Group V Phospholipase A$_2$-Derived Lysophosphatidylcholine Mediates Cyclooxygenase-2 Induction in Lipopolysaccharide-Stimulated Macrophages[1]

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Activation of macrophages and macrophage cell lines by bacterial LPS elicits a delayed phase of PG biosynthesis that appears to be entirely mediated by cyclooxygenase-2 (COX-2). In previous work, we found that a catalytically active group V secreted phospholipase A$_2$ (sPLA$_2$-V) was required for COX-2 induction, but the nature of the sPLA$_2$-V metabolite involved was not defined. In this study, we identify lysophosphatidylcholine (lysoPC) as the sPLA$_2$-V downstream mediator involved in COX-2 induction by LPS-stimulated macrophages. Inhibition of sPLA$_2$-V by RNA interference or by the cell-permeable compound scalaradial blocked LPS-induced COX-2 expression, and this inhibition was overcome by incubating the cells with a nonhydrolyzable lysoPC analog, but not by arachidonic acid or oleic acid. Moreover, inhibition of sPLA$_2$-V by scalaradial also prevented the activation of the transcription factor c-Rel, and such an inhibition was also selectively overcome by the lysoPC analog. Collectively, these results support a model whereby sPLA$_2$-V hydrolysis of phospholipids upon LPS stimulation results in lysoPC generation, which in turn regulates COX-2 expression by a mechanism involving the transcriptional activity of c-Rel. *The Journal of Immunology,* 2007, 179: 631–638.

T he release of arachidonic acid (AA)[3] from its phospholipid storage sites by the action of one or more phospholipase A$_2$ (PLA$_2$) is a key limiting step for the generation of the proinflammatory mediators known as the eicosanoids (1–4). The PLA$_2$ superfamily of enzymes is comprised of >30 distinct proteins, all of which hydrolyze membrane phospholipids at the sn-2 position of the glycerol backbone, releasing a free fatty acid and a lysophospholipid (5). The PLA$_2$ enzymes are systematically classified into 15 group types according to their primary sequence (5). However, from a biochemical point of view, the PLA$_2$ are usually categorized into four broad families, namely the Ca^{2+}-dependent secreted enzymes (secreted PLA$_2$ (sPLA$_2$)), the Ca^{2+}-dependent cytosolic enzymes (cytosolic PLA$_2$ (cPLA$_2$)), the Ca^{2+}-independent cytosolic enzymes (Ca^{2+}-independent PLA$_2$ (iPLA$_2$)), and the platelet-activating factor acetyl hydrolases. In major immunoinflammatory cells such as macrophages and mast cells, members of the two first families have been implicated in the release of AA for eicosanoid generation, in particular the group IV cPLA$_2\alpha$ (cPLA$_2\alpha$)

and the group V sPLA$_2$ (sPLA$_2$-V) (6–20). There is general consensus that cPLA$_2\alpha$ is the critical enzyme in AA release (21–23), whereas sPLA$_2$-V may participate by amplifying the cPLA$_2\alpha$-mediated process by various mechanisms (4, 24–26). Definitive genetic evidence for this model has been recently provided in murine peritoneal macrophages and mast cells by showing that disruption of the cPLA$_2\alpha$ gene nearly abrogates eicosanoid production (12, 27), whereas disruption of the sPLA$_2$-V gene leads to a 35–50% reduction in eicosanoid production (18, 20). These results also support the existence of a coordinate action between cPLA$_2\alpha$ and sPLA$_2$-V, although the molecular basis of this cross-talk still remains poorly understood. Many potential mechanisms have been proposed, ranging from the cPLA$_2\alpha$ regulation of sPLA$_2$ activity by gene induction (11, 28) to sPLA$_2$ regulation of cPLA$_2\alpha$ via calcium signaling (29) or during secretion of the sPLA$_2$ (30, 31), through binding to cell membrane proteoglycans or plasma membrane phosphatidylcholine (32–36).

