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A Phosphatidylcholine-Specific Phospholipase C Regulates Activation of p42/44 Mitogen-Activated Protein Kinases in Lipopolysaccharide-Stimulated Human Alveolar Macrophages

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This study uses human alveolar macrophages to determine whether activation of a phosphatidylcholine (PC)-specific phospholipase C (PC-PLC) is linked to activation of the p42/44 (ERK) kinases by LPS. LPS-induced ERK kinase activation was inhibited by tricyclodecan-9-yl xanthogenate (D609), a relatively specific inhibitor of PC-PLC. LPS also increased amounts of diacylglycerol (DAG), and this increase in DAG was inhibited by D609. LPS induction of DAG was, at least in part, derived from PC hydrolysis. Ceramide was also increased in LPS-treated alveolar macrophages, and this increase in ceramide was inhibited by D609. Addition of exogenous C2 ceramide or bacterial-derived sphingomyelinase to alveolar macrophages increased ERK kinase activity. LPS also activated PKCζ, and this activation was inhibited by D609. LPS-activated PKCζ phosphorylated MAP kinase kinase, the kinase directly upstream of the ERK kinases. LPS-induced cytokine production (RNA and protein) was also inhibited by D609. As an aggregate, these studies support the hypothesis that one way by which LPS activates the ERK kinases is via activation of PC-PLC and that activation of a PC-PLC is an important component of macrophage activation by LPS. The Journal of Immunology, 1999, 162: 3005–3012.

One of the early and important factors related to the development of the adult respiratory distress syndrome (ARDS)3 is the release of cytokines by alveolar macrophages (1–3). For sepsis-induced ARDS, endotoxin (LPS) is a major stimulus for the release of cytokines by alveolar macrophages and, thereby, an important factor in the development of ARDS (1–3). The best-defined receptor that is utilized by LPS to trigger expression of the cytokine genes in macrophages is CD14 (4, 5). In this setting, LPS interacts with CD14 complexed with the serum protein, LPS-binding protein. This interaction causes tyrosine phosphorylation of a number of cellular proteins and activation of multiple mitogen-activated protein (MAP) kinases (6–14). There are three important groups of MAP kinases, the p42/44 (ERK) kinases, the c-jun kinases, and the p38 kinases (15). The activation of these MAP kinase pathways appears to be linked to regulation and expression of cytokine genes as well as other important cellular functions (16–18). However, the mechanisms that result in activation of these kinase pathways by LPS are poorly understood. The focus of this study was to further delineate how LPS activates one of the kinase pathways, the ERK kinases, in alveolar macrophages.

The pathways by which the ERK kinases may become activated have been studied in a number of cell systems. These kinases are known to be directly activated by mitogen-activated protein kinase kinase, a kinase directly upstream of the ERK kinases (15). The mechanism by which MEK becomes activated varies, depending on the stimulus and the cell type. The best-described pathway involves the sequential activation of ras,raf-1,MEK, and the ERK kinases (15, 19, 20). Ueda et al. (21) described a ras-independent pathway that requiresraf-1 and PKCδ. Berra et al. (22, 23) used a dominant-negative mutant of PKCζ to block serum and TNF activation of the ERK kinases. These studies suggest that there are multiple pathways by which MEK and the ERK kinases can be activated. No studies have determined how LPS activates the ERK kinases, but several studies provided clues that have led to our hypothesis. A study by Grove et al. (24) showed that LPS activated a phosphatidylcholine-specific phospholipase C (PC-PLC) in macrophages. Several studies using other stimuli have linked activation of a PC-PLC to activation of the ERK kinases in other types of cells (25–27). Potentially, downstream of PC-PLC, ceramide may serve as an activator of PKCζ. This has been described in a study by Muller et al. (28), which used TNF as a stimulus. Collectively, these observations led us to hypothesize that LPS-induced activation of ERK kinases in alveolar macrophages would be mediated by sequential activation of PC-PLC, induction of cellular diacylglycerol (DAG) and ceramide, and activation of PKCζ. We further hypothesized that activation of a PC-PLC would play an important role in the production of cytokines by LPS-treated alveolar macrophages.

