Glycercyloprostaglandin Synthesis by Resident Peritoneal Macrophages in Response to a Zymosan Stimulus*

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Cyclooxygenase (COX-2) oxygenates arachidonic acid (AA) and 2-arachidonylglycerol (2-AG) to endoperoxides, which are subsequently transformed to prostaglandins (PGs) and glycercyloprostaglandins (PG-Gs). PG-G formation has not been demonstrated in intact cells treated with a physiological agonist. Resident peritoneal macrophages, which express COX-1, were pretreated with lipopolysaccharide to induce COX-2. Addition of zymosan caused release of 2-AG and production of the glycercyl esters of PGE₂ and PGF₂α over 60 min. The total quantity of PG-Gs (16 ± 6 pmol/10⁷ cells) was much lower than that of the corresponding PGs produced from AA (21,000 ± 7,000 pmol/10⁷ cells). The differences in PG-G and PG production were partially explained by differences in the amounts of 2-AG and AA released in response to zymosan. The selective COX-2 inhibitor, SC236, reduced PG-G and PG production by 49 and 17%, respectively, indicating a significant role for COX-1 in PG-G and especially PG synthesis. Time course studies indicated that COX-2-dependent oxygenation rapidly declined 20 min after zymosan addition. When exogenous 2-AG was added to macrophages, a substantial portion was hydrolyzed to AA and converted to PGs; 1 μM 2-AG yielded 820 ± 200 pmol of PGs/10⁷ cells and 78 ± 41 pmol of PGs/10⁷ cells. SC236 reduced PG-G and PG production from exogenous 2-AG by 88 and 76%, respectively, indicating a more significant role for COX-2 in the utilization of exogenous substrate. In conclusion, lipopolysaccharide-pretreated macrophages produce PG-Gs from endogenous 2-AG during zymosan phagocytosis, but PG-G formation is limited by substrate hydrolysis and inactivation of COX-2.

Cyclooxygenase (COX1; prostaglandin G/H synthase) catalyzes the first two steps in the conversion of arachidonic acid (AA) to prostaglandins (PG), prostacyclin, and thromboxane (1–4). The product of the COX reaction is the endoperoxide, PGH₂, which is then further metabolized to the terminal PGs through the action of various synthase enzymes. Two isoforms of COX have been identified and characterized (5). The isoforms differ considerably with regard to transcriptional regulation in that COX-1 is usually expressed constitutively, whereas COX-2 is induced in response to a variety of inflammatory and proliferative stimuli, including cytokines, growth factors, and tumor promoters. As a consequence of these varied expression patterns, COX-1 is believed to be primarily responsible for “housekeeping” functions such as gastric cytoprotection and regulation of platelet aggregation. In contrast, COX-2 is thought to be involved in the inflammatory response, pyrexia, and in the regulation of cellular proliferation (6–11). However, recent work has indicated that these distinct roles are probably oversimplified, leaving an ongoing question as to the exact physiological functions of each isoform (12).

Despite their differences in transcriptional regulation, COX-1 and COX-2 share 60% sequence identity, with nearly superimposable three-dimensional structures and highly similar active sites (13–16). Kinetically, the two isoforms are virtually indistinguishable when AA is used as substrate (17). However, subtle differences in the active sites have allowed the development of selective inhibitors for each isoform (18–22). These same differences allow COX-2 to use neutral ester and amide derivatives of AA as substrates more effectively compared with COX-1 (23–25). Among the neutral derivatives of AA that can serve as selective COX-2 substrates are the endocannabinoids, 2-arachidonylglycerol (2-AG) and arachidonylethanolamide (anandamide), and the lipoic acid, N-arachidonoylglycine (23–26). COX-2 converts these substrates to endoperoxide derivatives analogous to PGH₂, PGI₂, glycercyl ester and PGH₂ ethanolamide can be further metabolized by all of the terminal synthases except thromboxane synthase (27). Thus, a wide variety of potential PG glycercyl esters (PG-Gs) and ethanolamides (PG-EAs) are biochemically possible.

A number of reports have demonstrated biological activities for PG E₂-G, PGE₂-EA, and PGF₂α-EA (28–30), suggesting the possibility that these compounds serve specific physiological functions. Weber et al. (31) have demonstrated the in vivo production of PG-EAs in mice injected with anandamide; however, no PG-EAs were detected in animals in the absence of exogenous substrate. Because 2-AG is usually present at higher concentrations than anandamide in vivo, it is anticipated that PG-Gs should be more abundant that PG-EAs. However, detecting PG-Gs, especially in rodent models, will likely be challenging because of rapid hydrolysis by plasma esterases (32). Consequently, attention has been focused on production by intact cells in culture. Thus, RAW264.7 cells have been shown to produce PG-Gs in response to exogenous 2-AG or from endogenous 2-AG in response to an ionomycin stimulus (24). However, there has been no demonstration of PG-G or PG-EA production by primary cells using endogenous substrate in response to a physiological stimulus. Therefore, to develop a better understanding of the potential biological role of PG-Gs,
we have undertaken a study of PG-G formation by murine primary resident peritoneal macrophages (RPMs) in response to the phagocytosis of zymosan. Our results demonstrate that phagocytizing RPMs produce both PGE\textsubscript{2}-G and PGI\textsubscript{2}-G concomitantly with the analogous PGs. Although the levels of PG-Gs produced were low relative to those of PGs, the absence of significant PG-G hydrolytic activity allowed PGE\textsubscript{2}-G-to accumulate in RPM culture medium to levels shown to induce Ca\textsuperscript{2+} mobilization in RAW264.7 cells in culture (28).

