositol 3-kinase; PLD, phospholipase D; ERK, extracellular regulated kinase; IκB, inhibitory κB; L-NMMA, L-*N*-monomethylarginine; ρ-BPB, ρ-bromophenacyl bromide.

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cytokines such as IL-1β and TNF-α and its activation can lead to the release of arachidonic acid and subsequent production of various other proinflammatory mediators such as PGs, leukotrienes, and platelet-activating factors (24–26). sPLA$_2$ is also suspected to play an important role in sepsis. Recent studies of patients with sepsis revealed a strong correlation between the plasma levels of sPLA$_2$ and sepsis. sPLA$_2$ plasma levels were significantly higher in patients who died of sepsis than in those who survived the illness (27–29). Nevertheless, the biological role of sPLA$_2$ in septic shock remains unclear. More recently, several research groups have shown that PLA$_2$ regulates the cytokine production of macrophages and phagocytosis (29, 30). Furthermore, a PLA$_2$ inhibitor could simultaneously reduce NO production and superoxide generation in a certain cell type (31). However, PLA$_2$- and especially sPLA$_2$-mediated NO production by macrophages is still not sufficiently understood.

The purpose of this study is to determine whether the activation of macrophages by sPLA$_2$ is linked to iNOS expression and nitrite production and if these events are dependent on NF-κB activation. We found that sPLA$_2$ in combination with LPS was a potent activator of murine macrophages and stimulated iNOS expression and nitrite production. The role of the PLA$_2$ isoforms in LPS-stimulated nitrite production and iNOS expression was further elucidated by the use of type-specific inhibitors. In addition, we demonstrated that the sPLA$_2$-potentiated iNOS expression is associated with the activation of NF-κB. Our studies provide direct evidence that sPLA$_2$ is one of the effective molecules that mediates NO production of macrophages and that it does so in a NF-κB-dependent mechanism.

Materials and Methods

Reagents

Type II sPLA$_2$ enzyme was obtained from the cDNA transfectants or purified from human synovial fluid as previously described (24). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and enhanced chemiluminescence reagents were purchased from Amersham (Buckinghamshire, U.K.). RPMI 1640 and PBS were obtained from Life Technologies (Grand Island, NY). FCS was purchased from HyClone (Logan, UT). Rabbit polyclonal iNOS Ab and anti-rabbit IgG peroxidase-conjugated secondary Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (from *Escherichia coli* 0111:B4, gamma irradiated) and PDTC were obtained from Sigma (St. Louis, MO). PLA$_2$ inhibitors methyl arachidonyl fluorphosphate (MAFP), arachidonyltrifluoromethyl ketone (AACOCF$_3$), and 12-epi-scalaradial were purchased from Biomol (Plymouth Meeting, PA) and dissolved in DMSO before addition to cell cultures or enzyme assays; final concentrations of DMSO were 0.1% or less. Controls using DMSO alone were run in all cases.

Cell culture

The macrophage cell line Raw264.7 was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The cells were grown at 37°C, 5% CO_2 in fully humidified air and subcultured twice weekly. Cells were seeded on 12-well plates at 5×10^5 cells/well or 6-well plates at 1×10^6 cells/well. The cells were stimulated for various lengths of time ranging from 1 to 24 h in the presence of LPS with or without inhibitors. LPS was diluted with culture medium to a final concentration of 1 μg/ml.

PLA$_2$ activity assay and measurement of [5,6,8,9,11,13,14,15-^{3}H]arachidonic acid ([^{3}H]AA) release

PLA$_2$ activity of purified enzymes or transfectants supernatants was measured as acylhydrolysis of 1-palmitoyl-2-[1-^{14}C]linoleoyl L-3-phosphatidylethanolamine as previously described (24–26). The samples were incubated with the enzyme and substrate for 10 min at 37°C. Results are calculated as cpm or dpm free fatty acid hydrolyzed. For [^{3}H]AA release experiments, cells labeled with [^{3}H]AA (1 μCi/ml) were used, and the incubations were performed in the presence or absence of cytosolic PLA$_2$ (cPLA$_2$) inhibitors. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

NO assay

Synthesis of NO was determined by assaying culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen. Briefly, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) at room temperature for 10 min. The OD of the assay sample was measured spectrophotometrically at 570 nm. Fresh culture medium served as the blank in all experiments. Nitrite concentration was calculated from a standard curve derived from the reaction of $NaNO_2$ under assay conditions.

