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Protection from endotoxic shock in mice by pharmacologic inhibition of phosphatidic acid

(sepsis/endotoxin/tumor necrosis factor)

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ABSTRACT **Certain phosphatidic/plasmanic/plasmenic acid (PA) species function as lipid intermediates in cell activation and may function directly as intracellular signaling molecules. PA can also be dephosphorylated to 1,2-diradyl-*sn*-glycerol by phosphatidate phosphohydrolase. Treatment of various cell types, including murine P388 monocytic leukemia cells, with bacterial lipopolysaccharide rapidly stimulates large increases in PA and PA-derived diradylglycerol. Pentoxifylline, 1-(5-oxohexyl)-3,7-dimethylxanthine, inhibits lipopolysaccharide-stimulated formation of PA in P388 cells at high concentrations (IC_{50} = 500 μM). Lisofylline [1-(5*R*-hydroxyhexyl)-3,7-dimethylxanthine] is a unique metabolite of pentoxifylline in humans and is >800-fold more active as an inhibitor of PA formation than pentoxifylline (IC_{50} = 0.6 μM). Lisofylline does not inhibit lipopolysaccharide-induced activation of phosphatidylinositol-specific phospholipase C and generation of phosphatidylinositol-derived diradylgylcerol. Lisofylline but not pentoxifylline protects BALB/c mice from endotoxin lethality when administered 4 hr after lipopolysaccharide. This protective effect is independent of either agent's effect on suppression of plasma tumor necrosis factor α. These data suggest that inhibitors of PA formation may have significant clinical potential in the treatment of sepsis and septic shock.**

The lipid A component of lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is a potent cellular activator and is thought to mediate many manifestations of Gram-negative sepsis. LPS, also known as endotoxin, induces shock by stimulating the release of endogenous inflammatory mediators. The most important are thought to be tumor necrosis factor α (TNF-α) (1) and interleukin 1β (IL-1β) (2), but other factors play significant roles, including IL-6 (3), IL-8 (4), γ-interferon (5), leukemia inhibitory factor (6), tissue factor (7), and, possibly, platelet-activating factor (8). These endogenous mediators act in concert and are either additive or synergistic in their effects (9). Given the redundancy of inflammatory cytokines, it is not surprising that inhibitors of single components of the inflammatory cascade have shown only modest protection against endotoxin lethality when used as a single therapeutic (3, 8, 10, 11). Typically, those agents require administration either before or within 2 hr following a lethal dose of endotoxin to confer significant protection against lethality.

An alternative approach to agents that inhibit single components of the endogenous inflammatory mediator cascade is to suppress intracellular signal transduction pathways used by several of the inflammatory mediators involved in the systemic inflammatory response syndrome (SIRS). Recent studies have shown that IL-1β (13), TNF-α (14), and platelet-activating factor (15), as well as lipid A (16), may activate and signal at least in part through a common lipid intracellular signaling pathway involving phosphatidic/plasmanic/plasmenic acid (PA). Rapid increases in intracellular levels of specific species of both PA and diradylglycerol (DG) take place within seconds of exposure of cells to an activating stimulus. Here we provide evidence that LPS-induced PA in P388 cells may be derived from lyso-PA via the enzyme lysophosphatidate:acyl-CoA acyltransferase (LPAAT) and that a small-molecule inhibitor of PA generation markedly protects mice from the lethal effects of endotoxic shock.

MATERIALS AND METHODS

P388 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. Twenty-four hours before stimulation, cells were starved in Dulbecco's modified Eagle's medium with 0.5% fetal bovine serum overnight. The cells were resuspended in fresh medium (0.2 ml) in 5-ml test tubes held at 37°C. LPS (from *Salmonella abortus equi*, catalog no. L-1887; Sigma) was added at 10 ng/ml, and 5×10^7 cells were fixed in 500 μl of methanol at various times ranging from 5 to 300 sec thereafter. The pellets were extracted and analyzed as described (13, 16). Folch-extracted lipids were separated by HPLC with monitoring for UV absorption (13, 16). Simultaneous mass determination (to 1 nmol) was achieved by evaporative light-scattering detection in series with UV detection of lipid species.