**EXPERIMENTAL PROCEDURES**

**RPM Cultures**—Female CD-1 mice (25–30 g) were obtained from Charles River Laboratories (Wilmington, MA). The mice were killed with carbon dioxide, and the peritoneal cavities were lavaged with a total of 3 ml of ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS) (33). Peritoneal cells were collected by centrifugation of lavage fluid and resuspended at a concentration of 2–3 × 10\textsuperscript{6} cells/ml in a minimal essential medium (α-MEM) supplemented with Glutamax (Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS; Summit Biotechnologies, Fort Collins, CO) plus 100 units/ml penicillin and 0.10 mg/ml streptomycin (Sigma) (α-MEM/FCS). The cells were subsequently resuspended at 35-mm culture dishes at 3 ml/dish or onto 60-mm dishes at 6 ml/dish and incubated for 2 h at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. Nonadherent cells were removed by washing the plates four times with PBS, and the cultures were then incubated overnight in fresh α-MEM/FCS. The mean protein content of RPM cultures (35-mm dish) was 100 ± 10 µg/dish (8.2 ± 0.8) × 10\textsuperscript{5} cells/dish.

**Lipoyxosaccharide (LPS) and Zymosan Treatment of RPM Cultures**—Cultures of RPMs were washed twice with PBS at 37°C and then overlaid with 2 ml of fresh α-MEM/FCS with or without the desired concentration of LPS (E. coli 011:B4; Calbiochem). Cells were incubated for the desired time periods, washed twice again with PBS, and overlaid with 2 ml of serum-free α-MEM. Unopsonized zymosan A (Sigma) was prepared as described by Bonney et al. (34) and suspended at 200 mg/ml. Aliquots (0.1 ml) were added directly to the macrophage culture medium as desired, and cells were incubated as indicated for individual experiments.

For some experiments, cells were incubated with or without LPS, washed twice with PBS, and overlaid with α-MEM containing 0, 50, 100, or 200 nm SC236 (Calbiochem) or 0, 40, 80, or 120 µm RH80267 (BIOMOL Research Labs Inc., Plymouth Meeting, PA). After a 30-min incubation, zymosan was added as described above. SC236 and RH80267 were prepared as stock solutions in dimethyl sulfoxide diluted as appropriate and added to the culture medium to give a constant concentration of 0.1% dimethyl sulfoxide in all samples.

**Metabolism of Exogenous AA, 2-AG, and PGE\textsubscript{2}-G by RPM Cultures**—RPM cultures were prepared and incubated in the presence or absence of LPS as above. Cells were then washed twice with PBS and overlaid with 1 ml of serum-free α-MEM containing 0, 50, 100, or 200 nm SC236. Following a 30-min incubation, the medium was removed and replaced with 1 ml of α-MEM containing the same concentration of SC236 and 1 µM either AA or 2-AG (Cayman Chemical Co., Inc., Ann Arbor, MI). Cells were incubated for 30 min. For addition to the culture medium, AA and 2-AG were prepared as 1 mM stock solutions in dimethyl sulfoxide and added to the medium so that the total dimethyl sulfoxide concentration contributed by addition of ethyl acetate/hexane (90:10), and the organic extracts were evaporated to dryness under a stream of argon. The dried organic extracts were reconstituted in 1 ml of ethyl acetate/methanol/acetic acid (94:5:0.1, v/v/v), and each was applied to a dry silica solid-phase extraction column (Sep-Pak Vac, 100 mg; Waters Corp., Milford, MA). The columns were eluted with 3 ml of ethyl acetate/methanol/acetic acid (94:5:0.1, v/v/v). 4 ml of eluate was collected, evaporated to dryness, reconstituted in 100 µl of α-MEM, and analyzed for total PG and AA by silver ion coordination LC-MS/MS as described (36). In typical samples, both 2-AG and 1(3)-AG were detected. In most cases, 2-AG was the predominant isomer, and the relative ratio of 2-AG to 1(3)-AG was similar to that in the internal standard, which was added as 100% 2-AG-d\textsubscript{5} to the samples. Because nonspecific acyl migration of 2-AG to a mixture of 2-AG and 1(3)-AG occurred during sample workup, it was not possible to determine exactly how much of each isomer was present in the cells originally, so the two isomers were quantified together, and data are reported as total AG.

**Protein Immunoblotting for COX-2 Protein Expression**—For the determination of COX-2 expression, RPM cultures were incubated with the desired stimuli; the medium was removed; and the cells were then washed twice with ice-cold PBS. Cell monolayers were scraped into 200 µl of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 4 mM EDTA, 50 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.1 mM NaVO\textsubscript{4}, 0.2% Triton X-100, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 µg/ml antipain, 5 µg/ml leupeptin, 5 µg/ml chymostatin, and 5 µg/ml pepstatin (all components from Sigma)). Cell lysates were allowed to stand for 30 min on ice with occasional vortex mixing, and particulate material was then removed by centrifugation for 10 min at 16,000 × g. Samples were stored at −80°C until analyses could be completed.

The protein concentrations of 20-µl aliquots of macrophage cell lysates were determined using a BCA protein assay kit (Pierce) according to the manufacturer’s directions. Macrophage lysate samples containing 15 µg of protein were then subjected to SDS-PAGE and immunoblot analysis as described (12), with the exception that the rabbit polyclonal antibody directed against murine COX-2 (Cayman Chemical Co., Inc.) was used at a dilution of 1:5,000 instead of 1:3,000.

**Metabolism of 2-AG and PGE\textsubscript{2}-G by RPM Cell Lysates**—RPMs were isolated and plated onto 60-mm tissue culture dishes. Following washing and overnight incubations, cells were treated for 6 h with or without 100 ng/ml LPS as described above, washed twice with PBS, and scraped into 100 mM Tris-HCl containing 1 mM EDTA (pH 7.5) (total volume of 3 ml/dish). The resulting cell suspensions from four dishes were combined and homogenized by sonication using a Virsonic cell disrupter (ICN, Costa Mesa, CA) with a Vibrospect at power setting 35 for four 15-s bursts.