Western blot analysis

Raw264.7 cells were plated in six-well plates (1×10^6 cells/well) and treated with LPS for 18 h. The cells were washed with cold PBS, scraped off, and pelleted at $700 \times g$ at 4°C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and centrifuged. Supernatants were saved as the whole-cell lysates. The proteins (20 μg) were separated by 8% reducing SDS-PAGE and transferred in 20% methanol, 25 mM Tris, and 192 mM glycine to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20), and subsequently incubated with anti-iNOS Ab for 4 h. The membrane was then washed and incubated for 1 h with a secondary Ab conjugated to HRP. The membrane was then washed and developed using an enhanced chemiluminescence system.

Northern blot analysis

Raw264.7 cells (1×10^6 cells) were cultured for 6 h at 37°C with the indicated concentrations of sPLA$_2$ and/or LPS. The cells were then washed three times with PBS containing 2% BSA, and RNA was isolated using the RNeasy kit (Quiagen, Chatsworth, CA). Then, 2-μg aliquots of total RNA were denatured and fractionated by gel electrophoresis using a 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred by capillary action with 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to a nylon membranes (Amersham). The blots were incubated with specific DNA probes for iNOS or GAPDH, which had been labeled with [α-^{32}P]dCTP by random priming using the Prime-a-Gene kit from Promega (Madison, WI). The iNOS DNA probe corresponds to bases 1–800 of the rat iNOS-coding region. The GAPDH probe was used as an internal control for RNA loading.

Transfection assay

Mouse type II sPLA$_2$ cDNA was subcloned into the mammalian expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA). cDNA carrying or empty vector was transfected into human embryonic kidney 293 cells using the Lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Then, 2 μg of plasmid was mixed with 1 μl of Lipofectamine in 200 μl of Opti-MEM medium (Life Technologies) for 15 min and then added to cells that had grown to 40–60% confluence in 6-well plates. After incubation for 5 h, the medium was replaced with fresh culture medium. After an overnight incubation, the medium as replaced again with fresh culture medium and culturing continued. For analysis of transient expression, the cells were harvested 3 days after transfection and used immediately. To obtain stable transfectants, cells transfected with cDNA were cloned by serial dilution in 96-well plates in a culture medium containing 700 μg/ml G418. After continued subculturing for 4 wk, wells representing a single colony were selected, and the expression of sPLA$_2$ was confirmed by measuring PLA$_2$ activity released into the supernatants. The cells were pellets and lysed in lysis buffer containing protease inhibitors. The lysates were then analyzed by Western blot analysis with anti-iNOS Ab.

Nuclear extracts

Raw264.7 cells (1×10^6 cells) were incubated with sPLA$_2$ or LPS for 30 min as indicated. Cells were harvested in PBS containing 2% serum, washed twice with PBS, and resuspended in 400 μl of buffer (10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 10 mM KCl, 1 mM $ZnCl_2$, 0.2 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). After the cells were incubated on ice for 10 min and then lysed by the addition of 50 μl of 10% Nonidet P-40 (1.1% final concentration), the nuclei were harvested by centrifugation. The nuclear pellets were resuspended in 60 μl of extraction buffer (10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 300 mM NaCl, 1 mM $ZnCl_2$, 0.2 mM EGTA, 25% glycerol, 1 mM Na_3VO_4, 10 mM NaF, 0.5 mM DTT,

0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) and incubated for 15 min on ice. Nuclear debris was removed by centrifugation (13,000 rpm for 10 min), and the nuclear protein extract was used for gel-shift analysis. Protein concentration was determined by the Bradford method.

EMSA

Gel-shift analysis of nuclear extracts was performed using oligonucleotides containing the consensus sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG-3′; Santa Cruz Biotechnology) end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Typical binding reactions consisted of 10 μg of nuclear extract, 1 ng DNA probe, 2 μg/ml poly[d(I-C)] in a buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol and were incubated at 30°C for 20 min. Binding reactions were separated on 6% Tris-glycine nondenaturing polyacrylamide gels in a 2× Tris-glycine buffer system. The gels were transferred to Whatman paper (Tewksbury, MA), dried, and subjected to autoradiography.

