Bacterial Lipopolysaccharide Priming of P388D1 Macrophage-like Cells for Enhanced Arachidonic Acid Metabolism

PLATELET-ACTIVATING FACTOR RECEPTOR ACTIVATION AND REGULATION OF PHOSPHOLIPASE A₂*

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P388D₁ cells are stimulated by platelet-activating factor (PAF) to release arachidonic acid metabolites (Lister, M. D., Glaser, K. B., Ulevitch, R. J., and Dennis, E. A. (1989) J. Biol. Chem. 264, 8520-8528). While the release of prostaglandin E₂ (PGE₂) in response to PAF is only two to three times the constitutive PGF₂α production, bacterial lipopolysaccharides (LPS) are able to prime P388D₁ cells for enhanced arachidonic metabolism, increasing PAF-stimulating PGF₂α production to 9-12 times the constitutive PGF₂α production. The extent and rate of [³H]arachidonic acid release from prelabeled P388D₁ cells are also increased in primed cells relative to unprimed cells in response to PAF-stimulation. LPS from either *Salmonella* Re595 or *Escherichia coli* 0111:B₄ prime P388D₁ cells in a concentration-dependent manner but have themselves no ability to stimulate arachidonic acid metabolism. PAF priming is sensitive to inhibition by actinomycin D, while primed PAF-stimulation of PGF₂α production is blocked by cyclohexamide which implicates a protein which is rapidly turning over. Primed PAF stimulation is also inhibited by the phospholipase A₂ inhibitor manalogue and the tyrosine-specific protein kinase inhibitor genistein, but not by the kinase inhibitor H-7. These results suggest that priming amplifies signal transduction pathways for PAF, which results in increased arachidonate availability. The multiple levels at which primed PAF-stimulated PGF₂α production appears to be regulated are discussed.

The release of arachidonic acid and the production of eicosanoids is an early event in the activation of macrophages by many types of inflammatory stimuli. This has been extensively studied in murine resident peritoneal macrophages (1-5) and several murine macrophage-like cell lines (6-11). The release of arachidonic acid from the sn-2 position of membrane phospholipids is thought to be the rate-limiting event in the biosynthesis of the eicosanoids. This rate-limiting event is most likely controlled by a phospholipase type enzyme (12), the most direct mechanism being the action of a phospholipase A₂ (PLA₂) which would release arachidonate directly from the sn-2 position of membrane phospholipids (13). Characterization of the different types of phospholipases in these inflammatory cells has been an important first step in the elucidation of the mechanisms which regulate arachidonic acid release (14, 15).

The characterization of the phospholipase activities has revealed multiple forms present in both resident peritoneal macrophages (16, 17) and macrophage-like cell lines (14, 18, 19). The most detailed characterization is available on a membrane-associated, Ca²⁺-dependent PLA₂ from the P388D₁ cell line (14). This enzyme has been purified (20), kinetically characterized (21), and evaluated with potential phospholipase A₂ inhibitors (7) as has a soluble lysophospholipase from the same cell (22). Nonetheless, the mechanisms by which these enzymes are activated or regulated in the intact cell are still poorly understood.

In peritoneal macrophages, distinct differences in the activation mechanisms for receptor-mediated versus soluble stimuli have been observed (5). There appears to be a Na⁺ requiring event early in phagocytic/receptor-mediated activation of arachidonic acid release but not for activation by soluble stimulants such as PMA or Ca²⁺ ionophore A23187. Protein synthesis is also required for both PGF₂α production (23) and arachidonic acid release in peritoneal macrophages (5). The model proposed by Adem et al (5) for receptor-mediated arachidonic acid release consists of a sequential series of signals involving Na⁺ influx, protein synthesis, and finally an elevation in intracellular Ca²⁺. Therefore, in the macrophage, there are mechanisms which appear to control the ability of these cells to generate eicosanoids which are complex and stimulus dependent which ultimately lead to activation of PLA₂. A divergence in this receptor-mediated sequence of signals for arachidonic acid release is demonstrated by bacterial lipopolysaccharides (LPS) which are poor triggers for the release of arachidonic acid in macrophages (24), but are able to prime macrophages for enhanced arachidonic acid metabolism in response to various stimuli (24, 25). The mechanism of LPS priming of macrophages is poorly understood but affects many of the functional capacities of these cells (26, 27). Many of the actions of LPS in endotoxin shock may be mediated by macrophages (26) which produce potent mediators of shock including the eicosanoids and platelet-activating factor and tumor necrosis factor (28-30). LPS