Cell lysates were treated for 5 min at 37°C, and 2-AG was added as a stock solution of 1 mM in dimethyl sulfoxide to give a final concentration of 1 µM. The lysates were incubated for the desired time periods, and 0.5-ml aliquots were removed and combined with either 1 ml of acetone containing 10 ng of 2-AG-d\textsubscript{5} and 100 ng of AA-d\textsubscript{5} (Cayman Chemical Co., Inc.) and stored at −20°C for analysis. Alternatively, lysates were treated as described above, and aliquots of 0.5 ml (for samples containing 50 nm PGE\textsubscript{2}-G) or 0.2 ml (for samples containing 1 µM PGE\textsubscript{2}-G) were removed and combined with 1 ml of ethanol containing 5 pmol each of PGE\textsubscript{2}-d\textsubscript{5} and 6-keto-PGF\textsubscript{1α}-d\textsubscript{5}, plus 50 pmol each of PGE\textsubscript{2}-d\textsubscript{5} and 6-keto-PGF\textsubscript{1α}-d\textsubscript{5}. Following addition of 0.5 ml of PBS, the samples were acidified with 10 µl of acetic acid and extracted twice with 1 ml of chloroform. The extracts were evaporated to dryness, and the residues were subjected to direct LC-MS/MS analysis for PG and PG-G content or purified by silica solid-phase extraction, followed by LC-MS/MS analysis for total AG and AA content as described above.

Alternatively, lysates were treated as described above, and aliquots of 0.5 ml (for samples containing 50 nm PGE\textsubscript{2}-G) and 0.2 ml (for samples containing 1 µM PGE\textsubscript{2}-G) were removed and combined with 1 ml of ethanol containing 5 pmol of PGE\textsubscript{2}-d\textsubscript{5} and 50 pmol of PGE\textsubscript{2}-d\textsubscript{5}. PBS was added to each sample to bring the aqueous volume to 1 ml, and the samples were then acidified with acetic acid and extracted with chloroform as described above for analysis by LC-MS/MS for PGE\textsubscript{2}-G and PGE\textsubscript{2}- content.

**Data Analysis**—Statistical significance was determined using Student’s t test, with the minimal criterion for significance being p < 0.05.
RESULTS

Synthesis of PG-Gs from Endogenous Substrate by RPMs during Zymosan Phagocytosis—2-AG is a selective COX-2 substrate in vitro, so it was reasonable to expect that PG-G synthesis might occur during the response to stimuli such as LPS that induce de novo COX-2 expression. We first analyzed medium from RPMs incubated with 100 ng/ml LPS for periods of up to 24 h in the presence or absence of serum. Trace amounts of PG-Gs were detected in the medium of LPS-treated cells, but they were too low to be reliably quantified. Prior studies had demonstrated PG-G synthesis by RAW264.7 cells treated with ionomycin (24), suggesting a role for an intracellular Ca\(^{2+}\)/H\(_{11001}\) signal. Therefore, we chose unopsonized zymosan as a potential stimulus because it evokes a sharp increase in intracellular Ca\(^{2+}\) and a robust PG synthesizing response in RPMs (34, 37–40). Based on the assumption that high levels of COX-2 expression should favor PG-G formation, we pretreated RPMs with LPS for 6 h to induce maximal COX-2 protein levels (12) and then challenged them with zymosan. We used 160 \(\mu\)g of zymosan/35-mm culture dish and a 2-h incubation period because these conditions were previously shown to maximize PG synthesis by RPM cultures of the size and cellular density used here (37).

LC-MS/MS analysis of the culture medium from LPS-pretreated RPMs that were incubated for 6 h in the presence of 100 ng/ml LPS in α-MEM/FCS. Cells were then washed and placed in serum-free medium in the absence (A) and presence (B) of zymosan (160 \(\mu\)g/dish). Following a 2-h incubation, the medium was removed and analyzed for PG-Gs by LC-MS/MS. The results show the chromatograms obtained by selected reaction monitoring of the following transitions: m/z 482 to 391 (6-keto-PGF\(_{1\alpha}\)-G), m/z 487 to 396 (6-keto-PGF\(_{1\alpha}\)-G-d\(_{5}\)), m/z 444 to 391 (PGE\(_{2}\)-G), and m/z 449/396 (PGE\(_{2}\)-G-d\(_{5}\)). Data are presented as relative abundance, with the normalization factors (NL) designated on each chromatogram.

However, the medium from zymosan-stimulated cells clearly contained detectable quantities of PGE\(_{2}\)-G and 6-keto-PGF\(_{1\alpha}\)-G (the stable hydrolysis product of PGI\(_{2}\)-G) (Fig. 1B). These results demonstrate that zymosan induced PG-G as well as PG synthesis. PG-Gs were not detected in RPM cell lysates, suggesting that, like PGs, PG-Gs are primarily secreted from the cell. No PG-EAs were detected in RPM culture medium or cells. However, this finding was not surprising because intracellular anandamide levels are so much lower than 2-AG levels that the quantities of PG-EAs likely to be produced would be below the detection limit of our assay.