Materials and Methods

Isolation of human alveolar macrophages

Alveolar macrophages were obtained from bronchoalveolar lavage as previously described (29). Briefly, normal volunteers with a lifetime nonsmoking history, no acute or chronic illness, and no current medications

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Abbreviations used in this paper: ARDS, adult respiratory distress syndrome; MAP, mitogen-activated protein; ERK kinases, p42/44 kinases; MEK, mitogen-activated protein kinase kinase; PKC protein kinase C; PC-PLC, phosphatidylcholine-specific phospholipase C; D609, tricyclodecan-9-yl xanthogenate; DAG, diacylglycerol; MBP, myelin basic protein; PA, phosphatidic acid; lyso-PC, 1-1-palmitoyl-2-lyso-palmitoylphosphatidylcholine; PC, phosphatidylcholine; PLD, phospholipase D.

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underwent bronchoalveolar lavage. The lavage procedure used five 25-ml aliquots of sterile, warmed saline in each of three segments of the lung. The lavage fluid was filtered through two layers of gauze and centrifuged at 1500 \( \times g \) for 5 min. The cell pellet was washed twice in HBSS without Ca\(^{++}\) and Mg\(^{++}\) and suspended in complete medium, RPMI tissue culture medium (Life Technologies, Gaithersburg, MD) with 5% FCS (HyClone, Logan, UT), and added gentamicin (80 \( \mu \)g/ml). Cell number was determined using a Wright-Giemsa-stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages.

This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

### Immunoprecipitation of ERK kinase and PKC \( \zeta \)

Alveolar macrophages were cultured in complete medium with or without LPS (1 \( \mu \)g/ml, Sigma, St. Louis, MO), and/or tricyclodecan-9-yl xanthogenate (D609, 100 \( \mu \)M, Calbiochem, San Diego, CA) a relatively specific inhibitor of PC-PLC. (30–32). In some instances, cells were also exposed to C\(_2\) ceramide (16 \( \mu \)M, Biomol, Plymouth Meeting, MA) or bacterial-derived sphingomyelinase (0.4 U/ml, Biomol). After culture, cells were lysed on ice for 20 min in 500 \( \mu \)l of lysis buffer (0.05 M Tris (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF, 50 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin, 50 \( \mu \)g/ml pepstatin, 0.4 M sodium \( \alpha \)-vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate, all from Boehringer Mannheim, Indianapolis, IN). The lysates were then spun at 15,000 \( \times g \) for 10 min, and the supernatant was saved. Protein was measured, and 600 \( \mu \)g from each sample were removed for immunoprecipitation. The samples were cleared by incubation for 2 h with 1 \( \mu \)g/sample rabbit IgG and 10 \( \mu \)g/sample GammaBind Sepharose (Pharmacia, Piscataway, NJ). After centrifugation, the supernatants were transferred to a tube containing 3 \( \mu \)g/sample rabbit anti-ERK Ab (sc-154, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-PKC \( \zeta \) b (sc-216, Santa Cruz Biotechnology) bound to GammaBind Sepharose and rotated at 4°C overnight. The beads were subsequently washed three times with high salt buffer (0.05 M Tris (pH 7.4), 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer without protease inhibitors. The ERK or PKC \( \zeta \) complexes were either released with 2 \( \times \) sample buffer for Western analysis or used to determine kinase activity.

### ERK and PKC \( \zeta \) kinase activity

After immunoprecipitation of ERK or PKC \( \zeta \) from alveolar macrophages, the protein-containing pellet was washed twice with kinase buffer (20 mM MgCl\(_{2}\), 25 mM HEPES, 20 mM \( \beta \)-glycerophosphate, 20 mM sodium \( \alpha \)-vanadate, and 2 mM DTT). The pelleted samples were subsequently washed three times with kinase buffer, 10 mM \( \alpha \)-phosphatase, 50 mM ATP, 5 \( \mu \)Ci of \( \gamma \)-\( ^{32} \)P\( \alpha \)-ATP (BLU 002Z, DuPont/NEN, Boston, MA), and 10 \( \mu \)g of myelin basic protein (MBP, Sigma) or in one instance, 8 \( \mu \)g of MEK-1 (55-KDa polyhistidine-tagged fusion protein, Santa Cruz Biotechnology). The reaction was continued for 15 min (ERK kinase) or 30 min (PKC \( \zeta \)) at 25°C and then stopped by the addition of 40 \( \mu \)l/sample of 2 \( \times \) sample buffer. The beads were solubilized in an aliquot of octyl-