Septic shock was modeled by endotoxin injection of 6- to 8-week-old female BALB/c mice as in previous reports (10), under protocols approved by the Animal Care and Use Committee of the Biomembrane Institute, Seattle. Animals were injected intravenously with an approximate LD_{100} (10 μg/g) of *S. abortus equi*-derived endotoxin (Sigma) in phosphate-buffered saline (PBS). Lisofylline (CT-1501R; LSF) or pentoxifylline (PTF) was injected intraperitoneally (100 μg/g) three times per day (100 μl per injection). Control mice were injected at the same times with a similar volume of vehicle control (PBS). In addition, two experiments in the simultaneous (zero time) group shown in Fig. 5 were included in which the mice received only a single injection of LSF (20 mice total). Survival was followed for at least 120 hr with no changes in survival after 72 hr. Groups of 10 mice each were treated with PBS alone or LSF or PTF alone on the same schedule as the experimental mice. There were no adverse effects noted and survival was 100% over the course of the experiment (data not shown).

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Abbreviations: CM, ceramide; DG, diradylglycerol; PA, phosphatidic/plasmanic/plasmenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LPAAT, lysophosphatidate:acyl-CoA acyltransferase; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; PTF, pentoxifylline; LSF, lisofylline; FAB-MS, fast-atom-bombardment mass spectrometry.

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Particulate-free plasma was assayed for cytokine by commercial murine TNF-α ELISA (Genzyme) with normal mouse plasma used to generate standard curves. Each point is the average of two ELISA measurements made from EDTA-treated serum pooled from three mice. The data were compiled from two independent experiments.

RESULTS AND DISCUSSION

LPS interacts with macrophages by complexing with endotoxin-binding protein and binding to CD14 (17), by a pertussis toxin-sensitive p73 receptor (18), by a possible lectin receptor (19), and by serum-independent hydrophobic mechanisms (19). The intracellular signaling mechanisms triggered by LPS in these cells are not fully elucidated. Conflicting data exist regarding the effects of LPS in stimulating calcium flux, phosphatidylinositol (PI) hydrolysis, and subsequent DG synthesis and protein kinase C activation. We examined lipid second-messenger intermediates by HPLC and fast-atom-bombardment mass spectrometry (FAB-MS) analysis of HPLC phospholipid peaks following LPS stimulation in the murine monocytic leukemia cell line P388. The combined HPLC and FAB-MS methodology allowed simultaneous detection of mass changes in, and unambiguous identification of, DG, PA, PI, phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), ceramide (CM), and sphingomyelin (SM) from whole cell extracts (16). LPS stimulation of P388 cells induced synthesis of new PA species of m/z 679, 685–688, 698, 699, and 701 within 5 sec after stimulation, with an increase in PA of 250–400% (mean, 325%; Figs. 1–3). These species are highly enriched (see legend to Fig. 2) in 1-alkyl (plasmanic) and 1-alkenyl (plasmenic) molecules. The PA species were converted to 1,2-*sn*-DG within 5–30 sec following LPS stimulation (Figs. 1 *B–F*, 2, and 3). This DG subfraction was initially derived predominantly from PA and not PI, as demonstrated by an increase in DG species of m/z 599–619 (predominant species at 599, 605, 607, and 617–619 as opposed to species at 645, 647, and 649 derived from PI: cf. Figs. 2 and 4). However, PI-derived DG appeared after 30 sec of LPS stimulation and persisted to 2 min (Fig. 2*C*). Although no changes were noted on HPLC up to 30 sec following LPS stimulation in any of the other lipid signaling intermediates such as SM or CM (Fig. 1 *A–F*; peaks at 26–28 min following PC; ref. 13), an increase in the palmitoyl CM of m/z 522–523 at 60 sec with a reciprocal decrease in palmitoyl SM, m/z 704–707, was noted by mass spectrometric analysis of the peaks. PC mass was unchanged throughout this time period by UV absorption or evaporative light scattering, nor was there a FAB-MS-detectable increase in appearance of PC-derived DG peaks (Figs. 1 and 2; ref. 13) FAB-MS analysis of the HPLC fractions (Fig. 2) confirmed (*i*) the identity of the indicated peaks as PA and DG, in accord with previous findings in other cell types (13, 16); (*ii*) significant mass increases in unique PA species following LPS stimulation (Fig. 2 *A* and *B*); and (*iii*) the change of mass of other lipid signaling intermediates within this time period.