To determine optimal conditions for PG-G formation, RPM cultures were preincubated for varying time periods with LPS (100 ng/ml) and then challenged for 2 h with zymosan. PG-G levels in the culture medium were quantified by LC-MS/MS using synthetic deuterated internal standards. As shown in Fig. 2A, maximal levels of PG-G formation were reached after 4–6 h of LPS pretreatment. The relative proportion of PGE\(_{2}\)-G also increased from 45 to 90% during 0–24 h of incubation with LPS (data not shown). The maximal synthesis of PG-G production correlated with maximal COX-2 protein expression as determined by immunoblot analysis (Fig. 2, B and C). The increase in the relative proportion of PGE\(_{2}\)-G correlated with the induction of microsomal PG synthase-1 by LPS (12). LPS concentrations from 20 to 500 ng/ml effected the same qualitative and quantitative results, indicating that RPMs respond to
very low LPS concentrations with COX-2 and microsomal PGE synthase-1 expression. When RPMs were pretreated for 6 h with 100 ng/ml LPS and then challenged with varying quantities of zymosan (20–240 μg/dish), maximal PG synthesis occurred with zymosan at ~80 μg/dish. Collectively, these results confirmed that maximal PG synthesis resulted from pretreatment of RPMs for 6 h with 100 ng/ml LPS, followed by stimulation with zymosan at 160 μg/dish. These conditions were used for further experiments.

RPMs not pretreated with LPS also produced significant quantities of PGs (0-h sample in Fig. 2). These cells contained levels of COX-2 protein that were much higher than those observed in RPMs that had not been exposed to LPS or zymosan (in which COX-2 was nearly undetectable). These results suggest that zymosan induced COX-2 expression, consistent with prior reports (41–45), and led to the hypothesis that zymosan-induced COX-2 may be responsible for the PG-G synthesis in these cells.

Time Course of PG-G Formation in Zymosan-stimulated RPMs—To better characterize PG-G formation by zymosan-stimulated RPMs, the time course of the process was monitored concomitantly with that of PG formation. RPMs were preincubated for 6 h in the absence (control RPMs) or presence (LPS-pretreated RPMs) of LPS and then exposed to zymosan for varying periods of time. The culture medium was analyzed for PG-G and PG formation, and the cells were analyzed for substrate levels. As shown in Fig. 3A, zymosan phagocytosis caused an increase in cell-associated total AG (2-AG plus 13-AG) that was detectable as early as 5 min and statistically significant (p < 0.05) at 20 min. Levels reached a maximum at 60 min and then remained reasonably constant through the remainder of the 120-min incubation. AG levels were higher in control cells than in LPS-pretreated cells. In some experiments, cell lysates were also analyzed for PG-Gs, but none were detected at any of the time points, confirming that PG-Gs are not retained intracellularly.

Both control and LPS-pretreated RPMs produced PG-Gs in response to zymosan; however, there was a marked delay in the response of control RPMs compared with LPS-pretreated cells (Fig. 3B). Thus, PG-G levels increased significantly (p < 0.05) as early as 10 min in LPS-pretreated RPMs, but not until 40 min in control cells. The long delay in the time course of PG-G synthesis in control RPMs was consistent with the hypothesis that zymosan-dependent COX-2 induction is required before PG-G synthesis can occur. However, the most rapid rate of PG-G formation in control RPMs occurred between 20 and 60 min after zymosan addition, and immunoblot analysis indicated that COX-2 expression remained quite low throughout this time period (Fig. 3E). These results were more consistent with the hypothesis that PG-G formation by control RPMs is COX-1-dependent.

Zymosan also induced a marked increase in cell-associated AA levels in both control and LPS-pretreated RPMs (Fig. 3C). The time course of the AA increase was similar to that of the AG increase, with significant (p < 0.05) increases first detected at 20 min. Levels in control RPMs were higher than those in LPS-pretreated RPMs. However, unlike the levels of AG, the levels of AA dropped somewhat after 60 min in both cell populations. The increase in free AA was accompanied by a strong PG synthetic response (Fig. 3D). Consistent with prior reports (34, 37), the primary PGs were PGE_2 and 6-keto-PGF_1α, and only these two PGs were quantified. Therefore, values of total PG formation refer to the sum of these two products. As was observed for PG-G formation, PG formation was delayed in control RPMs compared with LPS-pretreated RPMs. Thus, PG levels increased significantly (p < 0.05) as early as 5 min after zymosan addition in LPS-pretreated cells, but not until 20 min after zymosan addition in control cells. Despite these differences, both cell populations produced the same total amount of PGs by the end of the 2-h incubation.

The data in Fig. 3 suggest that PG-G and PG synthesis was essentially complete 2 h after zymosan addition to the cells. Thus, this time point was used to compare the quantities of substrates and products in control versus LPS-pretreated RPMs. The results indicate that LPS-pretreated RPMs produced 16 ± 6 pmol of PG-Gs/10^7 cells, which represented a small (1.3-fold) but significant increase over the quantity produced by control RPMs (12 ± 5 pmol/10^7 cells). LPS pretreatment also significantly increased the proportion of PGE_2-G relative to 6-keto-PGF_1α-G (Fig. 4A). Zymosan-stimulated RPMs produced much greater quantities of PGs compared with PG-Gs (Fig. 4B). LPS pretreatment had no effect on the total quantities of PGs synthesized by the cells (21,000 ± 6,000 and 21,000 ± 7,000 pmol/10^7 cells in control and LPS-pretreated cells, respectively), although it did increase the relative proportion of PGE_2 to 6-keto-PGF_1α (Fig. 4B). LPS pretreatment also significantly lowered the levels of AA and AG in zymosan-stimulated RPMs, with the effect being more notable for AA.
Effects of SC236 on PG-G and PG Synthesis in Response to Zymosan—To better elucidate the relative roles of COX-1 versus COX-2 in zymosan-stimulated RPMs, control and LPS-pretreated cells were preincubated for 30 min with the selective COX-2 inhibitor, SC236, and then challenged with zymosan for 2 h in the ongoing presence of the inhibitor. The reported SC236 IC_{50} values are 5–10 nM for COX-2 and 17 nM for COX-1 using cell-free enzyme preparations (46, 47). For these experiments, we used SC236 concentrations of 50, 100, and 200 nM to delineate an inhibitor concentration that completely blocked COX-2 but had a minimal effect on COX-1 activity in the intact RPMs. As shown in Fig. 5A, the lowest concentration of SC236 significantly reduced PG-G synthesis in LPS-pretreated RPMs (49% inhibition), but not in control RPMs (26% inhibition). SC236 eliminated the increment in PG-G synthesis obtained from LPS pretreatment, so in the presence of all three concentrations of the inhibitor, there was no significant difference in zymosan-dependent PG-G formation in control versus LPS-pretreated cells (Fig. 5C).