Results

The effect of sPLA$_2$ on nitrite production and iNOS mRNA and protein expression in Raw264.7 cells

LPS by itself activates mouse macrophages to express iNOS and produce NO. To investigate whether sPLA$_2$ could induce NO production in the Raw264.7 cells, we monitored nitrite concentrations in the culture media of cells stimulated with a sPLA$_2$-enriched supernatant. The sPLA$_2$ enzyme was obtained from sPLA$_2$-cDNA-transfected cells as described in *Materials and Methods*. After appropriate selections, several transfectants stably expressing substantial levels of sPLA$_2$ had been isolated. While sPLA$_2$ activity was barely detectable in parental 293 cells (135 dpm), it was strongly detected in the sPLA$_2$-cDNA transfectants (15,500 dpm). As shown in Fig. 1*A*, after 18 h of incubating Raw264.7 cells with the sPLA$_2$ (0–400 ng/ml), we saw little effect on nitrite production. However, sPLA$_2$ in combination with LPS (1 μg/ml) stimulated nitrite production >20-fold. The fact that this NO production could be inhibited with L-*N*-monomethylarginine (L-NMMA), a competitive inhibitor of NOS activity, suggests that the sPLA$_2$-potentiated nitrite production in the LPS-stimulated Raw264.7 cells is dependent on the NOS-mediated arginine metabolism. Fig. 1*B* shows that sPLA$_2$ potentiated the production of nitrite in LPS-stimulated Raw264.7 cells in a dose-dependent manner. There was an agreement between the synthesis of nitrite and the level of iNOS. sPLA$_2$ itself did not cause induction of iNOS protein in these cells. Higher amounts of iNOS were expressed when the cells were treated with LPS. However, iNOS expression drastically increased in response to treatment with a combination of sPLA$_2$ and LPS (Fig. 1*C*). The effect of sPLA$_2$, in the presence of LPS, on iNOS mRNA accumulation in Raw264.7 cells was examined by Northern blot analysis. sPLA$_2$ and LPS both stimulated the expression of iNOS mRNA following a 6-h exposure. However, the combination of sPLA$_2$ and LPS stimulated in iNOS mRNA accumulation with synergy (Fig. 1*D*). GAPDH, used as a control, was detected in all samples.

As Fig. 2*A* shows, low doses of LPS induced nitrite production only to a small extend. However, when sPLA$_2$ was present in the presence of LPS, a dose-dependent increase of nitrite accumulation was seen in response to increasing amounts of LPS. This potentiation of NO synthesis was evident in cells treated with LPS and sPLA$_2$ in combination. The dose-dependence curve for LPS shows that saturation in the presence of sPLA$_2$ was obtained in LPS concentrations above 100 ng/ml. Although LPS also stimulated iNOS expression, this effect was potentiated by sPLA$_2$ (Fig. 2*B*). Taken together, the results in Fig. 1 demonstrated that sPLA$_2$ stimulates iNOS expression and nitrite production and that sPLA$_2$ potentiates the LPS effect on Raw264.7 cells.

To confirm this sPLA$_2$ effect, we measured stimulation of nitrite production and iNOS expression using type II sPLA$_2$ purified from human synovial fluid. The effects achieved with the purified sPLA$_2$ were similar to those obtained with the overexpressed sPLA$_2$ in terms of nitrite production in Raw264.7 cells and is shown in Fig. 3*A*. When we added the type II sPLA$_2$ enzyme (0–800 ng/ml) to the cells, nitrite production was induced, but the level was very low (Fig. 3*A*, *inset*). However, this effect increased by purified