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The abbreviations used are: PLA₂, phospholipase A₂; LPS, bacterial lipopolysaccharide; PGF₂α, prostaglandin E₂; PAF, platelet-activating factor (1-O-alkyl-2-O-acetyl-sn-glycerol-3-phosphocholine); PMA, phorbol 12-myristate 13-acetate; UNG, oleyl-acetyl-sn-glycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenbis(oxyethyleneminitilo)tetraacetic acid.
may also participate in a priming effect which allows for enhanced production of these mediators, exacerbating the acute onset in shock.

In this report, we demonstrate the priming of a murine macrophage-like cell P388D1, by bacterial lipopolysaccharide for enhanced arachidonic acid metabolism. The priming of the P388D1 cell is qualitatively different than that observed in resident murine peritoneal macrophages and appears to require RNA synthesis as priming is sensitive to inhibition by actinomycin D but not cyclohexamide. The priming of P388D1 cells enhances PGE2 production in response to Ca2+ ionophore A23187 and PAF but not to the protein kinase C activator oleoyl-acetyl-sn-glycerol (OAG). The priming of P388D1 cells, described herein should help us to better understand the stimulus-coupling mechanisms for arachidonic acid release in response to activation of the PAF receptor and aid in the elucidation of the relevant phospholipases involved in arachidonic acid release.

**EXPERIMENTAL PROCEDURES**

**Materials.**—P388D1 cells were obtained from the American Type Culture Collection (Bethesda, MD). PGE2 [5,6,8,11,12,14,15-3H] (specific activity 185 Ci/mmol), 1-palmitoyl-2-14C-palmitoyl phosphatidylcholine (specific activity 54 Ci/mmol), and arachidonic acid [5,6,8,9,11,12,14,15-3H] (specific activity 230 Ci/mmol) were from Du Pont-New England Nuclear. PGE2 and arachidonic acid standards were from Cayman Chemical (Ann Arbor, MI). PGE2 anti-sera was a kind gift of Dr. Lawrence Levine, Brandeis University (Waltham, MA). Fetal bovine serum (Lot No. A21212) was from Gemini Bio-Products, Inc. (Calabassas, CA). Manoalogue was generously provided by Dr. Edward D. Mihelich, Lilly Research Laboratories. BW755c was a gift from Wellcome Research Laboratories (Research Triangle Park, NC). L-659,989 was a kind gift of Dr. John Chabala, Merck Sharp & Dohme Research Laboratories. Actinomycin D and cyclohexamide were from Calbiochem (La Jolla, CA). Cytochalasin B, indomethacin, calcium ionophore A23187, OAG (oleoyl-acetyl-sn-glycerol), PAF (platelet-activating factor purified from bovine heart), enantio-PAF and PMA were from Sigma. Genistein was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). LPS from Salmonella Re 595 was provided by Dr. R. J. Ulevitch, Scripps Clinic and Research Foundation (La Jolla, CA).

Cell Culture—P388D1 cells were maintained in Iscove’s modified Dulbecco’s medium (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (heat-inactivated), HEPES (25 mM), glutamine (2 mM), and gentamycin (50 μg/ml) supplemented with nonessential amino acids at 37°C in a humidified atmosphere of 95% air, 5% CO2. Adherent cells were selected as previously described (7) and passaged every 3-4 days (doubling time ~28 h). For experiments, adherent cells were scraped and plated into 6-well dishes (Falcon) at 1 x 10^6 cells/well. Cells were allowed to adhere overnight in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). All experiments were performed in serum-free medium with penicillin/streptomycin.