SM mass was stable across observed time points in LPS-treated and drug-incubated cells until 60 sec, at which point there was a decrease in palmitoyl-derived SM (m/z 704–707) and a reciprocal increase in the corresponding CM (m/z 522–523). As seen in Figs. 1, 2*C*, and 4*B*, this did not correlate with the formation of PC-derived DG at 590–594 (1-palmitoyl 2-oleoyl and 1-palmitoyl 2-linoleoyl DG) or at 621 (1-stearoyl 2-oleoyl DG) where commonly found PC-derived DG species are usually discovered. This suggests that sphingomyelinase is directly stimulated by LPS rather than by formation of PC-derived DG. As the abolition of PA and DG fractions by LSF did not prevent the formation of the m/z 522–523 ceramide, we also conclude that these PA and DG species are not necessary to formation of CM in this system. In addition

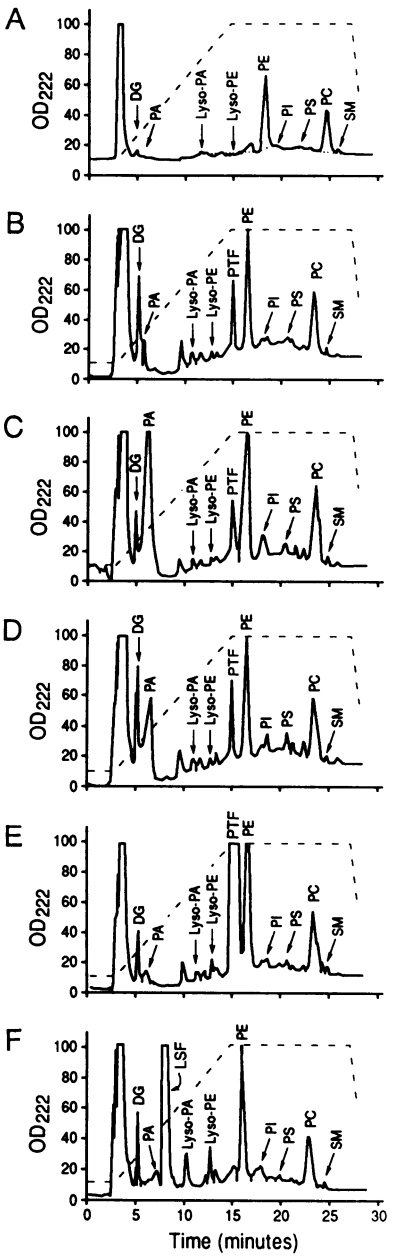

FIG. 1. HPLC analysis of changes in mass and composition of second-messenger signaling lipids in P388 cells following stimulation with LPS. (*A*) Unstimulated control cells, without LPS, PTF, or LSF. (*B–D*) Cells incubated with LPS and 10 μM PTF (indistinguishable from cells stimulated with LPS alone) for 5, 15, or 30 sec, respectively. (*E*) Cells in the presence of LPS and 1 mM PTF for 15 sec. (*F*) Cells in the presence of LPS and 10 μM LSF for 120 sec. No PA activation was found at any surveyed time point from 5 to 300 sec when either 1 mM PTF or >10 μM LSF was present.

to the above observations, predominant FAB positive-ion mass peaks at 759 and 761 (1-palmitoyl 2-oleoyl and 1-palmitoyl 2-linoleoyl PC) and at 788 (1-stearoyl 2-oleoyl PC) do not decrease over this time course, further reinforcing our observation that LPS does not activate PC-directed phospholipase C.