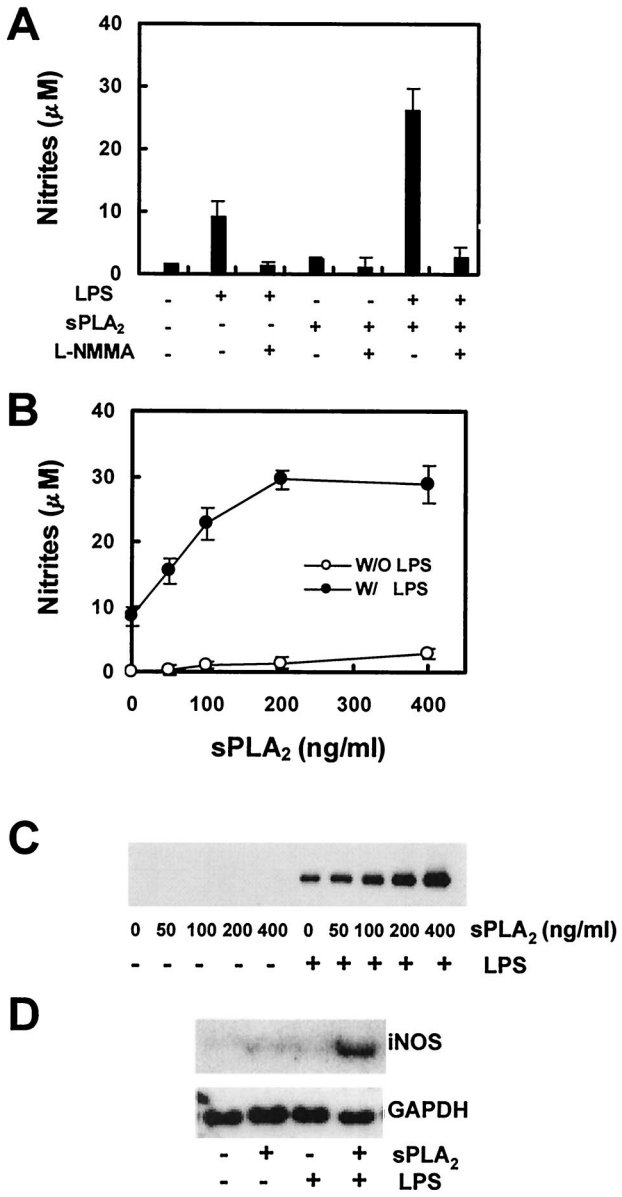

FIGURE 1. Effects of sPLA$_2$ and LPS on nitrite production, protein expression, and iNOS mRNA accumulation in Raw264.7 cells. *A*, Raw264.7 cells (1 × 10^6 cells/3 ml) were incubated for 18 h with the indicated concentrations of sPLA$_2$, LPS (1 μg/ml), and L-NMMA (0.5 mM). *B*, Cells were incubated with the indicated concentrations of sPLA$_2$ in the presence or in the absence of LPS (1 μg/ml). Nitrite production in the culture supernatant was determined as described in *Materials and Methods*. *C*, Raw264.7 cells (1 × 10^6 cells/3 ml) were incubated for 18 h with sPLA$_2$ (400 ng/ml) and LPS (1 μg/ml) concomitantly. The cells were isolated, and the expression of iNOS was determined by Western blot analysis as described in *Materials and Methods*. *D*, Raw264.7 cells (1 × 10^7 cells/10 ml) were cultured for 6 h in the presence of sPLA$_2$ (400 ng/ml) and LPS (1 μg/ml). Total RNA was isolated and probed for iNOS and GAPDH by Northern blot analysis as described in *Materials and Methods*. The results for nitrite are average values ± SE from five independent experiments. The iNOS protein and mRNA data are representative of three and two independent experiments, respectively.

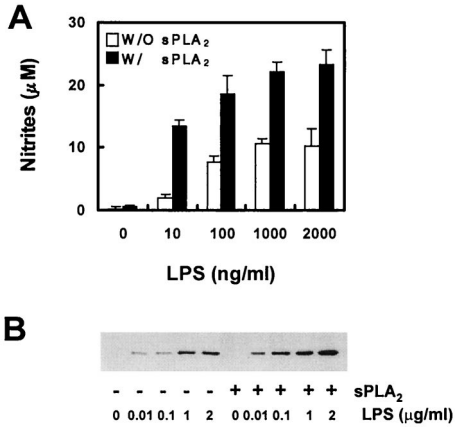

FIGURE 2. Dose-dependent induction of nitrite production and iNOS expression by sPLA$_2$ in LPS-stimulated Raw264.7 cells. Raw264.7 cells were incubated with the indicated concentrations of LPS and either culture medium or sPLA$_2$ for 18 h at 37°C. Nitrite production (*A*) was determined in the culture supernatant, and the level of iNOS protein (*B*) was determined by Western bolt analysis as described in *Materials and Methods*. Comparable results were obtained in three separate experiments.

sPLA$_2$ with synergism in a dose-dependent manner (Fig. 3*B*). Therefore, these results indicated that sPLA$_2$ is capable of stimulating Raw264.7 cells to produce of NO.

Effect of inhibitors of PLA$_2$ on LPS-induced nitrite production and iNOS expression in Raw264.7 cells

Because we have found that sPLA$_2$ raises the production of nitrite by Raw264.7 cells, we wanted to confirm the specificity of the PLA$_2$ type. Therefore, we stimulated the cells with LPS in the presence of selective cPLA$_2$ inhibitors, synthetic arachidonic acid analogue MAFP or AACOCF$_3$, and a specific sPLA$_2$ inhibitor, 12-epi-scalaradial. AACOCF$_3$ and MAFP inhibit cPLA$_2$-mediated phospholipid hydrolysis by binding tightly to the enzyme. 12-epi-scalaradial causes irreversible inhibition of sPLA$_2$ by forming a Schiff's base with a lysine residue on the surface of the enzyme (32). Nitrite production (Fig. 4*A*) as well as sPLA$_2$ activity (Fig. 4*C*) in response to LPS was inhibited in the presence of 12-epi-

