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Lipopolysaccharide-induced Cyclooxygenase-2 Expression in Human U937 Macrophages Is Phosphatidic Acid Phosphohydrolase-1-dependent*

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Cyclooxygenase (COX) has two isoforms, COX-1 and -2, which catalyze the key step in the conversion of cellular arachidonic acid into prostaglandins. In recent years, interest in COX-2 has significantly increased since it has been a target for the development of specific non-steroidal anti-inflammatory drugs. We report that COX-2 expression is up-regulated in phorbol ester (phorbol myristate acetate, PMA)-differentiated human U937 macrophage-like cells stimulated with lipopolysaccharide (LPS), whereas COX-1 is not up-regulated. We show that the LPS-induced up-regulation of COX-2 depends on the activity of the Mg\(^{2+}\)-dependent phosphatidic acid phosphohydrolase 1 (PAP-1). Inhibition of PAP-1 by bromoenol lactone, propranolol, or ethanol resulted in a decrease in LPS-induced COX-2 mRNA transcript production, COX-2 protein expression, and prostaglandin E\(_2\) release from U937 macrophages. To ensure that these results did not arise because of PMA treatment of the U937 cells, similar experiments were conducted with the P388D\(_1\) cell line, which does not require PMA differentiation. LPS increased the levels of endogenous cellular diacylglycerol (DAG) within 2 min of stimulation. This increase was observed to be sensitive to the PAP-1 inhibitors. Furthermore, phosphatidic acid phosphohydrolase activity assays showed that the bromoenol lactone-sensitive PAP-1 activity was translocated from the cytosolic fraction to the membrane fraction within 2 min of LPS exposure. Finally, DAG add-back experiments demonstrate that LPS-induced COX-2 expression is enhanced by the addition of exogenous DAG.

Bacterial sepsis and septic shock result from the overproduction of inflammatory mediators as a consequence of the interaction of the immune system with bacterial cell components, such as lipopolysaccharide (LPS)\(^3\) (1). LPS-activated macrophages produce and release a host of cytokines and eicosanoids in response to inflammatory stimulation, including prostaglandins (PGs). PGs are involved in a number of physiological processes, including inflammation, pain, and vascular permeability while also acting as major contributors of endotoxic shock (2).

Phospholipase A\(_2\) (PLA\(_2\)) comprises a superfamily of enzymes that catalyze the hydrolysis of the sn-2 ester bond in phospholipids, producing free fatty acid and lysophospholipid (3). PLA\(_2\)s regulate inflammation and intracellular signal transduction cascades by liberating arachidonic acid (AA) from membrane phospholipids. The AA that is released is subsequently metabolized by COX, producing prostaglandin H\(_2\) (PGH\(_2\)). There are two known isoforms of cyclooxygenase: COX-1 and COX-2. COX-1 is usually a constitutively expressed enzyme that primarily plays a housekeeping role and has been implicated in a number of physiological functions, including platelet aggregation and parturition (4), whereas COX-2 is usually not expressed under basal conditions in macrophages but is highly inducible by a number of pro-inflammatory agonists, such as LPS, interleukin 1\(\beta\), and tumor necrosis factor\(-\alpha\) (5, 6). In addition to inflammation, COX-2 expression is necessary for uterine contractions during the birthing process and has been further implicated in a number of pathological conditions, including fever, pain, and cancer (4, 6). The PGH\(_2\) produced by COX is further metabolized by specific downstream enzymes into prostaglandins, prostacyclins, and thromboxanes, which are then subsequently secreted (7). When released, eicosanoids function as ligands in receptor-specific autocrine and paracrine signal transduction pathways leading to a diverse range of physiological effects (8).

Two forms of phosphatidic acid phosphohydrolase (PAP) have been identified, a magnesium-independent transmembrane isoform, lipid phosphate phosphohydrolase (LPP), and a cytosolic magnesium-dependent isoform that translocates to the endoplasmic reticulum when it hydrolyzes its substrate, known as phosphatidic acid phosphohydrolase-1 (PAP-1). LPP is known to hydrolyze a host of substrates besides phosphatidic acid, including lysophosphatidic acid, sphingosine 1-phosphate, and ceramide 1-phosphate, whereas PAP-1 has been observed to have a preference for phosphatidic acid as substrate (9, 10). Furthermore, PAP-1 enzymatic activity requires magnesium. Cyclic AMP (cAMP) 3-dephosphorylates and activates PAP-1.