The substituted methylxanthine compound PTF is a weak inhibitor of LPS-induced PA and DG generation in P388 cells (Fig. 1 *B–E* and Fig. 3*C*; $IC_{50} \approx 500$ μM). PTF inhibits LPS- and TNF-α-mediated activation in a number of cell types (20) and has antiinflammatory effects in some animal models (21) but was ineffective at inhibiting regimen-related and cytokine-associated complications after bone marrow transplan-

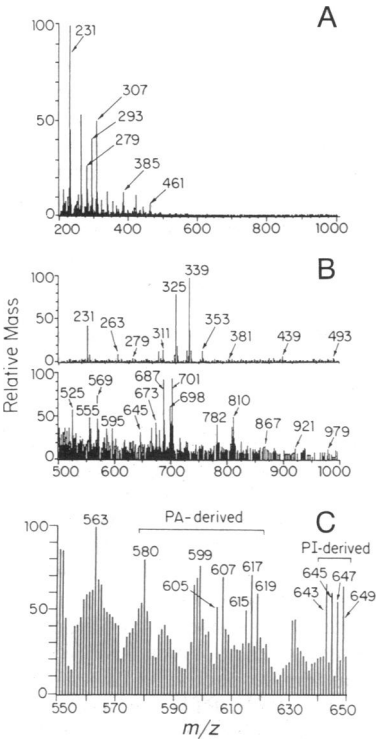

FIG. 2. FAB-MS of HPLC-separated and purified lipid species from Fig. 1. Purified lipid species were characterized by mass spectrometry as described (13). Each fraction was characterized at least three times by both FAB negative-ion and FAB positive-ion spectrometry for each P388 stimulation time point and lipid species [e.g., three separate collections for lipid species with HPLC elution at 4.5–5.5 min (DG) from P388 cells stimulated with LPS for 30 sec]. Lipid subspecies were identified as described (13, 16). Representative samples are shown. (*A*) PA (HPLC elution time, 5.5–7 min) from P388 cells either at zero time or not stimulated with lipid A. No significant mass is present at >600 *m/z*, suggesting a concentration of <250 pmol PA per 5×10^7 cells. (*B*) PA (5.5–5.7 min) (FAB negative ion) from P388 cells stimulated with LPS for 15 sec. Predominant species are identified at *m/z* 645, 1-myristoyl 2-oleoyl PA; 660, 1-*O*-hexadecyl 2-oleoyl PA; 673, 1-palmitoyl 2-oleoyl PA; 679, 1-*O*-(octadecadienyl) 2-linoleoyl PA; 685–688, 1-*O*-octadecyl 2-oleoyl/2-linoleoyl PAs; 697–699, 1-oleoyl/1-stearoyl 2-linoleoyl PAs; and 701, 1-stearoyl 2-oleoyl PA. Note the rich representation (>40% of total detected mass) of 1-alkyl and 1-alkenyl species (plasmanic and plasmenic acids, respectively). (*C*) DG (4.5–5.5 min) (FAB positive ion) from P388 cells stimulated with LPS for 30 sec. PA-derived DG was identified by the cluster of DG with removal of ≈80 Da (PO_3 group) from the corresponding PA fraction. PI-derived DG corresponded to the cluster with removal of 240–243 Da (inositol-PO_2) from the inositol monophosphate fraction, which had predominant peaks at 883–889, or removal of 337–339 Da from the inositol trisphosphate fraction, which had predominant peaks at 980–986. Predominant species are identified at *m/z* 580, 1-*O*-hexadecyl 2-oleoyl DG; 599, 1-*O*-(octadecadienyl) 2-linoleoyl DG; 605, 1-*O*-octadecyl 2-linoleoyl DG; 607, 1-*O*-octadecyl 2-oleoyl DG; 615, 1,2-dilinoleoyl DG; 617, 1-oleoyl 2-linoleoyl DG; and 619, 1-stearoyl 2-linoleoyl DG. Note the relative overrepresentation of 1-alkyl and 1-alkenyl 2-acylglycerol species in the PA-derived DG population. Also note that the peaks at 590–594 and 620–623, which are overrepresented in the presence of stimulation of PC-specific phospholipase C, are missing. These latter species could not be detected at any time point.