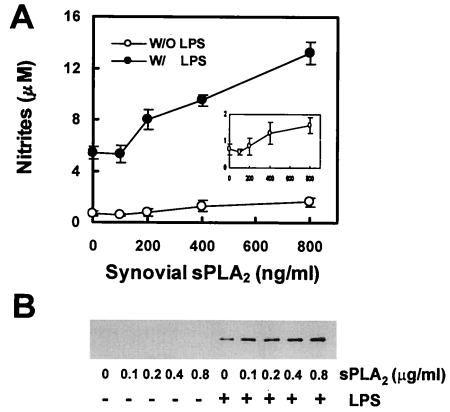

FIGURE 3. Effect of purified sPLA$_2$ on nitrite production and iNOS expression in LPS-stimulated Raw264.7 cells. Raw264.7 cells were incubated with the indicated concentrations of purified sPLA$_2$ in the presence or absence (*A*, *inset*) of LPS (1 μg/ml). Nitrite production in the culture supernatant was determined (*A*). Expression of iNOS (*B*) was determined by Western blot analysis as described in *Materials and Methods*. The results for nitrite are average values ± SE from three independent experiments, and iNOS expression data are representative of three independent experiments.

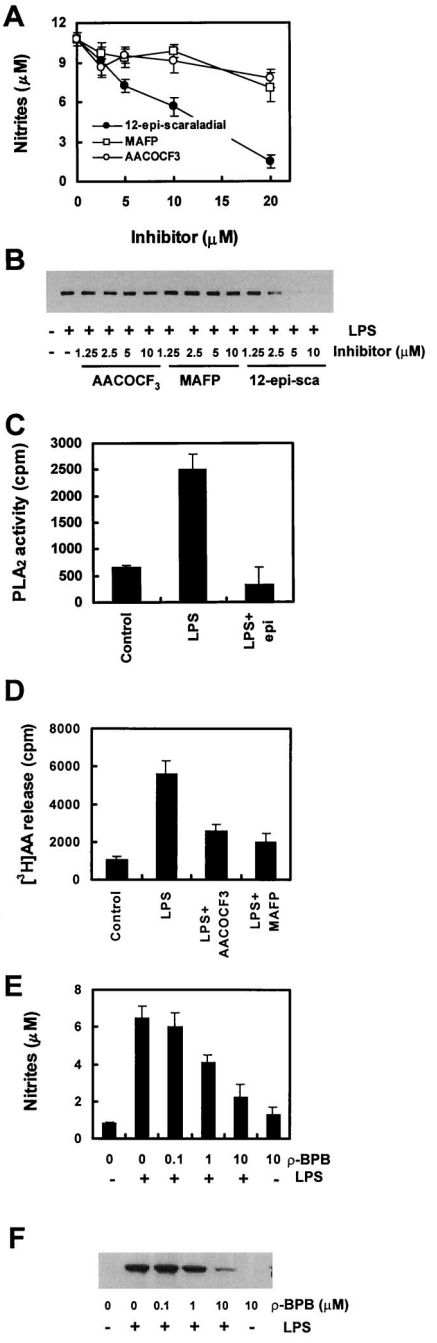

FIGURE 4. Effects of PLA$_2$ inhibitors on nitrite production, iNOS expression, and PLA$_2$ activity by Raw264.7 cells. Raw264.7 cells were pretreated with the indicated concentrations of MAFP, AACOCF$_3$, or 12-epi-scalaradial for 1 h followed by incubation with 1 μg/ml LPS for 18 h. Nitrite production (*A*) was determined in the culture supernatant. iNOS expression (*B*) was determined by Western blot analysis as described in *Materials and Methods*. *C*, The cells, pretreated with or without 10 μM 12-epi-scalaradial for 30 min, were challenged with LPS for 18 h. Afterward, supernatants were collected and assayed for PLA$_2$ activity. *D*, [^{3}H]AA release was assayed in LPS-treated cells. The cells were treated with 20 μM MAFP or 20 μM AACOCF$_3$ or neither for 30 min before the addition of LPS (1 μg/ml) for 18 h. The released [^{3}H]AA was determined as described in *Materials and Methods*. The cells were pretreated with the indicated concentrations of ρ-BPB for 1 h followed by incubation with LPS for 18 h. Nitrite production (*E*) was determined in the culture supernatant, and expression of iNOS (*F*) was determined by Western blot analysis as described in *Materials and Methods*. The results for nitrite and PLA$_2$ activities are average values ± SE from three independent experiments, and iNOS expressions are representative of three independent experiments.