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The abbreviations used are: LPS, lipopolysaccharide; PG, prostaglandin; PLA\(_2\), phospholipase A\(_2\); AA, arachidonic acid; COX-1,-2, cyclooxygenase 1 and 2; PAP, phosphatidic acid phosphohydrolase 1; LPP, lipid phosphate phosphohydrolase; NEM, N-ethylmaleimide; BEL, bromoenol lactone; PA, phosphatidic acid; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; PKC, protein kinase C; IMDM, Iscove’s modified Dulbecco’s medium; MAFP, methyl arachidonyl fluorophosphonate; PLD, phospholipase D.
nesium and is sensitive to the inhibition by N-ethylmaleimide (NEM), propranolol, and bromoenol lactone (BEL), whereas LPP has no divalent cation requirements for activity and is not inhibited by these agents (11–16). Agonist stimulation has been previously shown to induce the translocation of PAP-1 to the endoplasmic reticulum where it hydrolyzes phosphatidic acid (PA) to produce diacylglycerol (DAG) (17). Evidence has been presented that both the substrate and product of PAP-1, PA, and DAG, respectively, may be essential signaling molecules in normal macrophage function (17, 18). Previously, George Carman had succeeded in the partial purification and characterization of PAP-1 from Saccharomyces cerevisiae (19, 20). Recently, his laboratory has sequenced and cloned S. cerevisiae-derived PAP-1, identifying it as the homologue of mammalian Lipin 1, which has been associated with adipocyte development (21). In the present work, we will demonstrate that PAP-1 is necessary for the LPS-induced expression of COX-2 in the human U937 cell line.

The human U937 macrophage-like cell line has been a widely characterized model of the mammalian cellular response to various inflammatory stimuli. It has been shown that U937 cells respond to the presence of LPS with a marked increase in the rate of cellular AA metabolism and cytokine release into the extracellular space (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human promonocytic leukemia U937 cells and murine P388D1, cells were purchased from American Type Culture Collection (Manassas, VA). IMDM and RPMI 1640 cell culture medium were obtained from Invitrogen. Fetal bovine serum was from VWR International (Bristol, CT). Phorbol myristate acetate (PMA) and LPS, from Escherichia coli 0111:B4, were obtained from Sigma. Dioctoyl-DAG was from Biomol (Plymouth Meeting, PA). Human heart Poly(A) RNA was purchased from Clontech (Palo Alto, CA). [5,6,8,9,11,12,14,15-3H]AA (specific activity, 100 Ci/mmol), [9,10-3H]palmitic acid (specific acid, 47.5 Ci/mmole), and L-alpha-dipalmitoyl, [glycerol-14C(U)]phosphatidic acid (specific activity, 141 mCi/mmol) were obtained from PerkinElmer Life Sciences. MAPF, NS-398, aspirin, and the COX-1 and COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI). The specific cPLA2 inhibitor, pyrrophenone, was kindly provided by Dr. Kohji Hanasaki (Shionogi Research Laboratories of Shionogi & Co., Ltd.). The Group IVA PLA2 antibody was obtained from Cell Signaling (Beverly, MA), and the glyceraldehyde-3-phosphate dehydrogenase antibody was from BioTrend (Cologne, Germany). 20-cm × 20-cm × 250-μm K6 Silica gel TLC plates were from Whatman (Clifton, NJ).

**Cell Culture and Stimulation Protocol**—The normal growth medium of the U937 cells contained RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. For experiments the cells were seeded at a density of 5 × 105 cells/well in 12-well plates (Corning Inc.), and differentiation was initiated by the addition of PMA into the cellular medium at a final concentration of 100 nM and allowed to proceed for 48 h (23). The cells were then washed once and incubated in normal growth medium for an additional 24 h prior to the addition of LPS (1 μg/ml). When inhibitors were used, they were added to the medium 30 min before LPS was added. When DAG was added to the cells, it was added immediately after the addition of the LPS. The DAG was initially dissolved in Me2SO, and then further diluted into cellular medium contained prior to being dispensed into the medium of the cultured cells. Control cells were administered equivalent concentrations of Me2SO. Cell viability was assessed visually by the Trypan Blue dye exclusion assay (Invitrogen) and by using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI).

**P388D1**—Human promonocytic leukemia U937 cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO2. IMDM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated at a confluency 106/well in 12-well tissue culture plates, allowed to adhere overnight, and then used for experiments the following day. All experiments were conducted in serum-free IMDM medium.