Several lines of investigation have also indicated that sPLA$_2$ enzymes can control the induction of the cyclooxygenase-2 (COX-2) gene, thus influencing eicosanoid production in an alternate manner. The ability of sPLA$_2$-V to induce COX-2 expression has been clearly documented in LPS-treated P388D$_1$ macrophages (37) and, more recently, in Ag-treated murine mast cells (20). Other sPLA$_2$ enzymes in addition to sPLA$_2$-V have also been found to amplify COX-2 induction when transfected into HEK293 cells (26). In the P388D$_1$ macrophage-like cell system, the regulatory role that sPLA$_2$-V plays on COX-2 induction was found to depend on enzyme activity, thus suggesting that a lipid metabolite arising from the hydrolytic action of sPLA$_2$-V on cell membranes was involved (37). In this study, we describe studies that identify lysophosphatidylcholine (lysoPC) as the sPLA$_2$-V downstream metabolite involved in COX-2 induction in LPS-treated P388D$_1$ macrophages. In addition, our studies highlight a key role for the transcription factor c-Rel in COX-2 induction by lysoPC in P388D$_1$ macrophages.

Materials and Methods

Reagents

P388D$_1$ cells (MAB clone) (11) were provided by Y. Shirai and E. Dennis (University of California at San Diego, La Jolla, CA). RPMI 1640 medium and FBS were from Invitrogen Life Technologies (5, 6, 8, 9, 11, 12, 14). The [^{3}H]AA (sp. act. 214 Ci/mmol) and [1-^{14}C]oleic acid (OA) (sp. act. 53 mCi/mmol) were from Amersham Ibérica. LPS (*Escherichia coli* 0111: B4) was from Sigma-Aldrich. Rabbit anti-mouse COX-2 Ab, methyl arachidonyl fluorophosphonate (MAFP), and bromoenol lactone (BEL) were from Cayman Chemical. The 12-Epi-scalaradial and manoalide were from BIOMOL. Both 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phospho-choline (methyl-lysoPC) and radicicol were from Calbiochem. c-Rel Ab was from Santa Cruz Biotechnology. Pyrrophenone was a gift from T. Ono (Shionogi, Osaka, Japan). LY311727 was provided by E. Mihelich (Eli Lilly, Indianapolis, IN). Pure rat sPLA$_2$-V was provided by A. Aarsman (Utrecht University, Utrecht, The Netherlands). All other reagents were from Sigma-Aldrich.

Cell culture and labeling conditions

P388D$_1$ cells were maintained at 37°C in a humidified atmosphere at 95% air and 5% CO$_2$ in RPMI 1640 medium supplemented with 5% FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. P388D$_1$ cells were plated at 10^6/well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free RPMI 1640 medium. When required, radiolabeling of the P388D$_1$ cells was achieved by including 0.5 μCi/ml [^{3}H]AA or 0.1 μCi/ml [^{14}C]OA during the overnight adherence period (20 h). Labeled fatty acid that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 0.5 mg/ml albumin.

Primary cultures of peritoneal macrophages were established from resident cells from C57BL/6 male mice (University of Valladolid Animal House), as described previously (38, 39). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Small interfering RNA (siRNA) inhibition studies

The siRNA directed against sPLA$_2$-V was from Ambion (sequence 5′-CAC GAC UCC UUC UGU CCA AdTdT-3′). The cells (3 × 10^5/ml) were transiently transfected with oligonucleotide (5–20 nM) in the presence of 10 μg/ml lipofectamine (Invitrogen Life Technologies) under serum-free conditions for 6 h. Afterward, 5% serum was added and the cells were maintained at normal culture conditions for 20 h. Then the cells were used for experiments, as described above. A scrambled siRNA was used as a negative control.

PLA$_2$ activity measurements

The mammalian membrane assay described by Diez et al. (40) was used. Briefly, aliquots of P388D$_1$ cell homogenates were incubated for 1–2 h at 37°C in 100 mM HEPES (pH 7.5) containing 1.3 mM CaCl$_2$ and 100,000 dpm of [^{3}H]AA-labeled U937 cell membrane, used as substrate. When cPLA$_2$ activity was measured, the assay contained 25 μM LY311727 and 25 μM BEL to completely inhibit sPLA$_2$ and iPLA$_2$ activities. When sPLA$_2$ activity was measured, the assay contained 1 μM pyrrophenone and 25 μM BEL to completely inhibit cPLA$_2$ and iPLA$_2$ activities. These assay conditions have been validated previously (41–45).

Immunoblot analyses

Cells were serum starved and stimulated with 100 ng/ml LPS for the periods of time indicated in the presence or absence of the indicated inhibitors. Afterward, the cells were washed and then lysed in a buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 100 μM Na$_3$VO$_4$, 1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich) at 4°C. Protein was quantified, and a 4-μg aliquot was analyzed by immunoblot exactly as described previously (46, 47), with Abs against murine COX-2 (Cayman).