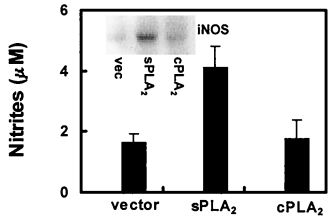

FIGURE 5. Effect of transiently expressed $sPLA_2$ on nitrite production and iNOS expression of transfected Raw264.7 cells. *A*, Raw264.7 cells were incubated with Lipofectamine complexes with $sPLA_2$, $cPLA_2$ cDNA/pCDNA 3.1, or empty vector for 72 h. After 72 h, nitrite production the medium was measured. *B*, Cell lysates from Raw264.7 cells transfected with the above cDNA were subjected to Western blot analysis with anti-iNOS Ab. The results for nitrite are average values ± SE from five independent experiments. iNOS expressions are representative of five independent experiments.

scalaradial in a dose-dependent manner and was completely inhibited at a 20-μM concentration of the inhibitor. However, $cPLA_2$ inhibitors MAFP and $AACOCF_3$ at a high concentration (20 μM) had little effect on the LPS stimulation of the cells (Fig. 4*A*), although $cPLA_2$ activities were decreased to almost control levels by both $cPLA_2$ inhibitors (Fig. 4*D*). To evaluate whether this was due to inhibition of iNOS expression, we monitored LPS-induced iNOS levels using immunoblot analysis. LPS-mediated iNOS expression was reduced in cells pretreated with 12-epi-scalaradial, while in the presence of MAFP or $AACOCF_3$ did not inhibit the LPS response (Fig. 4*B*). To strengthen this conclusion, we tested the effect of ρ-bromophenacyl bromide (ρ-BPB), the other structurally unrelated specific $sPLA_2$ inhibitor, on nitrite production and iNOS expression as well as $sPLA_2$ activity. ρ-BPB also strongly inhibited nitrite production and iNOS expression (Fig. 4, *E* and *F*) concomitantly with $sPLA_2$ activity (data not shown). These results suggest that the LPS-induced activation of iNOS is indeed the specific effect of $sPLA_2$. To confirm $sPLA_2$ specificity for the iNOS induction, cDNAs encoding mouse $cPLA_2$ and $sPLA_2$ were separately subcloned into mammalian expression vector (pcDNA 3.1) and used to transiently transfect Raw264.7 cells. We then measured PLA_2 activity and the amount of nitrites produced. The activity of PLA_2 increased about 4-fold in both transfectants as compare to empty vector transfectants. The production of nitrite was not detectable in untransfected Raw264.7 cells, while the nitrite production by the $sPLA_2$ transfectants was about 2-fold over the empty vector or $cPLA_2$ transfectants. Furthermore, expression of iNOS was also increased in the $sPLA_2$ transfectants compared with others transfectants, although empty vector and $cPLA_2$ transfectants both slightly induced iNOS expression (Fig. 5).

The role of NF-κB in $sPLA_2$-potentiated nitrite production, iNOS expression, and iNOS mRNA accumulation

Because $sPLA_2$ potentiated the production of NO in Raw264.7 cells, we wanted to see whether $sPLA_2$ could be involved in the LPS-mediated activation of NF-κB. One of the signaling molecules participating in the LPS-induction of iNOS expression is the transcriptional regulator NF-κB. The antioxidant, PDTC, a potent inhibitor of NF-κB activation, prevented LPS-induced iNOS expression in Raw264.7 cells. To determine whether NF-κB participated in $sPLA_2$-potentiated nitrite production and iNOS expression, the cells were pretreated for 1 h with 100 μM PDTC. After that, $sPLA_2$ or LPS and $sPLA_2$ plus LPS were added. The cells were then cultured for an additional 18 h. As seen in Fig. 6, *A* and *B*, the PDTC pretreatment prevented all LPS- and $sPLA_2$ plus LPS-induced nitrite production. Consistent with its inhibitory effects on nitrite production, PDTC also inhibited LPS- and $sPLA_2$ plus LPS-induced iNOS protein expression (Fig. 6*C*). We examined iNOS mRNA accumulation in macrophages treated with