**Preparation of RNA and Reverse Transcription**—Total cellular RNA was isolated from cells with the RNeasy Mini Kit from Qiagen (Valencia, CA), as described by the manufacturer’s procedure. Any remaining DNA in the extract was removed with the DNA-free kit from Ambion (Austin, TX). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen), following the manufacturer’s printed protocol. Oligo(dT) primers (Invitrogen) were used during the reaction to produce cDNA. The remaining RNA was removed from the cDNA by incubating the samples with 20 units of RNase H from Invitrogen at 37 °C for 20 min.

**Real-time Quantitative PCR**—Primers used for the PCR are as follows: glyceraldehyde-3-phosphate dehydrogenase forward primer (5′-CCACCCAGAAGACTGTGGAAT-3′) and reverse primer (5′-TTCCAGTCGGATGACCTT-3′); Group IVA PLA2 forward primer (5′-ACGAACTGCAATGGCCTT-3′) and reverse primer (5′-GGGACCATAAAGGTACCA-3′); COX-1 forward primer (5′-CAGTGCTCGTATGCCCAAAAT-3′) and reverse primer (5′-AGGACAGTTCAGGGAATG-3′); and COX-2 forward primer (5′-CGACCTTCCACGCATCGT-3′) and reverse primer (5′-CGCACCTTACGGGTATTG-3′). All of the primers were selected using the Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Genset Corp. (La Jolla, CA). All primers were tested by conventional PCR and shown to give only one product visually on 4% agarose gels.

Real-time quantitative PCR was performed on the ABI 7700 Sequence Detection System from Applied Biosystems (Foster City, CA) using SYBR Green PCR Master Mix detection as described in the manufacturer’s procedure. The quantitative PCR consisted of an initial hold at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amount of template cDNA used for each sample was 10 ng and 100 nM primer. Gene expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase.

**PGE2 Assay**—The cellular media was cleared of detached cells by centrifugation, and then the PGE2 release was quantitated using a monoclonal PGE2 EIA kit (Cayman Chemical, Detroit, MI).
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FIGURE 1. Time course of LPS-induced COX-2 mRNA transcript levels, COX-2 protein expression, and PGE₂ release in U937 macrophages. A, U937 macrophages were incubated in the absence (○) and presence (●) of 1 μg/ml LPS, and then subsequently COX-2 mRNA levels were determined at the indicated times over a 20-h period by quantitative PCR analysis. B, U937 macrophages were incubated in 1 μg/ml LPS for the indicated amounts of time before the cellular lysates were collected and blotted for COX-2 protein expression. C, U937 macrophages were incubated in the absence (○) and presence (●) of 1 μg/ml LPS, and then subsequently PGE₂ release was determined at the indicated times over a 20-h period. Representative experiments of at least three individual experiments are shown. Data are expressed as mean values ± S.D. of three individual replicates.

Ann Arbor, MI). The assays were conducted according to the manufacturer’s protocol.

Immunoblotting—Cells were washed twice with cold PBS, and scraped free with a rubber policeman in 75 μl of Complete Mini protease mixture solution (Roche Applied Science, Mannheim, Germany). Protein concentrations were determined and normalized using the Bio-Rad Protein Assay. 35 μg of total protein was loaded onto 4–12% Bis-Tris SDS-PAGE gels and then transferred onto nitrocellulose membrane. When blotting for the Group IVA PLA₂ the membrane was blocked using 3% bovine milk protein in PBS buffer containing 0.1% Tween 20 for 1 h before being probed with a Group IVA PLA₂ specific antibody overnight. The membrane was then washed three times in PBS containing 0.1% Tween 20 before addition of a rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Alpha Diagnostic) for 1 h. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit (Amersham Biosciences).

PA Phosphohydrolase Activity Assay—PA phosphohydrolase activity was determined from U937 and P388D₁ cellular lysates according to the method of Day and Yeaman (14) as modified by Balboa et al. (25). The substrate [¹⁴C]Glycercerol-labeled PA was presented as mixed micelles with Triton X-100 at a detergent/phospholipids molar ratio of 10:1. Assays were conducted at 37 °C for 1 h. The assay mixture contained 100 μM PA substrate (0.025 μCi/assay), 1 mM Triton X-100, 50 mM Tris-HCl, pH 7.1, 10 mM β-mercaptoethanol, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and the lysate to a final volume of 100 μl. The reaction was terminated by the addition of a chloroform/methanol system (60:40) to the reaction mixture. The organic phase was separated from the aqueous phase, placed into fresh microcentrifuge tubes, and dried in a vacuum centrifuge. The subsequent pellet was reconstituted in 20 μl of the (60:40) chloroform/methanol system, the resulting [¹⁴C]PA and [¹³C]DAG were then separated by TLC using an n-hexane/ether/water mixture (70:30:1). The TLC plate was then developed in an iodination chamber, and the DAG and PA spots were scraped and subsequently quantified by liquid scintillation counting. Additionally, to distinguish between the PAP-1 and LPP activities, the PAP-1-specific inhibitor, NEM, was added (8 mM) for a period of 10 min prior to addition of the substrate (11). When PAP-1 assays were performed on the cytosolic or membrane fraction of lysates, lystate preparations were centrifuged at a speed of 100,000 × g for 30 min, and the cytosolic and membrane fractions were then separated.