Measurement of extracellular fatty acid release

The cells were placed in serum-free medium for 1 h before the addition of LPS in the absence or presence of pyrrophenone or scalaradial, and in the presence of 0.5 mg/ml BSA. After the 24-h incubation period, the supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.
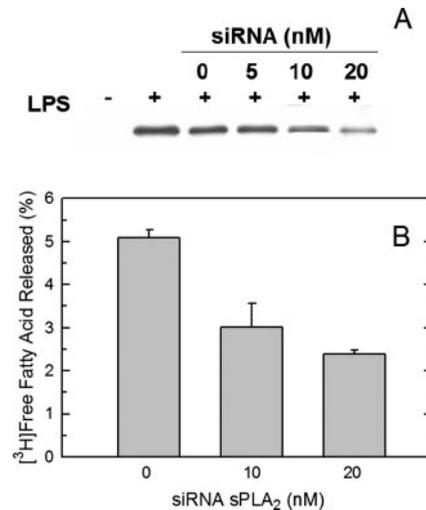
FIGURE 1. Inhibition of sPLA$_2$-V by siRNA. The cells were treated with the indicated concentrations of siRNA-targeting sPLA$_2$-V, exposed to LPS, and then assayed for COX-2 content (*A*). Cellular sPLA$_2$ activity in the homogenates (*B*) was assayed and described in *Materials and Methods*.

Transcription factor activity assay

Detection of transcription factor activity was performed with the BD Mercury TransFactor Profiling Kit, Inflammation 1 (BD Clontech), which uses an ELISA-based technique, following the manufacturer's instructions.

Other methods

Protein concentration was determined using the Bradford protein assay kit (Bio-Rad) with BSA as a standard. Data are presented as the mean ± SEM of at least three different experiments.

Results

Inhibition of sPLA$_2$-V blocks COX-2 expression in LPS-treated P388D$_1$ macrophages

In previous work, we have shown that inhibition of cellular sPLA$_2$-V activity by either antisense oligonucleotide technology or the pharmacological inhibitor LY311727 leads to a markedly reduced induction of COX-2 in LPS-treated P388D$_1$ cells (37). In these experiments, the antisense oligonucleotide and pharmacological approaches that we used failed to produce complete inhibition of cellular sPLA$_2$-V activity (37). Thus, the possibility exists that the partial suppression of COX-2 production that we generally observed (37) was due to incomplete inhibition of cellular sPLA$_2$-V. To address this issue, we devised new strategies to try to block cellular sPLA$_2$-V in a more efficacious manner. In the first place, we used siRNA technology. P388D$_1$ cells were transfected with siRNA-targeting sPLA$_2$-V, exposed to LPS, and then assayed for COX-2 content. Because we have been unable to find reliable Abs against murine sPLA$_2$-V, the efficiency of siRNA knockdown was judged by enzyme activity assay. Treatment with different concentrations of siRNA directed against sPLA$_2$-V induced a dose-dependent inhibition of COX-2 induction, as judged by immunoblot (Fig. 1*A*). However, as shown in Fig. 1*B*, the siRNA treatment only partially blocked sPLA$_2$-V expression, and a significant fraction of activity was still present at 20 nM, the highest siRNA concentration that did not promote loss of cell viability. Although these results provide further evidence for the regulatory role of sPLA$_2$-V on COX-2 expression, overall, siRNA technology produced no improvement over previous methods with regard to the abrogation of sPLA$_2$-V expression.
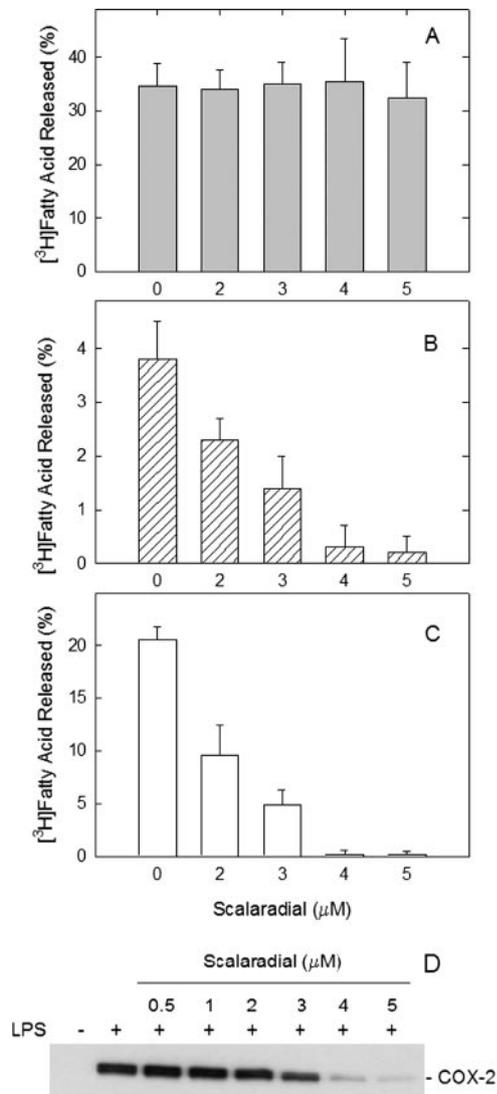
FIGURE 2. Scalaradial effects on PLA$_2$ activities and COX expression. The cells were exposed to 100 ng/ml LPS in the absence or presence of the indicated concentrations of scalaradial. After a 20-h incubation, homogenates were prepared, and the effect of scalaradial on cPLA$_2$ activity (*A*), sPLA$_2$ activity (*B*), and COX-2 protein content (*D*) was analyzed, as described in *Materials and Methods*. The effect of scalaradial on the activity of pure rat sPLA$_2$-V (50 ng/assay) is shown in *C*.