### Detection of DAG in phosphatidylcholine-labeled cells

To evaluate DAG in labeled cells, the following methods were utilized. Alveolar macrophages were cultured overnight in complete medium with added [1\( \rightarrow \)\( ^{3} \)C]palmitic acid (NEC061, DuPont/NEN), 0.1 \( \mu \)Ci/10\(^{4}\) cells, or 1\( \rightarrow \)\( ^{14} \)C]lysopalmityloxyphosphatidylcholine (lyso-PC, NEC683, DuPont/NEN), 0.1 \( \mu \)Ci/10\(^{4}\) cells. The cells were then washed with PBS and resuspended at 10\(^{6}\) cells/ml of complete medium and placed at 1 ml/tube in 1.5-ml microtubes. After incubation with LPS (1 \( \mu \)g/ml) or LPS and D609 (100 \( \mu \)M) for 5 min, lipids were extracted using the Bligh-Dyer method (34). After extraction, samples were dried under nitrogen gas and resuspended in 50 \( \mu \)l/sample chloroform-methanol (2:1). Samples were then spotted on a TLC plate, (LK 5D, Whatman, Clifton, NJ) and run in a solvent of hexane-diethyl ether-acetic acid (60:40:1). The DAG bands were found by comparison with the position of a known standard (1,2-dioleoyl-sn-glycerol, C18:1, cis-9, Sigma). Quantitation was done by analyzing the TLC plate on an AMBIUS radiosotope scanner (Scanalytics, Billerica, MA). Data are presented as fold increase, experimental sample/control sample. To confirm that the palmitoyl-1\( \rightarrow \)\( ^{14} \)C]lyso-PC label remained in the phosphatidylcholine (PC) fraction during the 24-h labeling incubation, we evaluated the distribution of the label immediately before the LPS stimulation. Alveolar macrophages were labeled overnight with palmitoyl-1\( \rightarrow \)\( ^{14} \)C]lyso-PC, and then lipids extracted using the Bligh-Dyer method. The major phospholipids (PC, sphingomyelinase, phosphatidylglycerol, phosphatidyethanolamine, phosphatidylserine, and phosphatidylinositol) were resolved using TLC in chloroform-methanol-acetic acid-water-acetone (40:25:4:2-4). The phospholipids that comigrated with known standards were scraped, and counts were determined using a liquid scintillation counter.

### Expression of cytokine mRNA

Whole-cell RNA was isolated using RNA Stat-60 according to the manufacturer’s instructions (Tel-Test “B,” Friendswood, TX). Cells were lysed in RNA Stat-60 solution, containing phenol and guanidinium thiocyanate. The mixture was then shaken vigorously, allowed to sit for 2–3 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. The RNA was removed from the upper aqueous layer, precipitated with isopropanol for 30 min, centrifuged at 12,000 rpm for 10 min at 4°C, and washed with 75% ethanol. The isolated RNA was fractionated in a 1.5% denaturing agarose gel containing 2.2 M formaldehyde. An RNA ladder (0.24–9.5 kb; Life Technologies) was included as a molecular size standard. RNA loading was confirmed by equivalent ethidium bromide staining in each lane. The RNAs were transferred to GeneScreen Plus (DuPont/NEN) as suggested by the manufacturer. IL-6 and TNF \( \alpha \) cDNA probes (generated by PCR with primers obtained from Clontech, Palo Alto, CA) were labeled with \( \gamma \)-\( ^{32} \)P\( \alpha \)-ATP (DuPont/NEN) by random primer method. Blots were prehybridized for 3 h at 42°C (10 ml formamide, 5 mM NaCl, 4 ml 50% dextran, 10% SDS, 1 M Tris (pH 7.0), and 0.4 ml of 50% Denhardt’s solution) and then hybridized with the labeled probe overnight at 42°C. The filters were washed twice with 1\( \times \) SSC at 25°C, twice with 1\( \times \) SSC plus 1% SDS at 65°C, and then once with 0.1\( \times \) SSC at 25°C. The filters were exposed to autographic film at \( -70^\circ \)C.