When quantifying the endogenous production of DAG, cells were incubated in 10% fetal calf serum RPMI medium supplemented with [9–10⁻³H]palmitic acid (1 μCi/ml) for 24 h, the times and exposed to a streptavidin-horseradish peroxidase-conjugated antibody (Vector) for 30 min at room temperature. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit. When blotting for the Lipin 1, 2, or 3 proteins the membrane was blocked with 5% milk protein in PBS buffer containing 0.1% Tween 20 for 1 h before being probed with a specific Lipin 1, 2, or 3 antibody (Alpha Diagnostic, San Antonio, TX) overnight. The membrane was then washed three times in PBS containing 0.1% Tween 20 before addition of a rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Alpha Diagnostic) for 1 h. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit (Amersham Biosciences).
cells were washed, and serum-free media was added for 1 h prior to beginning the experiment. Lipids were extracted according to the method of Bligh and Dyer (26). The extraction solution was placed into fresh microcentrifuge tubes and dried in a vacuum centrifuge. The resulting pellet was reconstituted in 20 μl of the (60:40) chloroform/methanol system, and the resulting [3H]DAG was separated by TLC using ether/water (70:30:1). The TLC plate was then developed in an atmosphere of (60:40) chloroform/methanol. The DAG and PA spots were scraped into an iodination chamber, and the DAG and PA spots were scraped off the TLC plate and subsequently quantified by liquid scintillation counting.

RESULTS

LPS Up-regulates Cyclooxygenase-2 Expression and Induces PGE2 Production in a Time-dependent Manner—Because LPS-induced PGE2 release is known to be dependent on the function of the PLA2 and COX enzymes, we measured their expression over time with LPS stimulation to determine whether changes in their levels are a mechanism for controlling PGE2 production. As has been previously described in the U937 cells (27), the levels of COX-2 mRNA transcript increased dramatically during the first 4 h of LPS stimulation, reaching maximal levels at 4 h, and then waning afterward (Fig. 1A). This result is corroborated by the LPS-induced COX-2 protein expression profile, in which the protein was undetectable until 2 h after LPS stimulation and then increased steadily until 20 h (Fig. 1B). Neither COX-1 nor Group IVA PLA2 were up-regulated on the protein or messenger RNA levels in response to the addition of 1 μg/ml LPS agonist (data not shown). Furthermore, 1 μg/ml LPS increased PGE2 release from U937 macrophages in a time-dependent manner. The PGE2 release started between 2 and 4 h after LPS stimulation and continued to increase with time for 20 h, as shown in Fig. 1C. There was no change in PGE2 production in control cells that were not exposed to LPS.

Inhibition of LPS-induced PGE2 Production and COX-2 Expression by BEL—U937 cells that had been preincubated with 25 μM BEL, a dual Group VI PLA2/PAP-1 inhibitor, prior to the addition of LPS had reduced COX-2 mRNA transcript levels relative to uninhibited cells (Fig. 2A). The inhibition by BEL was observed to affect not only the levels of COX-2 transcript, but also the amount of COX-2 protein being expressed (Fig. 2B) and the amount of PGE2 being produced by the cells in response to LPS stimulation (Fig. 2C). Because the BEL inhibitor is known to inhibit not only PAP-1, but also Group VI PLA2, it was imperative to discern which enzyme was responsible for the aforementioned phenomena. To clarify this issue, the chemical inhibitor MAFP, which inhibits the function of Group VI PLA2 but not PAP-1, was added to cell experiments as had been done with the BEL inhibitor. As can be seen in Fig. 2 (A and B), MAFP had no effect on the induction of COX-2, at both the mRNA transcript and protein levels. It should be noted that PGE2 release could not be determined for experiments conducted with MAFP, because addition of MAFP to the immunoassay gives a false-positive measurement.