**Priming and Stimulation of P388D1 Cells.**—P388D1 cells at 1 x 10^6 cells/well were placed in serum-free medium with or without inhibitors for 1 h prior to LPS priming. After 1 h in serum-free medium, LPS was added to a final concentration of 10 ng/ml. Cells were washed two times with serum-free medium and then stimulated with PAF (10 nM) in the presence of cytochalasin B (1 μM) for 4 h at 37°C. After stimulation, the medium was removed, centrifuged to remove cells, and frozen at -20°C until radioimmunoassay could be performed as previously described (7).

**Arachidonic Acid Release from Unlabeled Cells.**—P388D1 cells were prelabeled with [14C]arachidonic acid (5 αCi/10^6 cells) for 18 h at 37°C. The prelabeled cells were primed as described above and [14C]arachidonic acid release measured as previously described (7).

**Cyclooxygenase Assay.**—Cellular cyclooxygenase was measured in unprimed (control) and primed (100 ng/ml for 1 h) cells by the method described by Raz et al. (31). Briefly, ~5 x 10^6 cells were solubilized in 50 mM Tris, pH 8.0, 1.0% Tween 20, and 10 mM EDTA by sonication, in a final volume of 200 μl with a MSE model 100-watt sonicator (4 x 15-s pulses). The cell homogenate (20 μl) was added to siliconized tubes containing arachidonic acid (100 μM), epinephrine (1 mM), phenol (1 mM), and normal rabbit serum (1 ml/assay) in 50 mM KPO4, pH 7.5, 10 mM EDTA, 10 mM EOTA, 100 mM N-ethylmaleimide, and 0.6% Triton X-100 in a final volume of 100 μl. The assay was initiated by addition of cell homogenate and incubated for 30 min at 37°C. Total cellular cyclooxygenase activity was determined as PGE2 formed as measured by specific radioimmunoassay. Relative cyclooxygenase activity in intact P388D1 cells was determined by the application of exogenous arachidonic acid (2 and 10 μM, which are present when the standard error is greater than the symbol size. All data points are the means ± S.E. of triplicate samples from a representative experiment showing similar results to several other identical experiments. Standard error bars are present when the standard error is greater than the symbol size.

**RESULTS**

**LPS Priming—Stimulus Response**—The effect of LPS on the “priming” of P388D1 macrophage-like cells for enhanced PGE2 production in response to various stimuli is shown in Fig. 1. LPS-primed P388D1 cells produce three to five times as much PGE2 as unprimed (control) cells in response to Ca2+ ionophore A23187 and PAF stimulation. Priming is effective at enhancing PGE2 production for stimulants such as A23187 and PAF but does not enhance PGE2 production in response to stimuli which do not stimulate unprimed cells, such as OAG (7). In non-stimulated cells, LPS priming results in a 1.6-fold increase in PGE2 production as compared with unprimed cells. In contrast, priming increases PGE2 production significantly, suggesting that LPS priming enhances PGE2 production.

**FIG. 1. Effect of LPS priming on PGE2 production in response to various stimulators.** P388D1 cells were primed with 100 ng/ml LPS Re 595 for 1 h and then stimulated for 4 h with no stimulant, A23187 (0.5 μM), PAF (10 nM), or OAG (25 μM). PGE2 production was measured by specific radioimmunoassay.
by 3- and 3.6-fold in response to A23187 and PAF, respectively, as compared with unprimed stimulated cells. OAG treatment of primed P388D1 cells showed only a 1.1-fold increase in PGE₂ production which is not significantly different than the increase observed in non-primed stimulated cells. Priming results in increased levels of PGE₂ as well as the other arachidonic acid metabolites as compared with control cells, but no redistribution of metabolites among the prostaglandins is observed (data not shown).