### Release of cytokines

For these studies, alveolar macrophages were cultured in RPMI medium with 5% FCS for 24 h in the presence or absence of LPS and with and without D609, as described above. After the culture period, the supernatants were harvested and stored at \( -70^\circ \)C until assayed. The amounts of IL-6 and TNF in the supernatant of the cells were measured by ELISA (R&D Systems, Minneapolis, MN).
All of the cytokine measurements and densitometry data are shown as means ± SEM. Statistical comparisons were performed using a paired t test with a probability value of p < 0.05 considered to be significant.

Results

D609 blocks activation of the ERK 2 (p42) MAP kinase in alveolar macrophages

Although prior studies showed that LPS activates ERK kinases in murine macrophages and human monocytes (9, 10, 12), no studies have shown that LPS activates these kinases in human alveolar macrophages. Thus, we first showed that LPS activates the ERK 2 MAP kinase in alveolar macrophages (Fig. 1).

No prior studies have determined whether activation of a PC-PLC is linked to activation of the ERK kinases in LPS-stimulated cells. To evaluate this, we first determined whether propranolol, an inhibitor of PA phosphohydrolase, blocked ERK 2 activation (35). Propranolol did not inhibit LPS-induced ERK 2 activation (Fig. 2). This observation suggests that PLD-derived DAG is not linked to ERK 2 activation. We also used 2,3-bis-diphosphoglycerate, an inhibitor of PLD (36). This compound also did not inhibit LPS-induced ERK 2 activation (data not shown). This observation suggests that neither activation of PLD nor PLD-derived PA is linked to LPS-induced ERK 2 activation.

LPS induces activation of PC-PLC

We next determined whether LPS stimulates PC-PLC activity and whether this effect is inhibited by D609. We first determined whether LPS increased DAG mass and whether this increase could be blocked by D609. Alveolar macrophages were cultured with or without LPS (1 μg/ml) for 1–5 min and with and without D609 (100 μM) which was added to the cells 30 min before LPS. The cells were harvested, and DAG mass was determined. LPS increased DAG by 1 min after LPS exposure, with a significantly greater increase at 5 min (Fig. 3). The LPS-induced increase in DAG was inhibited by D609. These data show that LPS triggers release of DAG in alveolar macrophages and that DAG might be derived from PC.

Statistical analysis

All of the cytokine measurements and densitometry data are shown as means ± SEM. Statistical comparisons were performed using a paired t test with a probability value of p < 0.05 considered to be significant.

FIGURE 1. LPS increases ERK 2 kinase activity in alveolar macrophages, and this is inhibited by D609. A. Alveolar macrophages were cultured with and without LPS (1 μg/ml) and D609 (100 μM). ERK 2 protein was then immunoprecipitated from the cells and incubated with MBP under phosphorylating conditions. The top bands show an autoradiograph of radiolabeled MBP run out on a SDS gel. The lower bands show a Western blot of the immunoprecipitated ERK 2 proteins, showing equal loading of the kinase in the assay. B. Densitometry was performed on three experiments, and calculations were made using the mean gray level values, *, p < 0.05 for LPS compared with control and LPS compared with LPS and D609.

FIGURE 3. LPS increases amounts of DAG, and this is inhibited by D609. Alveolar macrophages were cultured with and without LPS (1 μg/ml) and D609 (100 μM). Lipids were extracted, and DAG mass was determined. The is an autoradiograph of a TLC plate, showing radiolabeled DAG after experimental treatment with DAG kinase and [γ-32P]ATP. Values are representative of three separate experiments.

FIGURE 2. LPS-induced ERK 2 kinase activity is not inhibited by propranolol. Alveolar macrophages were cultured with and without LPS (1 μg/ml), propranolol (100 μM), and D609 (100 μM). ERK 2 protein was then immunoprecipitated from the cells and incubated with MBP under phosphorylating conditions. This shows an autoradiograph of radiolabeled MBP run out on a SDS gel.
We next determined whether the DAG was secondary to PC hydrolysis, using a PC-specific label. To perform these experiments, we cultured alveolar macrophages overnight in the presence of [14C]lyso-PC or [14C]arachidonic acid. After removal of the unincorporated label, the cells were stimulated with LPS (1 μg/ml) for 0.25 to 5 min. The cells were then harvested, and DAG levels were determined by separating the lipids on TLC plates (Fig. 4). LPS increased amounts of DAG, and the DAG was derived, at least in part, from PC because increases in labeled DAG were found after using the lyso-PC label. The amount of DAG detected using this label was similar to that detected using [14C]arachidonic acid, as a label. We next determined the distribution of [14C]lyso-PC into major phospholipids. We observed that the majority of the label incorporated into PC and, to a lesser extent, sphingomyelin; only a small percentage of the label was found in the other phospholipids (Fig. 5). The amount of the label in the phospholipids other than PC is unlikely to account for the increases seen in DAG, especially considering that PC is the major phospholipid found in eukaryotic cells. Thus, these results, taken together, suggest that the majority of DAG is derived from PC.

