Group V Phospholipase A2-dependent Induction of Cyclooxygenase-2 in Macrophages*

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When exposed for prolonged periods of time (up to 20 h) to bacterial lipopolysaccharide (LPS) murine P388D1 macrophages exhibit a delayed prostaglandin biosynthetic response that is entirely mediated by cyclooxygenase-2 (COX-2). Both the constitutive Group IV cytosolic phospholipase A2 (cPLA2) and the inducible Group V secretory phospholipase A2 (sPLA2) are involved in the cyclooxygenase-2-dependent generation of prostaglandins in response to LPS. Using the selective sPLA2 inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxo)propane sulfonic acid (LY311727) and an antisense oligonucleotide specific for Group V sPLA2, we found that induction of COX-2 expression is strikingly dependent on Group V sPLA2, which was further confirmed by experiments in which exogenous Group V sPLA2 was added to the cells. Exogenous Group V sPLA2 was able to induce significant arachidonic acid mobilization on its own and to induce expression of the COX-2. None of these effects was observed if inactive Group V sPLA2 was utilized, implying that enzyme activity is crucial for these effects to take place. Therefore, not only delayed prostaglandin production but also COX-2 gene induction are dependent on a catalytically active Group V sPLA2. COX-2 expression was also found to be blunted by the Group IV cPLA2 inhibitor methyl arachidonyl fluorophosphonate, which we have previously found to block Group V sPLA2 induction as well. Collectively, the results support a model whereby Group IV cPLA2 activation regulates the expression of Group V sPLA2, which in turn is responsible for delayed prostaglandin production by regulating COX-2 expression.

Phospholipase A2 (PLA2) plays a key role in numerous cellular processes by regulating the release of arachidonic acid (AA) from membrane phospholipids. In turn, free AA can be converted into the prostaglandin (PG) precursor PGH2 by the action of cyclooxygenases (COX). These two reactions constitute the regulatory checkpoints of the pathway leading to PG biosynthesis in mammalian cells. A considerable body of evidence suggests that specific coupling between certain PLA2 and COX forms accounts for the differential regulation of the immediate and delayed responses (1–11). We have shown that the immediate platelet-activating factor-receptor-mediated phase of PGE2 production in LPS-primed P388D1 macrophages involves Group V sPLA2 coupling to COX-2 (3). More recently, we have discovered that the exact same coupling of Group V sPLA2 to COX-2 also regulates the delayed PGE2 response of P388D1 macrophages to LPS alone (4). Under the latter conditions, expression of both Group V sPLA2 and COX-2 was markedly induced and correlated with ongoing AA release and PG biosynthesis, respectively (4), suggesting that the AA produced by Group V sPLA2 was used by COX-2 to produce PGE2. Importantly, sPLA2 expression could be abolished by pretreating the cells with the cPLA2 inhibitor methyl arachidonyl fluorophosphonate, implying that a functionally active cPLA2 is required for the delayed PGE2 response to occur (4).

In the current study we extend our previous observations on the delayed phase of PGE2 in P388D1 macrophages to further investigate the regulatory relationships between the three effectors of the response (i.e. cPLA2, sPLA2, and COX-2). We now demonstrate tight coupling between sPLA2 and COX-2 for the delayed phase of PGE2 generation by showing that COX-2 induction by LPS is dependent upon a catalytically active Group V sPLA2.

EXPERIMENTAL PROCEDURES

Materials—Isocëve’s modified Dulbecco’s medium (endotoxin < 0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from HyClone Laboratories (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). (5,6,8,9,11,12,14,15-[3H]arachidonic acid (specific activity, 100 Ci/mmol) was from NEN Life Science Products and 1-palmitoyl-2-[14C]palmitoyl-sn-glycero-3-phosphocholine (specific activity, 54 mCi/mmol) was from Amersham Pharmacia Biotech. LPS (Escherichia coli 0111:B4) was from Sigma. Methyl arachidonyl fluorophosphonate (MAFP), p-bromophenacyl bromide, and mannoalide were from Biofilm (Plymouth Meeting, PA). Antibodies against murine COX isoforms were generously provided by Dr. W. L. Smith (Dept. of Biochemistry, Michigan State University, East Lansing, MI). The antibody against Group IV cPLA2 was generously provided by Dr. Ruth Kramer (Lilly Research Laboratories, Indianapolis, IN). The sPLA2 inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxo)propane sulfonic acid (LY311727) was generously provided by Dr. Edward Mihelich (Lilly). cDNA probes for murine COX-2 were from Cayman (Ann Arbor, MI). cDNA probes for murine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were from Ambion (Austin, TX). Pichia pastoris strains and vectors were obtained from Invitrogen (Carlsbad, CA).