tation in man (22). Analysis of the plasma from those patients revealed that when PTF was administered alone, all detectable first metabolite was the *S* enantiomer of the secondary alcohol 1-(5-hydroxyhexyl)-3,7-dimethylxanthine. However, when the metabolism of PTF in humans was altered by coadministering the quinolone antibiotic ciprofloxacin (a P450IA2 inhibitor), an apparent increase in antiinflammatory

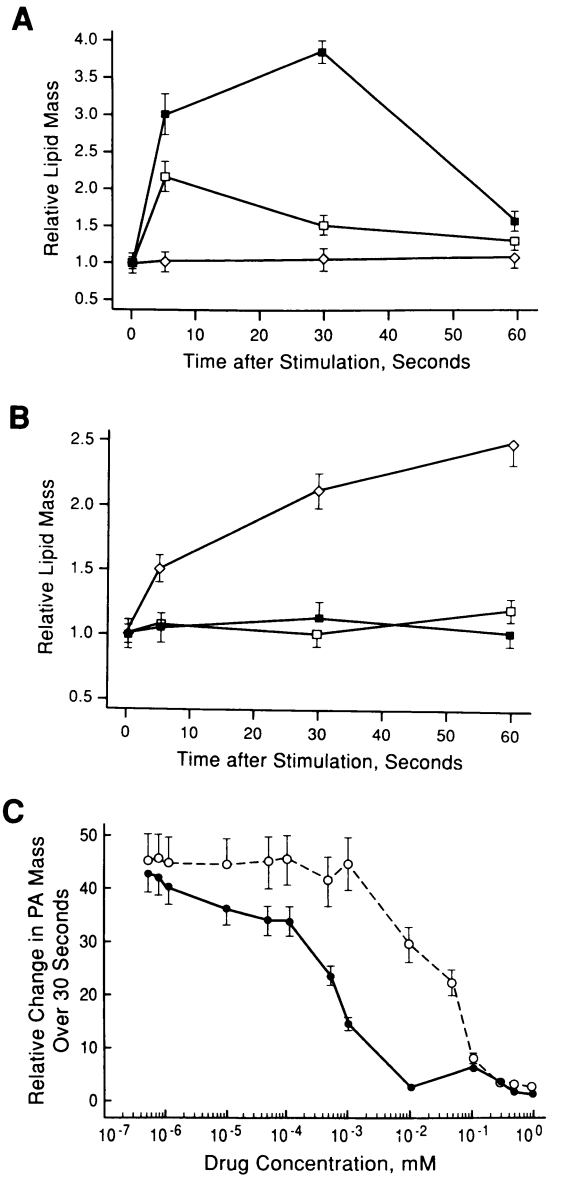

FIG. 3. Time course of PA and DG induction in P388 cells following LPS treatment and blocking of the increase by LSF and PTF. Cells were stimulated and treated as previously described, and lipids were extracted for HPLC separation. Mass was determined by evaporative light scattering in series with UV absorption. Estimates of initial PA mass (at rest) for the indicated number of cells ran from 250 to 500 pmol. These initial masses were set as 1.0, and the increase in mass following stimulation with LPS is expressed as multiples of this mass. (*A*) P388 cells were treated with LPS for various times and fixed and analyzed by HPLC. Shown are mass levels of PA (■), DG (□), and lyso-PA (◇) as measured by HPLC. (*B*) P388 cells were treated with LPS as in *A*, but with the addition of 10 μM LSF added 1 hr prior to LPS stimulation. (*C*) Dose response of the inhibition of PA generation in P388 cells measured at 30 sec following the LPS treatment with PTF (○) or LSF (●).