To ensure that PAP-1 is present in this cell line, U937 cell lysates were checked for the presence of Lipin 1 by Western blot. Lipin 1 is reported to run at a molecular mass of 140 kDa. The inhibition by BEL was observed to affect not only the levels of COX-2 transcript, but also the amount of COX-2 protein being expressed (Fig. 2B) and the amount of PGE2 being produced by the cells in response to LPS stimulation (Fig. 2C). Because the BEL inhibitor is known to inhibit not only PAP-1, but also Group VI PLA2, it was imperative to discern which enzyme was responsible for the aforementioned phenomena. To clarify this issue, the chemical inhibitor MAFP, which inhibits the function of Group VI PLA2 but not PAP-1, was added to cell experiments as had been done with the BEL inhibitor. As can be seen in Fig. 2 (A and B), MAFP had no effect on the induction of COX-2, at both the mRNA transcript and protein levels. It should be noted that PGE2 release could not be determined for experiments conducted with MAFP, because addition of MAFP to the immunoassay gives a false-positive measurement.
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FIGURE 3. Propranolol inhibits the LPS-induced production of COX-2 mRNA transcripts, COX-2 protein expression, and release of PGE₂. A, quantitative PCR analysis of the effect of propranolol (150 μM) on COX-2 mRNA transcript levels in U937 macrophages, in the absence (■) and presence (●) of 1 μg/ml LPS. B, Western blot analysis of COX-2 expression in U937 macrophages treated with propranolol (150 μM) prior to the addition of 1 μg/ml LPS for 20 h. C, effect of propranolol (150 μM) on PGE₂ release in the absence (■) and presence (●) of 1 μg/ml LPS. Representative experiments of at least three individual experiments are shown. Data are expressed as mean values ± S.D. of three individual replicates.

Additionally, the release of PGE₂ from LPS-stimulated macrophages was blocked in cells in a dose-dependent fashion, whereas n-butanol reduced PGE release comparably to that of 0.5% ethanol and t-butanol had no effect (Fig. 4C). Doses higher than 0.6% v/v of butanol were not utilized in cellular experiments due to lethal cytotoxicity to the macrophages at elevated concentrations.

DAG Production and PAP-1 Activity Are Reduced in PAP-1-inhibited U937 Cells—To ensure that the chemical inhibitors had the desired effect of inhibiting PAP-1 activity in the cultured U937 cells, cells were tritium-radiolabeled with palmitic acid to study the production of endogenous DAG in response to LPS, in the presence and absence of BEL. DAG production increased by 20% to 50% within the first 2 min of LPS stimulation, and then gradually returned to basal levels over 30 min following LPS stimulation (Fig. 5A). However, the addition of BEL to cells resulted in a dramatic reduction of the DAG spike 2 min after LPS stimulation, while having an insignificant effect on the basal DAG levels. These results suggest that the LPS-induced DAG spike can be attributed to the BEL-sensitive PAP-1, whereas the basal DAG level is not due to the activity of PAP-1. Presumably, this pool of DAG arises as a result of LPP activity associated with the basal metabolism of the cell or de novo DAG synthesis.

To confirm that the LPS-induced increase in DAG was due to PAP-1 activity, in vitro PAP assays of the cytosolic component of cellular lysates were performed to determine whether the levels of PAP-1 activity change after LPS stimulation. The PAP in vitro assay measures total PAP activity, which is the sum of all DAG produced from PA hydrolysis by PA-dephosphorylating enzymes, including PAP-1 and LPP. Furthermore, in an effort to distinguish PAP-1 activity from the total PAP activity, we have taken advantage of the fact that PAP-1 requires the presence of Mg²⁺ for full enzymatic activity and is inhibited by the addition of the chemical inhibitors BEL and NEM. It is known that LPP is a transmembrane protein localized to the membrane fraction of the cellular lysate; therefore, the only remaining PA-dephosphorylating enzyme present in the cytosolic component of the cellular lysate is PAP-1. Cells that had been stimulated with LPS were subsequently lysed, and the cytosolic and membrane components were separated and isolated by centrifugation (100,000 × g, 1 h, 4 °C). Because PAP-1 translocates from the cytosol to phospholipid membrane surfaces when it becomes activated, it was essential to first identify (Fig. 3A), an equally pronounced reduction of COX-2 protein expression (Fig. 3B), and near total abatement of PGE₂ release into the media (Fig. 3C).