Cell Culture and Labeling Conditions—P388D1 cells (MAB clone) (4) were maintained at 37 °C in a humidified atmosphere at 90% air and 5% CO2.

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1 The abbreviations used are: PLA2, phospholipase A2; cPLA2, cytosolic Group IV sPLA2, secretory Group V sPLA2, cyclooxygenase; AA, arachidonic acid; PG, prostaglandin; LPS, lipopolysaccharide; MAFP, methyl arachidonyl fluorophosphonate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxo)propane sulfonic acid.

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10% CO₂ in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and nonessential amino acids. P388D₁ cells were plated at 10⁵/well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove’s modified Dulbecco’s medium. Radiolabeling of the P388D₁ cells with [³H]AA was achieved by including 0.5 µCi/ml of [³H]AA during the overnight adherence period (20 h). Labeled AA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 1 mg/ml albumin.

Measurement of Extracellular [³H]AA Release—The cells were placed in serum-free medium for 30 min before the addition of LPS or exogenous sPLA₂ for different periods of time in the presence of 0.5 mM fetal bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Antisense Inhibition Studies in P388D₁ Cells—Transient transfection of P388D₁ cells with antisense oligonucleotide ASGV-2 or its sense counterpart, SGV-2, plus LipofectAMINE was carried out as described elsewhere (4, 12). Briefly, P388D₁ cells were transfected with oligonucleotide (125 nM) in the presence of 5 µg/ml LipofectAMINE (Life Technologies) under serum-free conditions for 8 h before treating the cells with or without 100 ng/ml LPS for 10 h after transfection.

Group V sPLA₂ Expression in P. pastoris and Purification—A pCH10 vector containing the gene encoding human Group V sPLA₂ (13) was a generous gift of Dr. A. Tischfeld (Dept. of Genetics, Rutgers University, Piscataway, NJ). The gene was transferred from the pCH10 vector to the P. pastoris expression vector pPIC9K. Because of difficulties in purifying the expressed enzyme, we opted to incorporate an N-terminal HisTag that could be removed by enterokinase. The construct was then transferred into the pPIC9K Pichia expression vector using the pPIC9 vector as a shuttle vector (14). The protease-deficient P. pastoris strain SMD1168 was transformed using 10 µg of the pGIII fragment of pPIC9K/G5HST that had been linearized with BgII using a spheroplasting procedure described previously (14). Briefly, colonies were picked and used to inoculate 10 ml of glycerol-containing BMGY medium and allowed to grow. For induction, cells were switched to BMM medium containing 1% methanol as a carbon source. After 2 days, the crude medium was assayed for PLA₂ activity as described below. The colony displaying the highest level of PLA₂ production was chosen for high level expression using a 5.0 l BioFlow 3000 fermentor (New Brunswick Scientific, Edison, NJ).

Following fermentation, the cells were removed by centrifugation at 4,500 × g for 10 min, and crude medium was stored at −20 °C. For purification, the medium was thawed and centrifuged again at 16,000 × g for 20 min to discard any remaining cells and precipitated proteins.

Inhibition of Endogenous Group V sPLA₂ Blocks COX-2 Expression in Activated P388D₁ Macrophages—We have recently shown that the selective sPLA₂ inhibitor LY311727 (18) is able to suppress long-term PGE₂ production in LPS-treated P388D₁ macrophages (4). We have taken these results as evidence that a catalytically active Group V sPLA₂ is needed for the cells to make PGE₂ at long stimulation times with LPS (4). We have found as well that the enzyme responsible for PGE₂ production in activated P388D₁ macrophages is exclusively COX-2. Albeit present in the cells, COX-1 is spared from the process (4). Thus a tight coupling between Group V sPLA₂ and COX-2 appears to exist for the regulation of PGE₂ production in activated P388D₁ macrophages. To further explore this coupling, we studied the effect of LY311727 on COX-2 gene expression. Unexpectedly, LY311727 was found to suppress COX-2 expression at both the mRNA (Fig. 1A) and the protein (Fig. 1B) levels.

Another method of inhibiting endogenous Group V sPLA₂ is to use antisense oligonucleotide technology (4, 12). An antisense oligonucleotide specific for Group V sPLA₂ (ASGV-2), which is identical to the one we have previously employed (4, 12), strongly inhibited COX-2 protein expression in LPS-treated cells, whereas the sense control (SGV-2) had no effect (Fig. 1C).

The above results indicate that Group V sPLA₂ is required for COX-2 expression in activated P388D₁ macrophages.

Effects of Exogenous Group V sPLA₂ on P388D₁ Cell Responses—We have recently succeeded in expressing Group V sPLA₂ in the P. pastoris yeast expression system. In this system, the enzyme is secreted to the extracellular medium in its catalytically active form. We have purified the enzyme from the supernatants to apparent homogeneity (see “Experimental Procedures”) and have studied its effect when added to the P388D₁ macrophages.