SC236 had little effect on the levels of cell-associated AA and total AG present 2 h after zymosan addition. The only significant effect was observed in the case of AA levels in LPS-pretreated cells, for which a concentration-dependent increase was observed (1.4-fold with 50 nM SC236 to 2.3-fold with 200 nM SC236) (data not shown).

Source of 2-AG for PG-G Biosynthesis—In prior studies of PG-G synthesis in ionomycin-stimulated RAW264.7 cells, the putative diacylglycerol lipase inhibitor, RHC80267, caused a significant reduction in PG-G formation (24). This suggested that the 2-AG used for PG-G formation in those cells most likely came from diacylglycerol that was released through the action of a phospholipase C or a phospholipase D plus phosphatidic acid phosphatase. We preincubated LPS-pretreated cells with RHC80267 (40–120 μM) and then exposed them to
zymosan for 2 h in the ongoing presence of the inhibitor. The results indicate that RHC80267 lowered PG-G formation by as much as 79% (Fig. 5C). However, the inhibitor also reduced PG formation by 62%. Analysis of total cellular AG levels showed that, at the lowest concentration of RHC80267, AG levels were elevated in the cells (Fig. 5D). As inhibitor concentrations increased, AG levels gradually decreased back to control (no inhibitor) values. The inability of RHC80267 to inhibit AG production and its inhibition of PG as well as PG-G formation suggest that it has nonspecific effects (including possibly COX inhibition) that minimize its utility as a probe for diacylglycerol lipase. Thus, the pathway of 2-AG formation for PG-G biosynthesis remains to be elucidated.

PG-G and PG Formations from Exogenous Substrates by RPM Cultures—The finding that LPS pretreatment had only a very modest effect on total PG-G synthesis and no effect on total PG synthesis during zymosan phagocytosis was unexpected because immunoblot analysis indicated strong induction of COX-2 in LPS-pretreated cells. Possible explanations included a restricted availability of substrate to the COX-2 enzyme or inactivation of the enzyme. To evaluate these hypotheses, the effect of LPS pretreatment on the ability of RPMs to metabolize exogenous substrates (2-AG and AA) was evaluated. In these experiments, cells were preincubated for 6 h in the presence (LPS-pretreated RPMs) or absence (control RPMs) of 100 ng/ml LPS, washed, and then overlaid with serum-free medium in the presence or absence of 50 nM SC236. After 30 min, the medium was removed and replaced with fresh serum-free medium containing either AA or 2-AG plus inhibitor as indicated. Because prior studies have shown that AA concentrations >1 μM are toxic to RPMs and that metabolism of exogenous AA by RPM cultures under serum-free conditions is complete within 30 min (48), the cultures were incubated with 1 μM 2-AG or AA for 30 min. The medium was then collected and analyzed for PG and PG-G metabolites by LC-MS/MS.

RPMs produced both PGE_2 and 6-keto-PGF_1α from exogenous AA, and LPS pretreatment resulted in a 1.8-fold increase in total PGs synthesized (Fig. 6A). The LPS-induced increment in PG formation was eliminated in the presence of 50 nM SC236, verifying that it was COX-2-dependent. PG-G formation was undetectable when AA was provided as substrate in control RPM populations. Trace quantities of PG-Gs were sometimes observed in LPS-pretreated cells, but these were too low to be reliably quantified.

The 1.8-fold increase in total PG formation from exogenous AA resulting from LPS pretreatment was in sharp contrast to the results obtained when RPMs utilized endogenous substrate in response to zymosan (no effect of LPS). One concern in interpreting these data is that the total quantities of PGs formed from 1 μM exogenous AA were significantly lower than those produced during zymosan phagocytosis, so a valid comparison cannot be made. However, when 20 μM exogenous AA was provided to the cells, the quantities of PGs produced (13,000 ± 1,000 pmol/10⁷ cells in control RPMs and 24,000 ± 3,000 pmol/10⁷ cells in LPS-pretreated RPMs) were very similar to those produced during zymosan phagocytosis and showed the same 1.8-fold increase as a result of LPS pretreatment that was observed at the lower AA concentration. These results further confirmed that the increase in PG synthesizing capacity from exogenous substrate observed in the LPS-pretreated RPMs was most likely due to the de novo expression of COX-2 in those cells rather than to an effect of low substrate concentration.

When RPMs were incubated with exogenous 2-AG, both PGs and PG-Gs were detected in the culture medium, with PGs being the predominant products (94% of the total products in control RPMs and 92% of the total products in LPS-pretreated cells) (Fig. 6, B and C). Total product formation from 2-AG was lower than that from AA. Thus, control and LPS-pretreated RPMs utilizing 2-AG produced 21 and 38% as much COX products, respectively, as when AA was provided as substrate. LPS pretreatment effected a marked increase in the utilization of 2-AG for both PG (3.2-fold increase) and PG-G (4.8-fold increase) formation, and in both cases, these increases were eliminated in the presence of SC236.