**PAF Dose Response in LPS-primed Cells**—In P388D1, cells stimulated with PAF (10 nM to 1 μM) only a 2-3-fold increase in PGE₂ production over constitutive PGE₂ production (7) could be observed which made it difficult to determine a dose-response relationship for PAF. However, in LPS-primed P388D, cells, PAF (10 nM to 1 μM) produced a 9-12-fold increase in PGE₂ production over constitutive production as shown in Figs. 1 and 2. In primed cells, a PAF dose-response was determined between 0.01 and 10 nM (Fig. 2). Concentrations of PAF between 10 nM and 1 μM gave no further increase in PGE₂ production. The apparent ED₅₀ for PAF-stimulated PGE₂ production in primed P388D cells is approximately 0.2 nM. This ED₅₀ value is consistent with a Kᵦ determined for PAF binding to P388D, cells of 0.08 nM (32). Enantio-PAF (D-PAF) is approximately 500-fold less potent than PAF (L-PAF) at stimulating PGE₂ production in P388D, cells. This demonstrates stereospecificity of the PAF receptor in P388D, cells for PGE₂ production in response to PAF. The PAF antagonist L-659,989 (33) inhibited PGE₂ production in LPS primed cells by 75% at 10 nM and 100 nM, respectively, when stimulated with 10 nM PAF.

**[³H]Arachidonic Acid Release in Primed Cells**—LPS-primed P388D cells, upon stimulation with PAF, release greater amounts of [³H]arachidonic acid from prelabeled cells and the rate of release appears to be increased (as per the 30-min time point) relative to release from unprimed cells (Fig. 3). The first time point taken (30 min) appears to be representative of the maximum release in both primed and unprimed cells. LPS-primed cells release approximately two to three times more [³H]arachidonic acid in response to PAF. This result is somewhat less than the 3-5-fold increase in PGE₂ production observed with LPS priming and may reflect the disparate labeling of phospholipid pools with exogenously applied [³H]arachidonic acid (7, 34).

**Effect of LPS Priming on Cyclooxygenase and Phospholipase A₂ Activities**—The level of cyclooxygenase activity, whether measured in a P388D, cellular homogenate or by application of exogenous arachidonic acid to intact cells, showed no enhanced activity as compared with unprimed (resting) cells. This was also true of the phospholipase A₂ activity present in the P388D, cellular homogenate when measured with vesicles of dipalmitylophosphatidylcholine at pH 9.0 with 5 mM Ca²⁺ (data not shown). Therefore, it appears that the activities of neither of the key enzymes involved in arachidonic acid metabolism in P388D, cells are altered appreciably by LPS priming.

**LPS Priming of P388D₁ Cells**—LPS priming of P388D₁ cells is concentration dependent from 10 to 1000 ng/ml of rough LPS Re 595 from Salmonella as well as smooth LPS from Escherichia coli 0111:B4 with respect to enhanced PGE₂ production in response to PAF (Fig. 4A). Some effects of LPS are also observed at concentrations as low as 1 ng/ml. The effect of the exposure time of P388D₁ cells to LPS (100 ng/ml) was evaluated to determine if prolonged exposure of P388D₁ cells to LPS down-regulated the primed response. The time course of LPS priming demonstrated that enhanced production of PGE₂ in response to PAF can be observed at 30 min of exposure to LPS and is maximal at 1-2 h of exposure (Fig. 4B). Between 2-4 h of LPS exposure, the P388D₁ cells become less responsive to the subsequent stimulation with PAF. After 4 h of exposure to LPS, PAF stimulation results in a response which is 30-35% of the maximal response observed after 1 h of LPS exposure. Therefore, prolonged exposure of P388D₁ cells to LPS (longer than 2 h) appears to down-regulate the primed state with regard to subsequent stimulation by PAF.

The stability of the LPS-primed state was determined by priming the cells for 1 h with 100 ng/ml LPS and then washing the cells free of LPS and allowing them to incubate for different time periods in serum-free medium before stimulation with PAF. As shown in Fig. 4C, the primed state of the P388D₁ cells was unstable and decreased to 20% of maximal after a 4-h incubation in serum-free medium. The time at which the primed state was reduced to 50% of its maximum was 1.75 h (see inset). Therefore, it appears that the LPS-primed state in P388D₁ cells is transient and deactivates with an apparent half-life of approximately 1.75 h in the absence of LPS or stimulus.