Fig. 1. Effect of inhibition of endogenous Group V sPLA₂ on COX-2 expression. A, effect of LY311727 on COX-2 mRNA levels as determined by Northern blot. Total mRNA from cells incubated with or without LPS (100 ng/ml, 18 h) and with or without LY311727 (50 µM), as indicated, was isolated and analyzed by Northern blot with probes specific for COX-2 or G3PDH. Ctrl, control. B, effect of LY311727 on COX-2 protein levels as determined by immunoblot. The effect of LY311727 (25 or 50 µM) on LPS-induced COX-2 protein is shown (100 ng/ml LPS, 18 h). The inhibitor was present throughout the entire 18-h incubation period. C, effect of a specific antisense oligonucleotide (ASGV-2) or its sense control (SGV-2) on COX-2 protein induction by LPS (100 ng/ml, 18 h).
Figure 2. Effect of exogenous Group V sPLA₂ induces AA release. A, synthetic (sGV) or recombinant (rGV) Group V sPLA₂ (200 ng/ml) was added to the cells in the absence (open bars) or presence (closed bars) of LY311727 (25 μM) for 1 h. Afterward, supernatants were assayed for [3H]AA release. Control denotes incubations in the absence of added enzyme.

macrophage cells. Within 1 h of addition to the cells, exogenous Group V sPLA₂ was already able to induce substantial AA release by itself (Fig. 2A). This finding is particularly interesting in view of the fact that exogenous Group IIA fails to elicit any AA release under similar experimental conditions (3). If the enzyme was preincubated with LY311727 before adding it to the cells, no AA release was observed, implying that the AA release response observed requires a catalytically active enzyme (Fig. 2).

Recently, we have succeeded in chemically synthesizing Group V sPLA₂ utilizing the same procedure previously used for Group IIA sPLA₂ (15). Under our assay conditions, the synthetic enzyme displayed the same specific activity as the recombinant enzyme obtained from P. pastoris. When this synthetic Group V sPLA₂ was added to the P388D₁ macrophages, essentially the same results as found with the recombinant enzyme were observed, i.e. the synthetic enzyme itself induced an AA release response that was inhibited by LY311727 (Fig. 2). These results show that the synthetic enzyme is indistinguishable from the recombinant one in terms of biological activity, just as synthetic Group IIA sPLA₂ was found to be biologically indistinguishable from its recombinant counterpart (15). Therefore we have used both the recombinant and synthetic enzymes for the experiments reported in this study.

When measurements were conducted over longer incubation times (up to 20 h), exogenous Group V was found to increase the AA release well above unstimulated controls at every time point tested (not shown). Interestingly, when added along with LPS, exogenous Group V sPLA₂ increased the AA release in an additive manner (not shown), which provides additional support to our previous proposal that Group V sPLA₂ levels determine long-term AA release in the LPS-treated macrophages (4). Prolonged incubation with exogenous Group V sPLA₂ did not affect cell viability, as judged by the trypan blue assay.

Likewise, exogenous Group V sPLA₂ was found to induce the expression of COX-2 at long incubation times at both the mRNA (Fig. 3A) and the protein (Fig. 3B) level. If the enzyme was preincubated with LY311727 before adding it to the cells, no effects were observed (Fig. 3, A and B). These effects were also not observed if the cells were incubated with inactive sPLA₂, which was prepared by treating the enzyme with the irreversible inhibitors p-bromophenacyl bromide (19) and mannoamide (19) (Fig. 3C). In vitro activity assays had demonstrated that under the conditions employed, both p-bromophenacyl bromide and mannoamide totally inactivated Group V sPLA₂ activity (not shown). The inactivated enzyme failed to induce AA release from the P388D₁ cells (not shown). Thus, these data indicate that a functionally active sPLA₂ is required not only to elicit AA release from the cells but also to induce the expression of the COX-2 gene.

cPLA₂ Inhibition Blocks COX-2 Expression—In a previous report we demonstrated that induction of Group V sPLA₂ was dependent upon a functionally active Group IV sPLA₂ (4). Because we have shown above that a functionally active Group V sPLA₂ is needed for expression of COX-2, it would be logical to anticipate that inhibition of the cPLA₂ also leads to a blockade in the induction of the COX-2 gene. Utilizing the conditions described previously (4), we confirmed that inhibition of cPLA₂ by methyl arachidonyl fluorophosphonate completely blocked COX-2 expression in the LPS-treated cells (Fig. 4). This inhibition could not be reversed by supplementing the incubation medium with exogenous AA (up to 100 μM), suggesting that either free AA is not responsible for COX-2 induction or COX-2 induction may depend on a specific eicosanoid whose production is not adequately mimicked by exposing the cells to exogenous AA.