Recent data have shown that, in addition to or instead of acting extracellularly, some sPLA$_2$s may actually effect phospholipid hydrolysis at an intracellular site before being released to the extracellular medium (30, 31). To account for possible intracellular site actions of sPLA$_2$-V, we assayed the effect of a cell-permeable sPLA$_2$ inhibitor, scalaradial. Scalaradial, an irreversible inhibitor of sPLA$_2$, has been widely used to investigate the involvement of this class of enzymes in a variety of cell functions, including AA release (48–50). However, studies with scalaradial may be complicated by the potential inhibitory effects of this compound on cPLA$_2$ activity, particularly when used at concentrations above 10 μM (49). To validate the use of scalaradial in our system, we first investigated conditions under which the drug blocked sPLA$_2$ activity without affecting that of cPLA$_2$. The cells were treated with different scalaradial conditions, homogenates were prepared, and sPLA$_2$ and cPLA$_2$ activities were measured using membrane-based assays, as described in *Materials and Methods*. In these experiments, cPLA$_2$ activity was assayed in the presence of BEL
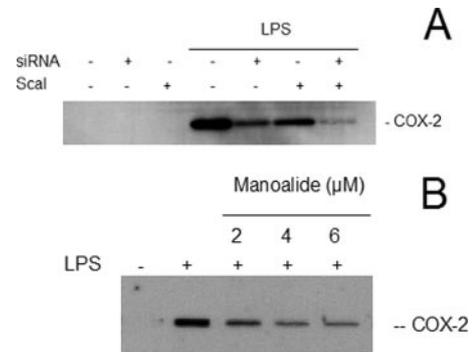

FIGURE 3. Inhibition of COX-2 expression by siRNA plus scalaradial and manoalide. *A*, The cells were treated with 20 nM siRNA-targeting sPLA$_2$-V, and exposed to 100 ng/ml LPS in the absence or presence of 3 μM scalaradial. COX-2 protein content was assayed by immunoblot. *B*, The cells were treated with 100 ng/ml LPS for 20 h in the absence or presence of the indicated concentrations of manoalide. Afterward, COX-2 protein content was measured by immunoblot.