**Effects of Manoalogue and BW755c on LPS Priming and Primed Stimulation**—The phospholipase inhibitor manoalogue and the dual cyclooxygenase/lipoxygenase inhibitor BW755c when present during LPS exposure had little effect on the ability of LPS to prime P388D₁ cells for enhanced PGE₂ production (data not shown). However, these compounds are effective inhibitors of primed PAF stimulation of PGE₂ production. Manoalogue has an apparent IC₅₀ of 0.4 μM in LPS-primed cells (Fig. 5), as compared to an apparent IC₅₀ of 1.0 μM for A23187 stimulation of unprimed cells (7) and BW755c at 10 μM resulted in 95% inhibition of PGE₂ production (data not shown). These results suggest that production of cyclooxygenase/lipoxygenase products may not be necessary for LPS priming of P388D₁ cells. This is also consistent with previous findings that LPS alone at concentrations up
LPS Priming of P388D₁ Cells

FIG. 4. A, LPS priming dose-response. P388D₁ cells were primed with either rough (R) LPS from Salmonella Re 595 (○, ○) or smooth (S) LPS from E. coli 0111:B4 (□, □). PGE₂ production in unstimulated primed cells (□, ○) and primed PAF-stimulated cells (●, ●); B, LPS exposure time effects on priming. P388D₁ cells were primed for various times with LPS Re 595 (100 ng/ml) and stimulated with PAF (10 nM). PGE₂ production in response to PAF (●) and without PAF (○); C, stability of the LPS-primed state. P388D₁ cells were primed with LPS Re 595 (100 ng/ml) for 1 h at 37 °C and then washed for various times before stimulation with PAF (10 nM) (●) or not stimulated (○). Inset, semi-logarithmic transformation of the decay of the primed state.

Effects of Actinomycin D and Cyclohexamide on LPS Priming—It has been shown that stimulatory doses of LPS result in the rapid (~30 min) accumulation of mRNA for both oncogenes (c-myc and c-fos) and competence genes (Jc and KC) along with the production of the early proteins, the seven short-lived proteins in the 38–85 kDa range (35). It was therefore of interest to evaluate both transcriptional (actino-

mycin D) and translational (cyclohexamide) inhibitors on the priming of P388D₁ cells with low doses of LPS.

As shown in Fig. 6A, actinomycin D reduced LPS priming to 58 and 16% of control at 1 and 3 μM, respectively. The temporal effects of actinomycin D on LPS priming. Actinomycin D (3 μM) was present 30 min prior to LPS priming (30 min Pre), during the first 30 min of LPS exposure (0–30' LPS priming) or during the second 30 min of LPS exposure (30–60' LPS priming). The effect of actinomycin D (3 μM) on primed PAF stimulation was determined as described.

Fig. 5. Manoalogue dose-response in primed PAF-stimulated PGE₂ release in P388D₁ cells. P388D₁ cells were preincubated with various concentrations of manoalogue for 1 h after LPS priming (100 ng/ml for 1 h at 37 °C) and prior to PAF (10 nM) stimulation also in the presence of manoalogue.

Fig. 6. A, effects of actinomycin D and cyclohexamide on LPS priming and primed PAF stimulation. Actinomycin D (1 and 3 μM) and cyclohexamide (10 μM) were present during LPS priming (LPS Priming) or were preincubated for 30 min after LPS priming before PAF stimulation in the presence of the inhibitor (Primed PAF Stimulation). B, temporal effects of actinomycin D on LPS priming. Actinomycin D (3 μM) was present 30 min prior to LPS priming (30 min Pre), during the first 30 min of LPS exposure (0–30' LPS priming) or during the second 30 min of LPS exposure (30–60' LPS priming). The effect of actinomycin D (3 μM) on primed PAF stimulation was determined as described.
significant ($P < 0.05$) decrease in the effect of actinomycin D is observed when present during the last 30 min of LPS exposure (26% of control when present during the first 30 min to 66% of control when present during the last 30 min). These data suggest an accumulation of mRNA during the initial exposure of P388D1 cells to LPS is essential for the development of the primed state.