DISCUSSION

We have recently identified in P388D₁ macrophages a pathway for delayed prostaglandin synthesis in response to LPS that involves the participation of three major effectors, namely Group IV cPLA₂, Group V sPLA₂, and COX-2 (4). Because of the existence of cross-talk between cPLA₂ and sPLA₂ (2, 3), the exact contribution of each of these enzymes to the bulk of AA release that is metabolized to prostaglandins during the delayed phase has not been easy to quantify.

However, several lines of evidence based on inhibition of the response by selective chemical inhibitors or antisense oligonucleotide technology suggest that the cPLA₂ behaves primarily as an initiator of the response, whereas the sPLA₂ plays an augmentative role by providing the bulk of the free fatty acid
used for prostaglandin production by COX-2. The strong correlation between induction of the Group V sPLA2 gene and the time courses of both AA release and PG production provides further support for the central role of this enzyme in this process. An essential requirement for sPLA2 in COX-2-dependent PG production has now been found in a variety of other cellular systems as well (6, 7, 20–22).

In this study we have provided further evidence to substantiate the notion that the sPLA2 is tightly linked to COX-2 in the chain of events leading to delayed PG production. Quite unexpectedly, we discovered that sPLA2 activation controls expression of the COX-2 gene. Hence, COX-2-derived PG production is critically dependent on a functionally active Group V sPLA2. Three different experimental approaches support this conclusion: (i) the specific sPLA2 inhibitor LY311727 greatly diminishes the induction of COX-2 protein and mRNA by LPS; (ii) antisense inhibition of Group V sPLA2 expression blocks COX-2 expression; (iii) exogenous Group V sPLA2 is able by itself to both activate the cells to release AA and to induce COX-2 expression.

We have previously reported that Group V sPLA2 expression is dependent on activation of the cPLA2 (4). As expected, here we have shown that COX-2 expression is also inhibited if the cPLA2 is inhibited. As a whole, these data demonstrate that the sequence of events leading to delayed PGE2 involves activation of the cPLA2 as the foremost event, followed by cPLA2-dependent induction and of the Group V sPLA2, which in turn controls the expression and activity of COX-2. Within this signaling cascade, two important points should be emphasized further. (a) Even though the cPLA2 appears not to be the main provider of free AA for PG production, its upstream position in the cascade clearly shows that it is the key enzyme in AA signaling in macrophages. It follows from this observation that any circumstance that leads to cPLA2 inhibition will prevent the cell from being able to generate PGs via COX-2 in response to stimuli. (b) The fact that COX-2 expression is dependent on both cPLA2 activation and Group V sPLA2 expression offers alternative avenues for the selective inhibition of COX-2 activity and conclusively supports our long-standing hypothesis (19, 23) that stimulated PG production can be efficiently controlled by controlling the upstream phospholipolytic step.

A striking feature of our study is that the effects of exogenous Group V sPLA2 on P388D1 cells reported here (AA release from cells and induction of COX-2 expression) appear to require the catalytic activity of the enzyme. In agreement with our data, Tada et al. (6) have reported very recently that exogenous Group IIA sPLA2 induces AA release from rat mast cells and fibroblasts by a mechanism that is dependent on enzyme activity (6). Our results using the irreversible inhibitors p-bromohiphenacyl bromide and manoalide suggest that enzyme activity is needed as well for the Group V sPLA2 to induce expression of COX-2. We cannot rule out at this time the possibility that at least part of the effect of sPLA2 on COX-2 expression may be due to the sPLA2 behaving as a ligand-like molecule if the inhibitors were preventing interaction of the enzyme with a putative binding site on the surface of the cell. Whether there are true receptors (i.e. of proteinaceous nature) on cells that account for some of the effects of exogenous sPLA2s reported in the literature remains to be clarified. To date, only one true protein receptor, the M-type receptor, which shows significant homology with the mannose scavenger receptor of phagocytes, has been cloned (24–26). This receptor binds certain sPLA2 forms with very high affinity, which suggests that these enzymes may be natural ligands for the receptor (27). Some cellular responses to exogenous sPLA2s, particularly Group IB, have been ascribed to interaction with the M-type receptor (28, 29). However, other putative ligand-like actions of Group IIA sPLA2 have been shown not to involve the M-type receptor (6, 30).

In summary, our data demonstrate that Group V sPLA2 regulates COX-2 expression in activated P388D1 macrophages by a mechanism that is best explained by the hydrolytic action of the enzyme on the outer surface of the cells. These findings add to the growing concept that Group V sPLA2 plays a fundamental role in AA release and PG synthesis in activated macrophage cells.