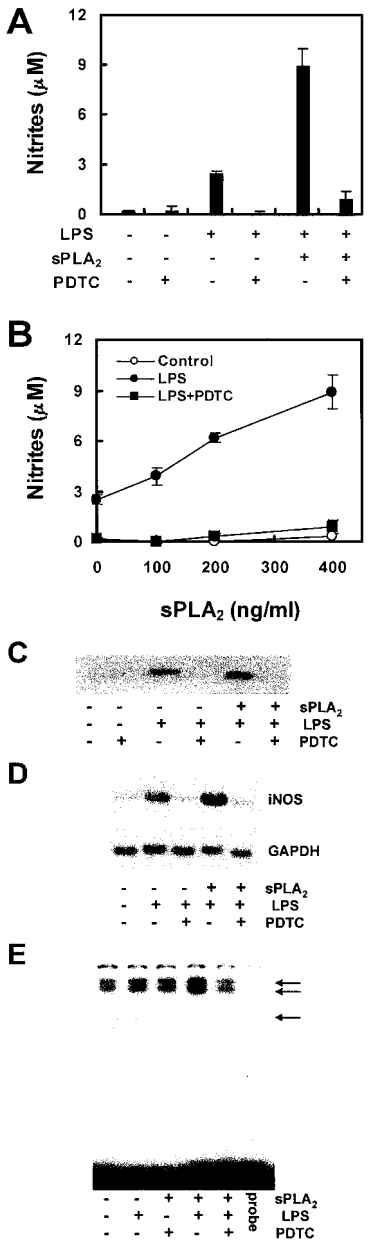

FIGURE 6. Effect of PDTC on nitrite production, iNOS expression, and mRNA accumulation in Raw264.7 cells. Raw264.7 cells were pretreated for 30 min with 100 μM PDTC and then incubated for 18 h with the indicated concentrations of $sPLA_2$ in the presence of LPS (1 μg/ml). Nitrite production (*A* and *B*) in culture supernatant was determined. Expression of iNOS (*C*) was determined by Western blot analysis as described in *Materials and Methods*. *D*, The cells were pretreated for 30 min with 100 μM PDTC and then incubated for 6 h with LPS (1 μg/ml) or LPS plus $sPLA_2$ (400 ng/ml) as indicated. Total RNA was isolated and probed for iNOS and GAPDH by Northern blot analysis as described in *Materials and Methods*. *E*, The cells were pretreated with 100 mM PDTC for 30 min before incubation with LPS or LPS plus $sPLA_2$. Then, nuclear extracts were prepared. NF-κB-specific DNA-protein binding activity in nuclear extracts was determined by EMSA as described in *Materials and Methods*. The results for nitrite are average values ± SE from three independent experiments. iNOS protein data are representative of three independent experiments. iNOS mRNA and EMSA data are representative of two independent experiments.

PDTC by Northern blot analysis. Fig. 6*D* show iNOS mRNA accumulation in Raw264.7 cells stimulated with LPS and sPLA$_2$ plus LPS. However, in the cells pretreated with the NF-κB inhibitor PDTC, the iNOS mRNA accumulation, even after LPS or sPLA$_2$ plus LPS treatment, dropped to basal levels. In addition, we used EMSA to investigate the involvement of NF-κB in the induction of iNOS. Raw264.7 cells were stimulated for 30 min with LPS or LPS plus sPLA$_2$. In nuclear extracts of unstimulated macrophages, two faint DNA-protein complexes were identified, the intensity of which increased following exposure of the cells to LPS. However, the intensity of bands is markedly increased on the treated cells with LPS plus sPLA$_2$. In addition, after treatment of the cells for 30 min with PDTC, the LPS plus sPLA$_2$-induced activation of NF-κB-specific DNA-protein complex formation was inhibited (Fig. 6*E*). These results suggest that the sPLA$_2$ plus LPS stimulation of iNOS mRNA transcription is dependent on NF-κB participation.

Discussion

Among the macrophage responses to LPS exposure is the expression of iNOS and increased production of NO (2, 6). Recent investigations have shown evidence that sPLA$_2$ enzymes may be important participants in the activation of macrophages by LPS (20–23, 33). It has also been reported that sPLA$_2$ enhances the response of leukocytes to LPS, which suggests a direct interaction of sPLA$_2$ with LPS (34). Rupprecht et al. have shown and suggested cross talk between sPLA$_2$ and iNOS in rat renal mesangial cells (35). Recently, Tsukahara et al. reported that the PLA$_2$ inhibitor quinacrine inhibited iNOS expression in alveolar macrophages and reduced lung injury in acute pancreatitis. They suggested that PLA$_2$ mediates NO production (36). Still, the mechanism by which PLA$_2$ stimulates iNOS expression is unknown.