Cyclohexamide (10 μM) partially prevented (70% of control) the LPS priming if present during the entire LPS exposure period as shown in Fig. 6A. Cyclohexamide present during the first 30 min of LPS exposure had no inhibitory effect on LPS priming and appears to slightly enhance (130% of control) the primed state in P388D1 cells (data not shown). This observation is similar to that observed with γ-interferon priming of peritoneal macrophages (35). These results suggest the possibility of a partial dependence of LPS priming in P388D1 cells on protein synthesis but a much less dramatic effect than that seen for transcription.

Effects of Actinomycin D and Cyclohexamide on Primed PAF Stimulation—Cyclohexamide (10 μM) inhibits primed PAF stimulation of PGE2 production by 88% (Fig. 6A) and reduces PAF-stimulated PGE2 production in primed cells to the level observed in non-primed cells. These data are consistent with the involvement of protein synthesis (a rapid turnover protein) in the stimulation of arachidonic acid metabolism by a number of various stimuli (5, 23) which now include PAF. Actinomycin D had only a partial inhibitory effect on primed PAF stimulation, 63% of control at 3 μM actinomycin D, suggesting the possibility of a partial involvement of transcription in primed PAF stimulation but a much greater role of translation in PAF stimulation of PGE2 production.

Effects of H-7, PMA, and Genistein on Primed PAF Stimulation—The role of protein kinase C in LPS priming of neutrophils is apparently unclear (36, 37), and LPS does not result in translocation or activation of this enzyme for priming of the respiratory burst. In the P388D1 cells, LPS priming for enhanced arachidonic acid metabolism is not inhibited by the protein kinase C inhibitor H-7 (10–50 μM) nor does H-7 inhibit primed PAF stimulation (data not shown). PMA, a phorbol ester protein kinase C activator, (1–10 μM) reduced LPS priming to 67% of control but did not affect primed PAF stimulation (data not shown). These results suggest a minimal contribution of protein kinase C to the priming of macrophages for enhanced PGE2 production and that protein kinase C activation is apparently not required for PAF stimulation of PGE2 production in P388D1 cells.

Genistein (38) is reported to be a selective tyrosine-specific protein kinase inhibitor with partial inhibitory effects on serine and threonine kinase activity (CAMP-dependent kinases). The dose-response for genistein effects on both LPS priming and primed PAF stimulation are shown in Fig. 7. Genistein had a small inhibitory effect on LPS priming in P388D1 cells (about 58% of control was observed at 30 μM genistein). However, genistein had a potent inhibitory activity against primed PAF stimulation with an apparent $IC_{50}$ of 7 μM and only 4% of control primed PAF stimulation was observed at 90 μM genistein. These results implicate a protein kinase activity in the PAF-stimulated arachidonic acid release in LPS-primed P388D1 cells.

DISCUSSION

The P388D1 cell produces arachidonic acid metabolites, predominantly cyclooxygenase products, in response to various stimuli (7). In the attempts to correlate the effects of various phospholipase inhibitors on the P388D1, Ca2+-dependent membrane associated PLA2, in vitro with inhibition of PGE2 and arachidonic acid release in intact P388D1 cells, the Ca2+ ionophore A23187 was used as a stimulus (7). However, a key point in studying the regulation of arachidonic acid release as the rate-limiting event for the biosynthesis of eicosanoids is the use of physiologically and pharmacologically relevant stimuli, i.e., receptor-mediated events. P388D1 cells respond to PAF to produce arachidonic acid metabolites (7) and possess high affinity PAF receptors (32); however, in resting cells (unprimed) only a 2-3-fold increase over constitutive PGE2 production is observed in response to PAF (7). In contrast, as shown in this report, LPS priming of P388D1 cells results in a 9–12-fold increase over constitutive PGE2 production in response to PAF, thus allowing more quantitative interpretations of inhibitor studies. With LPS priming it is possible to make clear the mechanisms governing both LPS priming and PAF-stimulated arachidonic acid metabolism in P388D1 cells.