Addition of short chain, primary alcohols into the media results in their uptake by the cultured cells and subsequent participation in the PLD-mediated transesterification of phosphatidylalcohols, which are not PAP-1 substrates (29). By competing with water, which is the normal PLD-mediated reaction nucleophile, the primary alcohol inhibits PAP-1 cellular activity by reducing the availability of the substrate phosphatidic acid. Cells were preincubated for 30 min in the presence of 2% (v/v) ethanol, 0.6% n-butanol, and 0.6% t-butanol, prior to the addition of LPS. The purpose of the tertiary alcohol, t-butanol, is to serve as a negative control, because it is not incorporated in PLD-mediated phospholipid hydrolysis due to steric hindrance. Fig. 4A demonstrates that the addition of ethanol or n-butanol to the supernatant resulted in a 50% reduction of COX-2 mRNA transcript levels in response to LPS stimulation. Importantly, t-butanol had no effect on the level of LPS-induced COX-2 mRNA transcript levels relative to that of the uninhibited cells. The LPS-induced COX-2 protein expression data corroborated the mRNA transcript results, as both ethanol and n-butanol were observed to reduce COX-2 protein expression by ~50% while t-butanol had no effect (Fig. 4B).
which time point following the addition of LPS agonist would yield the most pronounced change in PAP-1 activity. In Fig. 5B, the change in total PAP-1 activity that was observed in the cytosolic fraction of the cell lysate in response to the addition of LPS is shown relative to basal levels in the cytosolic fraction over the course of 30 min. The amount of total cytosolic PAP activity decreased by ~50% with LPS stimulation relative to basal activity during the first 2 min of stimulation and was not observed to return to basal activity levels over the 30-min time course of the experiment. Because 2 min of LPS stimulation was observed to yield the most dramatic DAG production and was seen to yield PAP-1 translocation, it was chosen as the representative time point from which to study chemical inhibition of PAP-1 activity in Fig. 5C.

To confirm that the decrease in PAP activity observed at 2 min of LPS stimulation was due to PAP-1 translocation from the cytosol, chemical inhibitor studies were performed on the basal activity. The NEM and BEL inhibitors were added directly to the cellular lysates prior to being assayed, whereas Mg\(^{2+}\) was simply not added to a third condition, which still included EDTA and EGTA to ensure that the PAP-1 activity could be accounted for as an increase in the total PAP activity of the membrane. Although the total PA hydrolase activity in the membrane fraction appeared to increase somewhat after LPS stimulation, unfortunately, the presence of LPP resulted in rates of PA hydrolysis that were 15 times greater in the membrane fraction than that had been observed in the cytosolic fraction. This factor made it impossible to accurately quantitate statistically significant changes in PAP-1 activity in the membrane fraction of cellular lysates.

Additionally, PAP activity was monitored in the cytosolic fractions of cellular lysates derived from cells that were challenged by increasing doses of LPS (Fig. 5D). Macrophages were administered LPS for 2 min prior to the lysates being collected, and the osmotic fraction was then assayed for PAP activity as described previously. PAP activity in the cell cytosol decreased in an LPS dose-dependent manner with increasing LPS.

**Exogenous DAG Induces COX-2 Expression and PGE\(_2\) Release**—To simulate the physiological activity of PAP-1 in the cell, 50 μM exogenous DAG was added to cells to determine whether the addition of DAG would induce COX-2 expression and increased release of PGE\(_2\). As shown in Fig. 6A, the addition of DAG to LPS-primed cells increased COX-2 mRNA transcript levels 2-fold relative to that of cells that were stimulated with LPS alone. The addition of DAG alone to cells without LPS appeared to have a negligible effect on COX-2 transcript levels. Concordantly, the addition of DAG to LPS-stimulated cells had a similar effect on COX-2 protein production, because DAG-primed LPS-stimulated cells expressed approximately twice as much COX-2 protein as cells that were stimulated with LPS alone (Fig. 6B). Furthermore, the addition of DAG enhanced the release of PGE\(_2\) production with LPS stimulation by increasing PGE\(_2\) 3-fold relative to that of cells that were treated with LPS alone (Fig. 6C). Addition of DAG to the cells alone had no significant effect on the release of the PGE\(_2\) from the cells. These data provide evidence that DAG accentuates the expression of COX-2 and subsequent release of PGE\(_2\) in response to the LPS priming of U937 cells. However, exogenous DAG addition appears not to be sufficient to elicit a pro-inflammatory response without LPS supplementation.