**LPS increases ceramide in alveolar macrophages**

Activation of PC-PLC can be linked to activation of sphingomyelinase activity (37, 38). To evaluate this, alveolar macrophages were incubated with and without LPS (1 μg/ml) for 30 s–30 min. The cells were then harvested, and amounts of ceramide were evaluated in cell lysates. D609 blocked the LPS-induced increase in ceramide to below baseline levels (Fig. 7). These data suggest that LPS activates a sphingomyelinase pathway in alveolar macrophages in a PC-PLC-dependent manner.

**Products of the sphingomyelinase pathway activate ERK kinase in alveolar macrophages**

To determine whether products of the sphingomyelin/ceramide pathway could induce ERK kinase activity in alveolar macrophages, the cells were cultured with medium alone (control), or with C2 ceramide (16 μM), or with bacterial-derived sphingomyelinase (0.4 U/ml) for 5 min or for 1 h. Both C2 ceramide and bacterial-derived sphingomyelinase triggered activation of the ERK kinase by 5 min (Fig. 8). This kinase activity was decreased by 1 h. Shown for comparison are the effects of LPS, which has a more prolonged time course. These studies show that components of the sphingomyelinase pathway can activate ERK kinases in alveolar macrophages.

We also asked whether D609 had a nonspecific effect on inhibition of ERK 2. To do this, we incubated alveolar macrophages with C2 ceramide and evaluated the effect of D609 on activation of ERK 2. D609 did not inhibit the activity of C2 ceramide (Fig. 9). This observation suggests that activation of PC-PLC is a proximal event to generation of ceramide. These data also show that D609 does not have a nonspecific effect on ERK 2 activation.
LPS activates PKCζ in alveolar macrophages, and this activation is blocked by D609

One means by which ceramide might activate the ERK kinases is via activation of an atypical PKC isoform (ζ or η) (39). In previous studies, we were able to detect PKCζ but not PKCη in alveolar macrophages (40). To determine activation of PKCζ, we cultured alveolar macrophages with and without LPS (1 μg/ml) and with and without D609 (100 μM). After 15 min, the cells were harvested, and PKCζ was immunoprecipitated from the cell lysates. Fig. 10 shows two different kinase activity assays, one using MBP as a substrate and the other using MEK as a substrate. We also looked at PKCζ autophosphorylation with similar results (data not shown). These experiments show that LPS causes activation of PKCζ and that this activation is blocked by D609. These experiments also show that LPS-activated PKCζ can phosphorylate the cellular substrate MEK, a kinase directly upstream of the ERK kinases.

D609 blocks LPS-induced release of cytokines

To determine whether D609 would interfere with a macrophage function that can be tied to ERK kinase activation (41), we evaluated the effect of D609 on cytokine release and mRNA production in LPS-treated alveolar macrophages. IL-6 and TNF are both proinflammatory cytokines that are released from LPS-activated alveolar macrophages. We evaluated the effect of D609 on IL-6 and TNF mRNA and protein release. LPS increased both IL-6 and TNF mRNAs and protein release, and these were blocked by D609 (Figs. 11 and 12). These studies show that activation of a PC-PLC can be linked to a physiologically relevant macrophage function (cytokine production after LPS exposure).

Discussion

This study was undertaken to define pathways by which LPS might activate the ERK kinases in alveolar macrophages. We hypothesized that LPS activates a PC-PLC, leading to production of DAG, an activator of sphingomyelinase activity. This, in turn, results in increased amounts of ceramide, an important effector molecule, which activates PKCζ. PKCζ activates MEK, which subsequently leads to ERK kinase activation. To test this hypothesis, we showed that LPS activates the ERK 2 kinase in alveolar macrophages and

FIGURE 7. LPS induction of ceramide is inhibited by D609. Alveolar macrophages were cultured with and without LPS (1 μg/ml) and D609 (100 μM). Lipids were extracted, and ceramide mass was determined. This shows an autoradiograph of the TLC plate, showing 32P-labeled ceramide from cellular lysates. Densitometry was performed on the autoradiograph, and fold increase was calculated as experimental values divided by control values. Values are representative of three separate experiments.