*PAF Priming and PAF*—The mechanisms by which bacterial lipo polysaccharides prime both neutrophils and macrophages for enhanced metabolic responses have been studied in detail, but still remain unclear. In general, the LPS priming of neutrophils for enhanced superoxide production does not result from the augmentation of the terminal enzymes in this system, e.g., NADPH oxidase, but rather appears to be a modulation (up-regulation) of the signal transduction mechanism (39–41). In the murine resident peritoneal macrophage, LPS priming also results in enhanced arachidonic acid metabolism in response to various stimuli (24). Herein, we report a response to LPS priming in a murine macrophage-like cell line, P388D1.

P388D1 cells exposed to low doses of LPS become primed for enhanced arachidonic acid metabolism. This enhanced metabolism is apparently stimulus-dependent, as responses to OAG or PMA do not become primed upon LPS exposure. This implies that responses which are coupled to selective transduction pathways for arachidonic acid release are those which are able to be primed by LPS exposure.

The enzymes which would most likely be involved in enhancing arachidonic acid metabolism in P388D1 cells or macrophages would be PLA2 and/or the cellular cyclooxygenase. In P388D1 cells, LPS priming results in increased [H]arachidonic acid release and increased PGE2 (immunoreactive) production. However, neither the total cellular cyclooxygenase activity nor the Ca2+-dependent PLA2, pH 9.0 optimum, activity was found to be different in primed cells. This is consistent with a change in the signal transduction mechanism(s) leading to a greater availability of substrate (arachidonic acid). This effect (priming) may be the result of a greater efficiency in the translation mechanism(s) or receptor ligand interactions which result in arachidonic acid release,
rather than increased enzyme levels to account for the increased product observed after LPS priming.

PAF-stimulated PGE₂ production in LPS-primed cells was 9-12 times the constitutive PGE₂ production. The dose-response curve for primed PAF-stimulated PGE₂ production gave an ED₅₀ value of 0.2 nM PAF which is in good agreement with the reported Kᵣ of 0.98 nM for PAF binding (32). The PAF response was also stereospecific, D-PAF being 500-fold less potent, and the selective PAF antagonist 1-659,989 (33) effectively inhibited PAF-stimulated PGE₂ production. These results demonstrate that the PGE₂ production in P388D₁ cells upon PAF stimulation is a receptor-mediated event. Therefore, evaluation of different inhibitors with PAF stimulation appears to be more physiologically relevant as PAF is an important agonist for macrophages and the concentrations of PAF being studied are in a more physiologically relevant range (i.e. ≤10 nM).

Regulation of LPS Priming—The mechanism of LPS priming still remains unclear. Early studies demonstrated that LPS priming of neutrophils was not sensitive to inhibition by cyclohexamide (42) and therefore a distinct event from induction of cellular protein synthesis. It was then suggested that an increase in resting Ca²⁺ levels may account for the priming effect (43, 44). In macrophages, LPS priming results in the myristolation of a set of cellular proteins which are also substrates for protein kinase C (45). A sequential set of events starting with LPS-induced myristolation of cellular proteins, membrane association of the myristolated proteins, and phosphorylation by protein kinase C has been proposed to account for the increased arachidonic acid release by LPS priming (46).

Although these mechanisms may be involved in LPS priming, we have observed dependence of LPS priming on transcriptional events (mRNA production) which are sensitive to actinomycin D inhibition. This is consistent with the observed increases in mRNA levels for oncogenes and competence genes during the first 30 min of LPS exposure (35). These results also demonstrate a lesser dependence of priming on protein synthesis which is also observed in human neutrophils (42). Actinomycin D inhibition of LPS priming is likely due to inhibition of the transcription of mRNA which is subsequently translated to produce the effector protein(s) which may be involved in the observed enhancement of arachidonic acid metabolism. Cyclohexamide only partially prevents this priming when present during LPS exposure. However, the translational events which lead to the primed state may be occurring later, after LPS exposure. Due to the unstable nature of the primed state in P388D₁ cells (t½ = 1.75 h) it was not possible to design experimental conditions which could distinguish cyclohexamide effects on translation which prevent priming from the observed deactivation of the LPS-primed state. Therefore, the observed deactivation of the primed state may reflect the disappearance of an unstable protein induced upon LPS exposure or the degradation of message induced by LPS priming. Further studies on the nature of LPS priming in P388D₁ cells and the message induced upon LPS exposure which eventually regulates arachidonic acid metabolism would be necessary to distinguish the possible mechanisms responsible for the development of the primed state.