**Inhibition of LPS-induced COX-2 Protein Expression in the P388D\(_1\) Cell Line by BEL, Propranolol, and Ethanol**—To confirm that the results garnered in the U937 macrophages were not merely an anomaly of the cell line, or the result of direct...
PKC activation by the addition of PMA to the cells, similar experiments were conducted on the P388D1 macrophage cell line, which does not require the addition of PMA for cellular differentiation. All three inhibitors significantly reduced LPS-induced COX-2 protein expression, COX-2 transcript levels, and the release of PGE2 into the supernatant (Fig. 7), confirming that the results are not simply an artifact of the U937 macrophages. Interestingly, one difference between the cell lines was that BEL and propranolol more potently inhibited LPS-induced COX-2 expression in P388D1 macrophages than in U937 macrophages. BEL and propranolol were observed to significantly reduce LPS-induced PGE2 release and COX-2 expression even when added to the cells at dosages as low as 1 and 25 μM, respectively (data not shown).

Consistent with the LPS stimulation-induced DAG production results garnered from U937 macrophages, endogenous DAG production in P388D1 macrophages increased immediately after the addition of LPS, with maximal production at 2 min following LPS stimulation (data not shown). Finally, the addition of exogenous DAG to LPS-stimulated P388D1 macrophages resulted in augmented COX-2 protein expression and transcript levels relative to macrophages that had been stimulated with only LPS, which is also consistent with results that had been obtained in experiments conducted with the U937 macrophages.

seemingly contradictory reports have been described in which the chemical inhibition of PAP-1 in interleukin 1β-stimulated WISH cells or LPS-stimulated RAW 264.7 cells resulted in enhanced COX-2 expression (34, 35). These contradictory results are difficult to interpret, although it may signify that the effect of the PLD/PAP-1 signaling pathway on the expression of COX-2 protein depends on the agonist stimulating the cells as well as the specific cell line.

Phospholipid-derived DAG has been identified as a signaling molecule in the activation of various enzymes involved in macrophage differentiation and inflammatory activation, including PKC, protein kinase D, RasGRF, and chimerins (36, 37). The chronology of DAG production in U937 macrophages from the hydrolysis of membrane phospholipids occurs in two distinctive waves. First, a transient wave attributed to phospholipid hydrolysis by phospholipase C arises within seconds of LPS stimulation. Subsequently, a second, more prolonged DAG wave is then produced by the joint actions of PLD and PAP-1. PLD hydrolysis of phospholipids results in PA release from membrane stores, which are then subsequently dephosphorylated by PAP-1 into DAG (38). The activation of PLD in differentiated U937 cells ultimately results in the enhanced release of AA from cells by a pathway involving a PLA2-mediated mechanism (39). Our results support a PLD/PAP-1 regulatory mech-
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In this work, PAP-1 has been shown to be a required signaling component in the LPS-stimulated up-regulation of COX-2 protein in the human U937 macrophage-like cell line. LPS stimulation resulted in increased COX-2 mRNA transcript levels and protein expression levels, while having no effect on the levels of COX-1 mRNA and protein. Chemical inhibition of PAP-1 in U937 macrophages with BEL and propranolol resulted in reduced levels of LPS-stimulated COX-2 mRNA transcript levels, COX-2 protein expression, and prostaglandin release from U937 macrophages. Propranolol has long since been known to be an inhibitor of PAP-1; however, recent advances by the Turk laboratory have shown that BEL has the ability to inhibit by binding thiol functional groups, which would explain the BEL sensitivity of PAP-1 (16). PAP-1 has been previously described as sensitive to thiol inhibitors, such as NEM and chlorpromazine. Because BEL is known to be an inhibitor of not only PAP-1, but also another possible pro-inflammatory enzyme, the Group VI PLA2, a second Group VI PLA2 inhibitor, MAFP, was utilized in the study to verify that the Group VI PLA2 was not responsible for the COX-2 induction. Further evidence was garnered by the cellular deprivation of PAP-1 substrate PA by the addition of ethanol and α-butoanol into the supernatant, which yielded inhibition of the LPS-stimulated pathway, whereas the negative control t-butanol had no effect. Because U937 cells are differentiated by PMA, a known activator of PKC, which is directly involved in the LPS signal transduction cascade by phosphorylating PLD, the results described in the U937 cells were confirmed by repetition in the murine P388D1 macrophage cell line, which requires no chemical additives to differentiate the cells into an adherent morphology. The fact that the results were consistent in the P388D1 cells suggest that PAP-1 may be a putative enzyme in a number of processes, because it has been shown to be involved in a number of different stimulation pathways leading to the expression of COX-2, including with LPS alone, PMA alone, and with the two in combination.