efficacy was noted (23). When coadministered with ciprofloxacin, approximately 10% of the detectable first metabolite of PTF appeared as the *R* enantiomer (LSF) (23). In view of this association of increased antiinflammatory effects when PTF was coadministered with ciprofloxacin, we reasoned that LSF might be a more potent inhibitor of PA generation than PTF. As shown in Figs. 1*F* and 3 *A–C*, the IC_{50} for PA inhibition by LSF was ≈0.6 μM, suggesting that it is 800 times more potent than PTF at inhibiting LPAAT activity. At or above their IC_{50} values both LSF and PTF

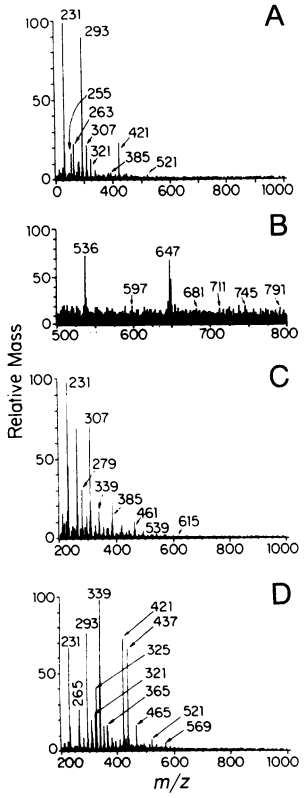

FIG. 4. MS of PA, DG, and lyso-PA fractions after stimulation of P388 cells with LPS in the presence of LSF. HPLC-purified lipid fractions, obtained as described above, were repurified and then analyzed by FAB-MS. (*A*) PA (5.5–7 min) fraction after stimulation for 30 sec with LPS in the presence of 10 μM LSF. Note loss of mass peaks > 600 and similarity to unstimulated PA fraction shown in Fig. 2*A*. (*B*) DG (4.5–5.5 min) fraction from 30-sec stimulation with LPS plus 10 μM LSF. Note loss of PA-derived DG peaks (*m/z* 580, 599, 605, 607, 615, 617, and 619), but persistence of PI-derived DG peaks at original mass levels. (*C*) Lyso-PA (10.5–11.5 min) fraction from 30 sec of LPS stimulation without LSF present. The *m/z* 421 peak, by coupling and base/acid sensitivity criteria, is identified as 1-*O*-octadecenyl lyso-PA. (*D*) Lyso-PA (10.5–11.5 min) fraction from 30 sec of LPS stimulation plus 10 μM LSF. Note significant accumulation of lyso-PA species, including 1-*O*-octadecenyl lyso-PA and 1-stearoyl lyso-PA (*m/z* 437), as well as accumulation of significant lyso-PA breakdown products (*m/z* 339, 325, and 265).

increased lyso-PA mass in the P388 cells by >300% (Figs. 3*B* and 4 *C* and *D*), suggesting that these compounds may specifically inhibit an LPAAT previously described after stimulation with either lipid A or IL-1 (13, 16). FAB-MS examination of HPLC-derived lipids showed that LSF resulted in abrogation of specific PA formation and DG species of *m/z* 599–621 derived from that PA (Fig. 4 *A* and *B*) but had no effects on the synthesis of PI-derived DG of *m/z* 645–649 (Fig. 4*B*). P388 cells stimulated with LPS demonstrated no significant baseline formation of specific lyso-PA (Figs. 3*A* and 4*C*), whereas preincubation of cells with LSF resulted in a significant increase in lyso-PA mass (Figs. 3*B* and 4*D*). These data strongly indicate that LSF does not inhibit PI-specific phospholipase C, since PI-derived DG continued to form, or phospholipase A_2, since formation of lyso-PA was unaffected. In addition to the above, the observed production of palmitoyl CM (*m/z* 522–523) 60 sec after stimulation with LPS, which appears to involve direct activation of sphingomyelinase, was not inhibited by LSF. In contrast to the effects on PA generation, LSF is an extremely weak inhibitor of cAMP phosphodiesterase. For instance, the IC_{50} for inhibition of cAMP phosphodiesterase activity assayed by both a radiometric (Amersham) and a fluorescence method for measurement was ≈1 mM for both PTF and LSF (W. Harris, personal communication).