In our current study, we examined the effect of sPLA$_2$ on macrophage activation and the mechanism by which sPLA$_2$ activates iNOS. Treatment of Raw264.7 cells with LPS stimulated iNOS expression and nitrite production. Alone, sPLA$_2$ also stimulated iNOS expression in Raw264.7 cells, but only slightly. However, in combination with LPS, sPLA$_2$ raised iNOS expression and nitrite production to high levels. The effect of PLA$_2$ on iNOS expression appears to be PLA$_2$ type specific. While sPLA$_2$ inhibitors, 12-epi-scalaradial and ρ-BPB, inhibited LPS-induced iNOS expression in the cells, cPLA$_2$ inhibitors, MAFP or AACOCF$_3$, did not inhibit nitrite production and iNOS expression. In addition, when cDNAs encoding either sPLA$_2$ or cPLA$_2$ were transfected into the cell, sPLA$_2$ transfectants stimulated nitrite production significantly more than cPLA$_2$ or empty vector transfectants. In the process of responding to LPS, an early crucial step is the nuclear translocation of NF-κB, which in turn induces the transcriptional activation of genes for various inflammatory cytokines. The sPLA$_2$-potentiated iNOS expression in Raw264.7 cells also required the activation of NF-κB. Our studies have shown that LPS- or LPS plus sPLA$_2$-induced iNOS expression, mRNA accumulation, and nitrite production can be prevent by treatment of the cells with the NF-κB inhibitor PDTC. In addition, PDTC inhibited LPS plus sPLA$_2$-induced NF-κB-specific DNA-protein binding and IκBα degradation by Raw264.7 cells (data not shown). These results suggest that sPLA$_2$ may participate in the iNOS induction, which then leads to the functional activation of NF-κB.

Our study showed that sPLA$_2$ induces iNOS expression and NO generation of macrophages, which contribute to sepsis. These conclusions are based on the observations of the direct effect of potent inhibitors with high selectivity against either sPLA$_2$ or cPLA$_2$. The role of sPLA$_2$ in endotoxic shock has been widely studied (27, 28). Both activity and protein levels of this enzyme are enhanced in the serum of patients with endotoxic shock, and both increase after the production of proinflammatory cytokines including TNF-α and IL-1β. It is also well known that circulating PLA$_2$ causes tissue injury such as damage to the alveolar surfactant or, by reacting with cell membranes, releases inflammatory mediators such as eicosanoids and platelet-activating factor. Therefore, we hypothesize that increased levels of PLA$_2$, especially type II, raise iNOS expression in macrophages and thus mediate sepsis or inflammation. This speculation is supported by several studies. Kurose et al. reported that an increased production of NO in rat Kupffer cells was proceeded by activated NF-κB, and the PLA$_2$ inhibitor quinacrine significantly attenuated the increase in NF-κB and NO production (37). Furthermore, in a study of an animal model of inflammation, when the rat air pouch was stimulated with zymosan, the levels of nitrites, sPLA$_2$ in exudates, and NOS activity in polymorphonuclear leukocytes and monocytes increased (38).

At present, we have preliminary data concerning the upstream targets of iNOS. Some reports have shown a possible role for tyrosine kinase and phosphatidylinositol 3-kinase (PI 3K) in the process of macrophage activation and LPS-induced iNOS induction (39–41). Inhibition of PI 3K by LY294002 results in down-regulation of iNOS expression, mainly through a mechanism that involves activation of NF-κB. Furthermore, Chen et al. reported that LPS activates phospholipase D (PLD) via tyrosine phosphorylation by protein kinase C and NF-κB activation, iNOS expression, and finally NO release (42). We are currently examining the effects of signaling molecules such as tyrosine kinase, PI 3K, PLD, and extracellular regulated kinase (ERK) on sPLA$_2$-potentiated iNOS expression in Raw264.7 cells. The PI 3K inhibitor LY294002 and the PLD inhibitor 1-butanol attenuate the sPLA$_2$-potentiated effects as well as LPS effects, whereas the mitogen-activated protein kinase/ERK (MEK)/ERK inhibitor PD98059, which abrogates MEK/ERK activation by LPS, has little effect on iNOS expression (data not shown). The results suggest that the signal transduction pathways leading to iNOS and to MEK/ERK activation diverge downstream of PI 3K and PLD activation. This is in contrast to the situation in epithelial cell invasion by *Listeria monocytogenes*, in which both PI 3K and ERK are activated (43).

sPLA$_2$ is a proinflammatory mediator found to be highly elevated both in the circulation and locally in tissues and in association with a number of pathologic conditions such as sepsis. The main proinflammatory effect of sPLA$_2$ is thought to be the generation of arachidonic acid as a precursor for eicosanoids. Our results suggest a potentially new role for sPLA$_2$, namely in the potentiation of iNOS and NF-κB-regulated expression of genes involved in LPS signal transduction. The sPLA$_2$- or LPS-mediated increase in NF-κB and the cellular consequences should be of interest in the search for inhibitor compounds for the treatment of inflammatory conditions.

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