At the end of the 30-min incubation of RPMs treated with AA, the cell-associated levels of the fatty acid remained elevated (670 ± 120 pmol/10⁷ cells versus resting values of 130 ± 70 pmol/10⁷ cells in control RPMs and 610 ± 250 pmol/10⁷ cells versus resting values of 120 ± 50 pmol/10⁷ cells in LPS-pretreated RPMs). Addition of AA had no effect on cellular AG levels. When RPMs were treated with 2-AG, cell-associated AG levels remained markedly elevated at the end of the 30-min incubation (1,200 ± 400 pmol/10⁷ cells versus resting levels of 38 ± 15 pmol/10⁷ cells in control RPMs and 1,000 ± 300 pmol/10⁷ cells versus resting levels of 24 ± 8 pmol/10⁷ cells in LPS-pretreated RPMs). Addition of 2-AG also resulted in elevations in cell-assos-
associated AA levels (260 ± 150 pmol/10⁷ cells in control RPMs and 130 ± 70 pmol/10⁷ cells in LPS-pretreated RPMs); however, these differences were not statistically significant.

As in the case of zymosan-induced PG-G formation, the synthesis of PG-Gs from exogenous 2-AG in control RPMs was unexpected. Furthermore, the synthesis of PG-G from exogenous substrate in control RPMs could not be attributed to zymosan-induced COX-2 expression. Immunoblot analysis demonstrated the presence of low levels of COX-2 protein in these cells. However, the finding that LPS pretreatment resulted in a 14-fold increase in COX-2 signal intensity (data not shown) but only a 4.8-fold increase in PG-G formation was inconsistent with the conclusion that all of the PG-G formation was COX-2-dependent in control RPMs. The finding that 50 nM SC236 eliminated the 4.8-fold increment in PG-G synthesis observed with LPS pretreatment suggests that PG-G synthesis was primarily COX-2-dependent in LPS-pretreated cells. Furthermore, the 43% inhibition of PG-G synthesis by SC236 in control RPMs suggests that at least a portion of PG-G synthesis in those cells was attributable to low level COX-2 expression. However, it is also notable that even 200 nM SC236 did not completely abolish PG-G formation in either cell population (data not shown), indicating that some PG-G synthesis was COX-1-dependent, as was observed for zymosan-dependent PG-G formation.

Biosynthetic Pathway for PGs Synthesized from Exogenous 2-AG by RPM Cultures—The finding that RPMs synthesized PGs from exogenous 2-AG led to the question of the biosynthetic pathway used to generate these products. PGs could arise from the hydrolysis of 2-AG, followed by the COX-dependent oxygenation of the resulting free AA. Alternatively, 2-AG could be converted to PG-Gs, the hydrolysis of which would yield free PGs. The fact that PG synthesis from 2-AG was augmented more (3.2-fold) by LPS pretreatment than was PG synthesis from AA (1.8-fold) suggests that PG synthesis from 2-AG was more highly COX-2-dependent than was PG synthesis from AA. This favors the hypothesis that 2-AG is directly converted to PG-Gs, followed by hydrolysis, a conclusion that would be of particular interest because it suggests that PG-G synthesis in the zymosan-treated RPMs might have been underestimated due to metabolism. To test this hypothesis, RPM cultures were preincubated in the presence or absence of LPS and then exposed to PGE₂-G at initial concentrations of 50 nM and 1 μM for periods of up to 2 h regardless of whether or not the cells had been LPS-pretreated or whether or not zymosan was included (data not shown). One possible explanation for this failure of RPMs to hydrolyze PGE₂-G is that the required enzymes are intracellular and therefore not accessible to extracellular substrate. Therefore, PGE₂-G at 50 nM and 1 μM was also incubated with lysates of RPMs (0.10 mg/ml protein). However, again, no significant hydrolysis occurred. These data indicate that PG-G synthesis followed by hydrolysis cannot readily account for the quantities of PGs produced from exogenous 2-AG by RPMs. They also indicate that PG-Gs are stable in RPM cultures.

**FIG. 5.** Effect of SC236 and RHC80267 on PG and PG-G formation by RPMs in response to zymosan. RPMs in 35-mm dishes were incubated for 6 h in the absence (control (CON)) and presence (LPS) of 100 nM LPS in α-MEM/FCS for SC236 experiments. Alternatively, all dishes were incubated for 6 h with LPS for RHC80267 experiments. Cells were then washed and overlaid with serum-free α-MEM containing the indicated concentrations of SC236 (A and B) and RHC80267 (C and D). After a 30-min incubation, zymosan (160 μg) was added to each dish, and the cells were incubated for 2 h. The medium was removed for LC-MS/MS analysis of PG and PG-G content (A–C), and the cells were scraped into methanol for LC-MS/MS analysis of AA and total AG (D). The results are the mean ± S.D. from three experiments in which duplicate determinations were made. * the results were significantly different (p < 0.05) from LPS-pretreated RPMs in the absence of SC236 or RHC80267.
experiments (results are the mean ± S.D. from three separate experiments or four experiments in which duplicate determinations were made. *, the results were significantly different (p < 0.05) from control RPMs in the absence of SC236; †, the results were significantly different (p < 0.05) from LPS-pretreated RPMs in the absence of SC236.

culture medium, so the quantities detected are a reasonable reflection of total synthesis.

To test the hypothesis that PG synthesis from 2-AG requires 2-AG hydrolysis, followed by oxygenation of free AA, RPM lysates (0.062 ± 0.012 mg/ml protein for control lysates and 0.054 ± 0.017 mg/ml for LPS-pretreated lysates) were incubated with 1 μM 2-AG at 37 °C for up to 1 h, and the reaction mixtures were monitored for 2-AG, AA, PG, and PG-G levels over time. The results indicate that 2-AG rapidly disappeared when incubated with lysates from RPMs (Fig. 7, A and B). The kinetics were similar whether or not the cells had been pretreated with LPS. At the end of the 1-h incubation, ~4% of the 2-AG remained, and ~20% of it could be accounted for as free AA. PGs and PG-Gs were produced from 2-AG in the presence of both lyase samples; however, the quantities of both product classes were higher for lysates from LPS-pretreated cells (2.1-fold for PGs and 2.8-fold for PG-Gs). Total COX products accounted for only ~2.2% and 4.5% of the added 2-AG for lysates from control and LPS-pretreated RPMs, respectively. The quantities of both product classes were much lower than the quantities of either 2-AG or AA throughout the incubation period with either lysate sample, and PG-G composed only 12 and 15% of the total products formed by control and LPS-pretreated lysates, respectively. The data clearly show that RPMs contain the enzymes necessary to efficiently hydrolyze 2-AG to AA and support the conclusion that 2-AG hydrolysis precedes oxygenation in the synthesis of PGs from 2-AG. The data also demonstrate that 2-AG and/or its hydrolysis product are subject to additional metabolic fates in RPM lysates. These may include incorporation into more complex lipids and metabolism by lipoxygenases.