In this study, we have observed an increase in the cellular endogenous concentrations of DAG within 2 min of LPS stimulation of the macrophages, and this increase is consistent with reports from other laboratories (41–43). The translocation of PAP-1 activity from the cytosolic fraction of the lysate to the membrane in the time course data is consistent with the temporal rise of endogenous DAG in the cells. Very importantly, the DAG spike was sensitive to the addition of BEL, whereas the PAP-1 activity could be blocked by the addition of the chemical inhibitors BEL or NEM, or removal of magnesium from the activity assay. Taken as a whole, these results demonstrate that PAP-1 is being mobilized from the cytosol in response to LPS signal transduction and is responsible for the generation of a DAG spike at 2 min following the addition of the stimulant. The fact, that BEL abates the formation of the DAG spike while also decreasing the basal cytosolic total PAP activity by an amount that can be attributed to the LPS-stimulated increase, further suggests that PAP-1 is responsible for these phenomena.

To simulate the activity of the endogenous PAP-1 enzyme in the cell during LPS transduction, exogenous DAG was added to the cells immediately after LPS priming, and was observed to bolster the induction of COX-2, while also increasing the release of eicosanoids into the supernatant. This suggests that the DAG produced by PAP-1 hydrolysis rather than the enzyme itself is the critical component in the LPS-stimulated signaling cascade leading toward COX-2 expression. The fact that DAG alone did not have a significant effect upon PGE2 release and COX-2 production in the absence of LPS supplementation suggests that DAG production is implicated in the pro-inflamma-

FIGURE 6. DAG supplementation to LPS-stimulated U937 macrophages augments COX-2 mRNA transcript production, COX-2 protein expression, and PGE2 release. A, quantitative PCR analysis of the effect of 50 μM DAG supplementation on COX-2 mRNA transcript levels in the absence [■] and presence [□] of 1 μg/ml LPS. B, Western blot analysis of COX-2 expression in U937 macrophages treated with 50 μM DAG prior to the addition of 1 μg/ml LPS for an incubation of 20 h. C, effect of 50 μM DAG on PGE2 production in the absence [■] and presence [□] of 1 μg/ml LPS. Representative experiments of at least three individual experiments are shown. Data are expressed as mean values ± S.D. of three individual replicates.
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A. 

B. 

C. 

FIGURE 7. Addition of BEL, ethanol, or propranolol to P388D1 macrophages results in the inhibition of the LPS-induced production of COX-2 mRNA transcripts, COX-2 protein expression, and release of PGE2. A, quantitative PCR analysis of the addition of BEL (5 μM), ethanol (2.0%), or propranolol (100 μM) on COX-2 mRNA transcript levels in P388D1 macrophages, both in the absence (■) and presence (○) of 1 μg/ml LPS. B, Western blot analysis of COX-2 expression in P388D1 macrophages treated with BEL (5 μM), ethanol (2.0%), or propranolol (100 μM) prior to the addition of 1 μg/ml LPS for 20 h. C, effect of BEL (5 μM), ethanol (2.0%), or propranolol (100 μM) on PGE2 release in the absence (■) and presence (○) of 1 μg/ml LPS. Representative experiments of at least three individual experiments are shown. Data are expressed as mean values ± S.D. of three individual replicates.

individual effects of the PA and DAG molecules on cell signaling. The production of PA in U937 macrophages has been shown to occur in two distinct waves, one derived from PLD activity occurring temporally before PAP-1 hydrolysis, and the second derived from DAG kinase activity, which utilizes the DAG that is produced by PAP-1 activity (42). Furthermore, PA and DAG are very easily interconverted by the actions of PAP and DAG kinases. The phosphatidic acid substrate of PAP-1 is derived from the action of PLD on phospholipids or DAGK phosphorylation of DAG. The existence of such a feedback mechanism would explain the durations of the two DAG pools in stimulated cells; the first being short and fleeting, the second longer. The production of DAG by the coupled activities of PLD and PAP-1 may lead to further PKC activation or to the activation of RasGRP. The induction of RasGRP, an activator of the mitogen-activated protein kinase pathway, would ultimately lead to activation of transcriptional factors, including AP-1 and NF-κB, which can result in the induction of COX-2 expression (24, 40, 46). Currently, our laboratory is conducting further research aimed at understanding both the regulation of PAP-1 leading to COX-2 expression as well as the relationship between PAP-1 function and the up-regulation of COX-2 protein expression.

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