Translational Dependence of PAF Stimulation—Responses to receptor-mediated stimulation of arachidonic acid metabolism in macrophages have been shown to be dependent on protein synthesis (5, 23). This is in contrast to non-physiological stimulation (e.g. Ca²⁺ ionophore A23187) of arachidonic acid release which does not need protein synthesis (5).

Herein we demonstrate that primed PAF stimulation of P388D₁ cells is also dependent on protein synthesis (sensitive to inhibition by cyclohexamide). The rapid release of arachidonic acid in response to PAF stimulation suggests that this cyclohexamide-sensitive event may depend on a rapid turnover protein which is induced upon receptor activation. This rapidly produced protein may be a product which is related to the LPS-induced transcriptional events occurring during LPS priming. This relationship is currently under investigation.

Protein Kinase Regulation of PAF Stimulation—Genistein, a selective tyrosine-specific protein kinase inhibitor (38), appears to be a potent inhibitor of primed PAF stimulation of arachidonic acid metabolism in P388D₁ cells. The release of arachidonic acid when P388D₁ cells are primed and subsequently stimulated with PAF is most likely mediated by a phospholipase A₂-like enzyme (7). The regulation of cellular PLA₂ may be complex considering the ubiquity of this type of enzyme and its general role in cellular homeostasis; therefore, regulation of PLA₂ activity by a protein kinase would be an attractive mechanism. The effects of genistein and the lack of effect of H-7 suggest that a tyrosine-specific protein kinase may be this regulating kinase activity, but this does not exclude the possibility of other kinases, other than protein kinase C, or other nucleotide-dependent enzymes also being involved in this regulation. This would be the first correlation of tyrosine-kinase inhibition with inhibition of arachidonic acid metabolism and possibly the PLA₂ liberating the arachidonic acid. The mechanism by which the kinase regulates the release of arachidonic acid would depend on identification of the kinase substrate which regulates the PLA₂ activity be it the enzyme (PLA₂) itself or another part of the stimulus-response coupling mechanism.

Regulation of phospholipid metabolism by a tyrosine-specific protein kinase has been suggested for phospholipase C activity and growth factor responses (47). The tyrosine-specific protein kinase is also known for its involvement in insulin action. Therefore, the regulation of arachidonic acid release via regulation of the PLA₂ or its activation mechanism seems a particularly attractive mechanism to regulate the biosynthesis of eicosanoids during cell activation in inflammatory and other responses.

Proposed Signal Transduction Mechanisms in P388D₁ Cells—We have described herein inhibitor studies designed to better understand both the LPS priming and PAF-mediated arachidonic acid metabolism in P388D₁ cells. As shown in Fig. 8, the possible mechanism for LPS priming and PAF-stimulated arachidonic acid release are summarized. LPS...
phospholipase A2 (20, 21) is the most likely phospholipase candidate and PGEz production in both unprimed (7) and primed Ulevitch for critical discussion regarding the interpretation of these results.

Events, but this would have to be more rigorously evaluated in further experiments. The protein kinase is an attractive mechanism for regulation of arachidonic acid release, possibly of the cellular phospholipase A2, but its substrate would have to be determined to understand how the release of arachidonic acid is being regulated.

There are undoubtedly other pathways of phospholipid metabolism being activated in these cells (e.g. phospholipase C hydrolysis of phosphatidylinositides, production of diacyl glycerol and inositol phosphates, or possibly phospholipase D activation); however, their relevance to arachidonic acid release awaits further purification and characterization of the enzymes and the evaluation of specific inhibitors. The effects observed with manno-oligosaccharide on labeled arachidonate release and PGE2 production in both unprimed (7) and primed cells (this study) with different stimuli suggest that a phospholipase A2 (20, 21) is the most likely phospholipase candidate responsible for the majority of arachidonic acid release.

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