FIGURE 8. C2 ceramide and sphingomyelinase increase ERK 2 kinase activity in alveolar macrophages. Alveolar macrophages were cultured with C2 ceramide (16 μM), bacterial-derived sphingomyelinase (0.4 U/ml) or LPS (1 μg/ml). ERK 2 protein was then immunoprecipitated from the cells and incubated with MBP under phosphorylating conditions. This shows an autoradiograph of radiolabeled MBP run out on a SDS gel. Values are representative of three experiments.

FIGURE 9. D609 does not block C2 ceramide induced ERK 2 kinase activity in alveolar macrophages. Alveolar macrophages were cultured with C2 ceramide (16 μM) with and without D609 (100 μM). ERK 2 protein was then immunoprecipitated from the cells and incubated with MBP under phosphorylating conditions. This shows an autoradiograph of radiolabeled MBP run out on a SDS gel.

FIGURE 10. LPS increases PKCζ activity in alveolar macrophages, and this is inhibited by D609. A. Alveolar macrophages were cultured with and without LPS (1 μg/ml) or D609 (100 μM). PKCζ was then immunoprecipitated and incubated with either MBP (top bands) or MEK-1 (lower bands) under phosphorylating conditions. This is an autoradiograph of radiolabeled MBP (top) or MEK (bottom) run out on a SDS gel. B. Densitometry was performed on the autoradiographs from three experiments that utilized MBP as a substrate; p < 0.05 for LPS compared with control and LPS compared with LPS and D609.
that this activation is inhibited by D609, a relatively specific inhibitor of PC-PLC. We next showed that LPS increases amounts of DAG and ceramide and that both of these effects of LPS are inhibited by D609. Our present studies indicate that LPS induction of DAG in alveolar macrophages is derived, at least in part, from a PC-containing phospholipid. Thus, we conclude from these experiments that the LPS-induced DAG is derived from hydrolysis of PC via activation of PC-PLC. In separate studies, the addition of exogenous C2 ceramide or bacterial-derived sphingomyelinase activated ERK 2 kinase in alveolar macrophages, and this activation was not inhibited by D609. Finally, we showed that LPS activates PKC ζ, and that PKC ζ from LPS-treated cells can activate MEK. The LPS-induced activation of PKC ζ also was inhibited by D609. Collectively, these studies strongly suggest that LPS activates the ERK kinases in alveolar macrophages via sequential activation of a PC-PLC, sphingomyelin hydrolysis, and PKC ζ. We have not proved that this is the only pathway by which LPS activates ERK kinases, but our studies strongly support the hypothesis that this is an important effector mechanism by which LPS activates ERK kinases in alveolar macrophages. We further show that LPS activation of a PC-PLC is linked to important macrophage functions, because inhibition of PC-PLC interferes with cytokine production in LPS-treated alveolar macrophages.

Several studies support the idea that hydrolysis of PC is coupled to activation of ERK kinases. Cai et al., using NIH 3T3 cells, have shown that the addition of exogenous PC-PLC induces phosphorylation of MEK, which subsequently activates ERK kinases (25). In fibroblasts (Rat-1), van Dijk et al. (26) have shown that stimulation of PC-PLC activity results in activation of the ERK kinases. Work by Sands et al. (42) suggests that PC hydrolysis might be a relevant signaling pathway in endotoxin-exposed macrophages. Using murine macrophages, LPS treatment increased DAG, which was due to activation of PC-PLC. These studies are consistent with our finding that LPS activates ERK kinases in alveolar macrophages via an activation of PC-PLC.