and LY311727 to ensure that endogenous iPLA$_2$ and sPLA$_2$ activities do not contribute to the activity measured. Similarly, the sPLA$_2$ assay was conducted in the presence of pyrrophenone and BEL to eliminate interference from cPLA$_2$ and iPLA$_2$ activities. Fig. 2*A* shows that, when used at concentrations up to 5 μM, scalaradial had no effect on cPLA$_2$ activity. Conversely, when assayed in the same range of concentrations, sPLA$_2$ activity was inhibited in a dose-dependent manner. Almost complete inhibition was observed at scalaradial concentrations above 3 μM (Fig. 2*B*). To further validate the specificity of the assay, experiments were also conducted using pure sPLA$_2$-V. The amount of enzyme used was 50 ng/assay, which is in the physiological range (51). Fig. 2*C* shows that scalaradial inhibited pure sPLA$_2$-V with a concentration-dependence curve that was similar to that observed when the cellular homogenate was used as a source of enzyme (Fig. 2, cf *B* and *C*). The effect of scalaradial on COX-2 expression by LPS-treated macrophages is shown in Fig. 2*D*. Scalaradial blocked COX-2 induction in a concentration-dependent manner, which corresponded well with that of inhibition of endogenous sPLA$_2$. The good correspondence between the dose-response effects of scalaradial on sPLA$_2$ activity and COX-2 expression, along with the finding that cPLA$_2$ activity is not impaired at these same concentrations, suggest that scalaradial-induced COX-2 expression is directly related to inhibition of cellular sPLA$_2$ activity.

To further assess the specificity of action of scalaradial, an experiment was designed in which cells treated with siRNA-targeting sPLA$_2$-V, and hence expressing lesser amounts of enzyme, were exposed to 3 μM scalaradial, a concentration that exerts little effect on COX-2 protein levels in cells expressing normal amounts of sPLA$_2$-V (see Fig. 2*D*). As shown in Fig. 3*A*, the reduction of LPS-induced COX-2 in sPLA$_2$-V-deficient cells was further diminished by treating the cells with scalaradial. Thus, cells expressing sPLA$_2$-V at lower levels than normal cells require lower scalaradial doses for quantitative inhibition of COX-2 protein levels. Together, these data provide further evidence that the scalaradial effect on COX-2 occurs via inhibition of sPLA$_2$-V.

The effect of another sPLA$_2$ inhibitor, manoalide (52), on LPS-induced COX-2 production is shown in Fig. 3*B*. This compound, at concentrations that do not interfere with cellular cPLA$_2$ activity as judged by in vitro assay (i.e., 6 μM and below), promoted a dose-dependent inhibition of COX-2 expression. Because manoalide is structurally unrelated to scalaradial (52), these data provide additional independent evidence for the role of cellular sPLA$_2$ in regulating COX-2 induction in response to LPS.
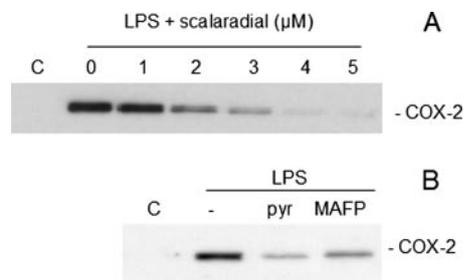
FIGURE 4. COX-2 expression in LPS-stimulated murine peritoneal macrophages. The macrophages were treated with 100 ng/ml LPS for 20 h in the absence or presence of the indicated concentrations of scalaradial (*A*) or 1 μM pyrrophenone (pyr), or 10 μM MAFP (*B*). Afterward, COX-2 protein content was measured by immunoblot.

COX-2 expression in mouse peritoneal macrophages

To assess whether the inhibitory effect of scalaradial on the induction of COX-2 in the LPS-treated P388D$_1$ macrophage-like cells is physiologically relevant and not just a peculiarity of the cell line used, we extended our studies to murine peritoneal macrophages. In agreement with the P388D$_1$ cell data, scalaradial markedly inhibited COX-2 expression in LPS-treated peritoneal macrophages in a concentration-dependent manner (Fig. 4*A*).

The effect of inhibiting cPLA$_2$$\alpha$ on COX-2 induction was also assayed in the murine peritoneal macrophages. For this purpose, we used the inhibitors MAFP and pyrrophenone. Although MAFP is a dual cPLA$_2$/iPLA$_2$ inhibitor (7, 53), pyrrophenone specifically inhibits the cPLA$_2$$\alpha$ (54, 55). Fig. 4*B* shows that both inhibitors, used at concentrations that completely abrogate cellular cPLA$_2$ activity (41–45), markedly blunted COX-2 production in the LPS-stimulated mouse macrophages. This is fully consistent with our previous work in the P388D$_1$ cells, in which a functionally active cPLA$_2$$\alpha$ was found to be necessary for the induction of sPLA$_2$-V and subsequent COX-2 production in response to LPS (37).