If PA derived from lyso-PA is an important intracellular signaling intermediate for LPS-induced cellular activation, then inhibitors of PA formation may protect against endotoxic shock. Moreover, a more active inhibitor of PA formation (LSF) should demonstrate greater *in vivo* activity than a less active inhibitor (PTF). To test these hypotheses, BALB/c mice were treated with an approximate LD_{100} dose of *S. abortus* endotoxin and mortality was measured at 72 hr. Mice were treated with either LSF or PTF at 100 μg/g intraperitoneally three times a day for 3 days beginning either immediately (0 hr) or 2 or 4 hr after administration of the endotoxin. PTF and LSF have similar pharmacokinetic profiles at these doses in mice. For instance, for 100 μg/g, the peak plasma concentrations were 65.7 and 77.6 μg/ml, half-lives were 0.067 and 0.103 hr, and area-under-the-curve values were 9.23 and 11.6 μg·hr/ml for LSF and PTF, respectively (W. Gordon, personal communication). The survival data from 10 independent experiments are summarized in Fig. 5*A*. LSF conferred significant protection when administered up to 4 hr after LPS. Survival was 68% when LSF was given simultaneously, 55% when LSF started after 2 hr, and 37% when LSF started after 4 hr, compared with 3% for the LPS-treated mice not given LSF ($P < 10^{-4}$, 10^{-4}, and 10^{-3} for 0, 2, and 4 hr, respectively; two-tailed Fisher's exact test). In contrast to LSF, PTF was unable to protect mice when administered beginning 4 hr after LPS ($P < 10^{-3}$ compared with LSF; $P = 0.318$ compared with control).

To determine whether the observed protective effects of LSF resulted from secondary inhibition of proinflammatory cytokines, levels of TNF-α were measured in the plasma of mice after LPS administration. TNF-α levels peaked at 1 hr (Fig. 5*B*) and gradually declined for the next 5 hr. Treatment of the mice with LSF or PTF equivalently decreased TNF-α at all time points measured. Peak levels of TNF-α were decreased approximately 2.5- and 2.6-fold in the LSF- and the PTF-treated mice, respectively. Thus even though PTF reduced circulating levels of TNF-α to an equivalent degree as LSF, it failed to protect against death when administered starting 4 hr after LPS. These data suggest that inhibition of TNF-α is not the sole determinant of outcome in sepsis, particularly if initiation of therapy is significantly delayed. It can be argued that delayed onset of intervention is more relevant to the clinical situation in humans than is simultaneous or prophylactic administration of a cytokine inhibitor. In fact, using the same model system, but an approach that targets only a single cytokine, others have observed 20% survival of mice given a TNF-α receptor–IgG chimeric molecule 1 hr after a lethal dose of LPS but 0% survival when administration was delayed for 2 hr (10).