**Effect of LPS Pretreatment on PG and PG-G Synthetic Rates in Zymosan-stimulated RPMs**—The data in Fig. 6 demonstrate that LPS pretreatment clearly increased both PG and PG-G synthetic capacity in RPMs. However, when cells utilized endogenous substrates, LPS pretreatment affected no and only a 1.3-fold increase in PG and PG-G synthesis, respectively. This is particularly surprising in view of the much more rapid initial rates of PG-G and PG formation observed in LPS-pretreated compared with control RPMs, as shown in Fig. 8. Using the data from Fig. 3, the average rates of PG-G and PG synthesis were calculated for each interval between time points by dividing the quantity of product produced during that interval by the length of the interval. The results indicate that, after 20 min of zymosan treatment, the rates of PG-G and PG synthesis in LPS-pretreated RPMs were 5.1- and 2.9-fold greater than in control RPMs, respectively. However, product synthesis rates peaked at 20 min in LPS-pretreated cells, but not until 40 min in control RPMs. Thus, the rates of PG-G and PG synthesis were actually lower in LPS-pretreated cells than in control cells after 60 and 40 min, respectively. As Fig. 8 also shows, the AG and AA levels remained elevated until at least 60 min in both cell populations, so the decreased rates of product formation did not correlate with gross decreases in substrate availability. These results indicate that LPS pretreatment significantly increased PG-G and PG synthesizing capacity in RPMs, but that this was largely eradicated by a much briefer period of maximal product synthesis.

**DISCUSSION**

The finding that 2-AG is a selective substrate for COX-2 that leads to the formation of PG-Gs has generated considerable excitement regarding the potential importance of these compounds as a new class of lipid mediators. The recent report of Ca2+ mobilization induced by PGE2-G in RAW264.7 cells suggests the possibility that there is a specific receptor for this PG-G that is distinct from the receptors for its corresponding free acid, PGE2 (28). However, the conclusion that PG-Gs are of biological importance requires the demonstration that a primary cell population produces these compounds from endogenous substrates in response to a physiologically relevant stimulus and that the compounds are released into the extracellular environment at concentrations adequate to elicit the response. Because RPMs contain unusually high concentrations of AA in their phospholipid pool, express high levels of constitutive COX-1 and inducible COX-2, and demonstrate robust PG synthesis in response to a variety of stimuli, they were chosen as a model system to explore PG-G synthesis (37, 49–56).
The results described above demonstrate that RPMs do produce PGE$_2$-G and PGI$_2$-G in response to the phagocytosis of zymosan. PG-Gs were detected only in the culture medium, and the fact that RPMs lack PG-G hydrolytic activity ensures that the compounds can survive to mediate extracellular signaling. It should be noted, however, that because of the difference in volume (~3 orders of magnitude) between the medium and cells in RPM cultures, a 100-fold concentration gradient would

**Fig. 7.** Hydrolysis and oxygenation of 2-AG by RPM lysates. RPMs in 60-mm dishes were incubated for 6 h in the absence (A and C) and presence (B and D) of 100 ng/ml LPS. The cells were washed and scraped into 100 ml Tris-HCl (pH 7.5) containing 1 mM EDTA. Following sonication, the lysates were warmed to 37 °C, and 2-AG was added to a final concentration of 1 μM. At the indicated times, 0.5-ml aliquots were removed and added to the appropriate internal standard solutions on ice for the LC-MS/MS determination of total AG and AA (A and B) and PG and PG-Gs (C and D). The results are the mean ± range from two separate experiments in which duplicate determinations were made. C and D are plotted on the same scale for ease of comparison of control versus LPS-pretreated lysates.

**Fig. 8.** Rates of PG-G and PG synthesis by RPMs during zymosan phagocytosis. The data from Fig. 3 were used to calculate the average rate of PG-G (A and B) and PG (C and D) synthesis in control (A and C) and LPS-pretreated (B and D) RPMs during zymosan phagocytosis. Average rates were obtained by calculating the quantity of product formed during each interval between time points and dividing by the length of time of that interval. Also plotted are the levels of AG (A and B) and total AA (C and D) present in the cells at each time point.
be required to reliably detect PG-Gs in the cells. Thus, our data do not completely rule out the intracellular accumulation of some PG-Gs, which could act as yet unknown nuclear or cytoplasmic receptors.

PG-G formation occurs concomitantly with PG formation, but quantities of PG-Gs produced are substantially lower than those of PGs. The average concentration of PG_E2-G achieved during zymosan phagocytosis in LPS-pretreated RPMs was 0.4 nM, a value well above the EC_{50} of 1 pM reported for PG_E2-G-dependent calcium mobilization in RAW264.7 cells (28). Because the EC_{50} values for PGs are generally in the 100 pM to 10 nM range (57), it is possible that PG-Gs are substantially more potent than PGs and that they can be biologically effective at much lower concentrations compared with PGs. It should be noted, however, that product concentrations achieved in these in vitro studies are a function of the volume of the culture medium and may not reflect concentrations achieved in vivo. Furthermore, effective in vivo concentrations will be the influenced by the presence of metabolizing enzymes in the surrounding tissue. Clearly, a complete assessment of the biological relevance of PG-G synthesis by RPMs must await further characterization of the putative PG_E2-G receptor and a more extensive characterization of PG-G formation in intact organisms.