Our observation that LPS activation of a sphingomyelinase pathway by PC-PLC-generated DAG is supported by studies evaluating TNF and IL-1 signaling in a variety of cell types (reviewed by Kolesnick and Golde (43)). Several studies have linked generation of DAG to activation of a sphingomyelinase activity and subsequent ceramide generation. Of special relevance to our studies are the observations of Lozano et al. (39) and Muller et al. (28) which reported that the addition of exogenous sphingomyelinase to cells activates PKC ζ (Lozano), and that PKC ζ is activated by ceramide (Muller). These studies are consistent with our findings for both ceramide generation and PKC ζ activation in LPS-treated alveolar macrophages.

These results are also in agreement with prior studies, which link PC-PLC to PKC ζ and ERK kinase activation. Bjorkoy et al. (44) have shown in NIH 3T3 cells that PKC ζ activation is a necessary downstream component of PC-PLC cell transformation. A study by van Dijk et al. (26) used an inhibitor of PKC (Ro31-8220) to block PC-PLC-induced MAP kinase activation. They showed that expression of a dominant negative mutant of PKC ζ.

**FIGURE 11.** D609 blocks cytokine mRNA production in LPS-treated alveolar macrophages. Alveolar macrophages were cultured with and without LPS (1 μg/ml) or D609 (100 μM). After 3 h of culture, whole cell RNA was isolated and Northern analysis was performed. Blots were probed with radiolabeled cDNA specific for IL-6 (top bands) and TNF (middle bands), and an autoradiograph was obtained. Ethidium bromide staining of ribosomal bands is included (bottom bands) to show equal loading of the RNA in the blot.

**FIGURE 12.** D609 blocks release of IL-6 and TNF protein from LPS-treated alveolar macrophages. Alveolar macrophages were cultured with and without LPS (1 μg/ml) or D609 (100 μM). After 24 h in culture, the supernatant was removed, and cytokine levels were determined by ELISA. Statistical analysis was done using a paired t test. *, p < 0.05; **, p < 0.01.
unlike down-regulation of other PKC isoforms (α, δ, and ε), inhibited the MAP kinase activation by PC-PLC. Berra et al. (23) also showed that a dominant negative mutant of PKCζ dramatically impaired the activation of both MEK and MAP kinase in serum- and TNF-treated COS cells. These studies are consistent with our finding that PKCζ plays a central role in ERK kinase activation by LPS in alveolar macrophages. DAG, phosphatidic acid, or ceramide have all been shown to stimulate PKCζ in vitro (26, 28, 45). The observation that neither propranolol nor 2,3-bis(diphasphoglycerate inhibits LPS-induced ERK 2 activation argues that PKA is of little importance in this system. In addition, the observation that ceramide inhibits DAG kinase suggests that PC-PLC-derived PA might be limiting and therefore of lesser importance in LPS induction of PKCζ activity (46).

Aside from LPS activation of MEK via PKCζ, a number of studies suggest a role for Raf-1 in ERK kinase activation. One possible parallel pathway could include LPS-PC-PLC-DAG-novel PKCζ isoform-raf-1 MEK-ERK. This pathway is suggested in studies where dominant-negative mutants of raf-1 inhibited signaling through PC-PLC (25). Other studies demonstrate that LPS causes phosphorylation and activation of Raf-1, which was temporally associated with MEK and ERK (12). These data suggest that LPS also could activate ERK kinases through a raf-1-dependent pathway.

To our knowledge, prior studies have not demonstrated that LPS induces ceramide, which was observed in our studies to be PC-PLC dependent and capable of activating the ERK kinase pathway. Ceramide can also directly activate Raf-1 (47). Thus, our data suggest a third possible pathway: LPS-PC-PLC-DAG- ceramide-raf-1 MEK-ERK. A recent paper by Luberto et al. (48) suggests a related possibility for the sequence of events involved in LPS signaling. This study suggests that PC-PLC might also function as a sphingomyelin synthase and/or that D609 cannot distinguish between the enzymatic activities of sphingomyelin synthase and PC-PLC. Thus, it is possible that activation of PKCζ and ERK 2 could result from activation of a sphingomyelin synthase rather than a PC-PLC. Thus, further studies are necessary to determine whether LPS-induced activation of ERK 2 is linked to a PC-PLC or a sphingomyelin synthase. A novel conclusion of these studies is that activation of a PC-PLC or a sphingomyelin synthase by LPS is critical for activation of the ERK kinases. The importance of this observation is strengthened by the recent observations of Machleidt et al. (49), who showed that inhibitory ceramide and arachidonic acid is critical for mitogenic signal transduction.

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