The specific membrane receptor(s) or binding site(s) for LPS which may activate LPAAT and increase PA levels in P388 cells is not known. However it probably involves a non-CD14-mediated interaction, since lipid A has been shown to rapidly activate PA metabolic pathways in non-CD14-expressing mesangial cells (16), tonsillar B cells (24), and human endothelial cells (14). The role of PA and its precursor lyso-PA as both a direct cytokine and early intracellular second messenger is well established. PA may directly activate atypical protein kinase C species (25) or alternative kinases (26) and may have effects on interactions between Ras protein and GTPase-activating protein (27). PA added exogenously to cells is a potent mitogen (28), stimulates calcium flux (29) and phospholipase C activity (30), and induces expression of several protooncogenes and growth factors (30). In fact, it has been suggested that lipid A may serve as a functional mimetic to PA, since either ring moiety of lipid A may act as a phosphomonoester containing a long-chain (C_{14}–C_{20}) fatty acyl chain which can assume planar

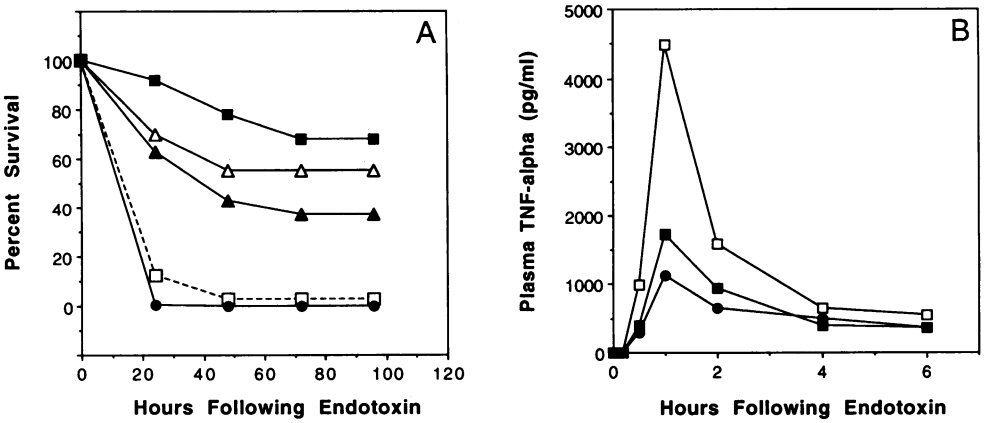

FIG. 5. (*A*) Inhibition of LPS-induced lethality in mice by LSF. Shown is a cumulative percent survival of mice from up to 10 independent experiments. Mice were treated with LPS (10 μg/g, i.v.) (□). Mice received their first treatment of LSF immediately following the LPS (■) or 2 hr (△) or 4 hr (▲) later. The mice were treated with LSF three times per day thereafter and survival was monitored. Also shown are data for mice treated with PTF 4 hr after LPS (●); these mice were treated like the LSF-treated mice with the substitution of PTF for LSF. LPS only, $n = 10$, 105 mice; $t = 0$ hr, $n = 7$, 65 mice; $t = 2$ hr, $n = 3$, 20 mice; $t = 4$ hr, $n = 4$, 35 mice; PTF, $n = 2$, 20 mice (where n is the number of experiments and t is the time of drug administration following endotoxin in hours). (*B*) Plasma levels of TNF-α in mice treated with LPS only (□) or LPS plus either LSF (■) or PTF (●). The drugs were given as a single i.p. injection (100 μg/g) immediately following injection of the LPS.

conformations similar to PA (16). The significance of LSF in decreasing PA-derived DG generated by LPS is not clear, insofar as no direct association of these unique DG forms and protein kinase activation has been directly demonstrated; in fact alkyl and alkenyl acylglycerols have been previously implicated as possible inhibitors of protein kinases (31). Certainly, the effects of LSF do not derive from blockade of PI-specific phospholipase C, as demonstrated by continued formation of PI-derived DG in the presence of the compound; phospholipase A_2, as demonstrated by unimpeded formation and accumulation of lyso-PA; or sphingomyelinase, as demonstrated by continued formation of the m/z 522–523 CM. Finally, blocking LPS-generated PA and DG formation may not only be important for blocking direct signaling of LPS but may at least in part block IL-1 (13) and TNF-α (14)-generated intracellular second-messenger signaling and activation as well. Thus, the efficacy of LSF may also involve its capacity to further block signaling of downstream modulators of inflammation and tissue injury.

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