Relative Contributions of COX-1 and COX-2 to PG-G and PG Synthesis—Based on prior studies with purified enzymes, we anticipated that control RPMs, which express high quantities of COX-1 but very little COX-2, would produce little to no PG-Gs from endogenous or exogenous substrates. Consequently, the synthesis of significant quantities of PG-Gs by zymosan-stimulated control RPMs was unexpected. Zymosan induced COX-2 expression in these cells, but the appearance of the enzyme did not correlate with the time course of PG-G formation. Furthermore, the selective COX-2 inhibitor, SC236, failed to totally suppress PG-G synthesis in these cells. Based on findings with SC236, we conclude that 50–75% of the PG-G synthesis by control RPMs is COX-1-dependent.

Experiments using purified enzyme in vitro have demonstrated that COX-1 has low activity with 2-AG (24), so a certain amount of COX-1-dependent PG-G synthesis in control RPMs was understandable. However, the fact that LPS pretreatment of RPMs resulted in only a 1.3-fold increase in zymosan-stimulated PG-G formation was unexpected. Equally unexpected was the failure of LPS pretreatment to cause any increase in PG synthesis in cells responding to zymosan. SC236 had little effect on zymosan-dependent PG synthesis in LPS-pretreated RPMs, and it eliminated the small increase in PG-G synthesis observed in these cells compared with control cells. These results suggest that the COX-2 induced by LPS pretreatment contributed almost nothing to total zymosan-dependent PG synthesis and was responsible for only the 30% increase observed in PG-G synthesis.

The apparent lack of involvement of COX-2 in the response of LPS-pretreated RPMs to zymosan suggested the possibility that the COX-2 was sequestered from the substrates or that the enzyme was inactive. To test the latter hypothesis, we compared the ability of control and LPS-pretreated RPMs to synthesize PGs and PG-Gs from exogenous AA and 2-AG. The results indicate that LPS pretreatment increased PG synthetic capacity from AA by 1.8-fold and both PG and PG-G synthetic capacity from 2-AG by 3.2- and 4.8-fold, respectively. Furthermore, studies using SC236 indicated that these increases could be attributed to the newly expressed COX-2. These results support the conclusion that the COX-2 protein expressed in response to LPS pretreatment was active.

The conclusion that active COX-2 was present in LPS-pretreated RPMs is further supported by our finding that the rates of zymosan-dependent PG and PG-G synthesis were initially much higher (2.9- and 5.1-fold, respectively) in LPS-pretreated RPMs than in control RPMs. However, the rate of zymosan-dependent product synthesis decreased very rapidly in LPS-pretreated cells, falling below the rate in control cells, so the total quantities of PGs and PG-Gs produced do not reflect the augmentation in product synthetic ability that appears to be initially present in the cells. The rapid decrease in product synthetic rates cannot readily be accounted for by a decrease in the availability of substrates, although we cannot rule out the possibility that a selected pool of substrates was exhausted. It is perhaps more likely either that COX-2 activity was down-regulated via a physiological mechanism or that the enzyme was inactivated by exposure to high concentrations of hydroperoxide (4, 58–62). Such loss of enzyme activity appears to selectively involve COX-2 because, as noted above, the experiments using SC236 suggest that its contribution to zymosan-dependent product synthesis was low.

Relative Quantities of PG-Gs Versus PGs—Studies using purified COX-2 in vitro have indicated that 2-AG is used as efficiently as AA as a substrate by this enzyme (24). Therefore, the large difference in the quantities of PG-Gs versus PGs produced by zymosan-stimulated RPMs was somewhat unexpected. Multiple factors contribute to these differences. First, the levels of AA are ~10-fold higher than the levels of AG during zymosan phagocytosis. Thus, a minimal 10-fold difference in relative product formation would be expected on this basis alone. Second, as described above, a significant proportion of the PG-Gs produced in response to zymosan are synthesized by COX-1, an enzyme that utilizes AA with much greater efficiency than 2-AG. Finally, experiments using exogenous substrates demonstrated that, even when pure 2-AG is provided as substrate to LPS-pretreated RPMs, and COX-2-dependent product formation occurs, PG formation is favored over PG-G formation by ~10-fold because of hydrolysis of 2-AG, followed by oxygenation of the resultant AA. Therefore, it is clear that the relative utilization of substrate in the intact cell is different from that of purified enzyme in vitro.

Our results clearly demonstrate that murine RPMs produce PG-Gs in addition to PGs in response to a zymosan stimulus and that the concentrations of PG-Gs accumulating in the medium of these cells reach levels known to trigger Ca^{2+} mobilization in RAW264.7 cell line. However, our results also demonstrate that PG synthesis is strongly favored over PG-G synthesis and that a significant proportion of PG-Gs produced, even in LPS-pretreated cells, is due to COX-1 rather than COX-2. A number of factors favor PG over PG-G formation in zymosan-stimulated RPMs. First, zymosan strongly activates cytosolic phospholipase A_2, resulting in much higher concentrations of AA compared with 2-AG. Second, COX-1 is strongly expressed in these cells and is in competition with COX-2 for the substrates. Finally, the rapid decrease in the rate of PG-G and PG formation in LPS-pretreated cells suggests down-regulation or inactivation of COX-2, thus limiting its role in both PG-G and PG formation. From these data we can conclude that the quantities of PG-Gs produced by zymosan-stimulated RPMs do not represent the optimal quantities of products that might be formed under all conditions. Cells that express lower quantities of COX-1 and stimuli that activate phospholipase C or D without cytosolic phospholipase A_2 in the context of high levels of COX-2 expression will be particularly interesting to study.

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