Effect of scalaradial on the release of fatty acids by LPS-stimulated P388D$_1$ cells

Unlike cPLA$_2$$\alpha$, sPLA$_2$-V does not preferentially hydrolyze AA-containing membrane phospholipids, being able to significantly affect the release of other fatty acids such as OA. In this regard, we previously documented that LPS stimulation of OA release in P388D$_1$ cells requires the hydrolytic action of sPLA$_2$, regulated by cPLA$_2$$\alpha$ (13). In keeping with these data, the LPS-stimulated release of OA from P388D$_1$ was prevented by scalaradial, confirming the involvement of sPLA$_2$ (Fig. 5*A*). AA release under these conditions was also inhibited by scalaradial, albeit to a lesser degree, i.e., 20–30% (Fig. 5*B*). This level of inhibition is consistent with the recent studies by Arm and colleagues (20) with mice lacking sPLA$_2$-V, who estimated a contribution of sPLA$_2$-V to delayed eicosanoid production of ~35%. As expected, cPLA$_2$$\alpha$ inhibition by pyrrophenone strongly inhibited both OA and AA release (Fig. 5), consistent with this enzyme being regulatory for OA release (13, 23), and the primary effector of AA release (22, 23).

Identification of the sPLA$_2$ metabolite involved in COX-2 production

Collectively, the above results indicate that sPLA$_2$-V-mediated phospholipid hydrolysis occurs during LPS stimulation of P388D$_1$ cells, and that inhibition of sPLA$_2$-V by scalaradial blocks both fatty acid release and COX-2 induction. To study the possible relationship between free fatty acids and COX-2, we studied the effect of exposing the cells to either OA or AA on COX-2 pro-
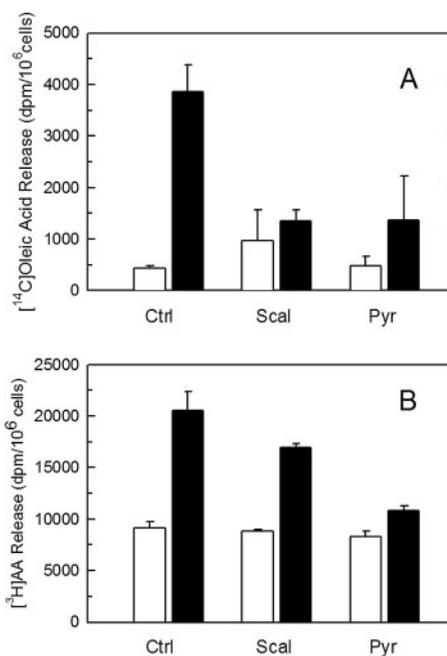

FIGURE 5. Effect of scalaradial on the release of OA or AA by LPS-stimulated cells. Cells, labeled with either [^{14}C]OA (*A*) or [^{3}H]AA (*B*), were incubated for 20 h in the absence ($\square$) or presence ($\blacksquare$) of 100 ng/ml LPS, and in the absence (Ctrl) or presence of 4 μM scalaradial (Scal), or 1 μM pyrrophenone (Pyr), as indicated. Radioactive fatty acid content in the supernatants was estimated by scintillation counting.

duction. Incubation of the cells with various concentrations of OA or AA (up to 100 μM) for various times (up to 24 h) did not induce COX-2 expression in the P388D$_1$ cells. Next, we studied whether the inhibitory effect of scalaradial on LPS-induced COX-2 production could be reversed by the presence of OA or AA. To this end, the cells were incubated with various concentrations of exogenous OA or AA (up to 100 μM), treated with LPS in the presence or absence of scalaradial, and assayed for COX-2 production by immunoblot. The fatty acids were either added 1 h before or at the same time as the LPS. No reversal of the inhibition of COX-2 production by scalaradial could be observed under any condition (results not shown). Collectively, these results indicate that free fatty acids are not the sPLA$_2$-V metabolites involved in COX-2 production.

To study the possible involvement of an oxygenated metabolite of AA instead, LPS-induced COX-2 production was studied in the presence of a variety of general cyclooxygenase or lipoxygenase inhibitors, namely indomethacin, aspirin, ebselen, and baicalein. These compounds were used at concentrations up to 25 μM, and were added to the cells 30 min before the addition of LPS. Neither of the inhibitors tested exerted any effect on the LPS-induced COX-2 production, thus ruling out the participation of an AA metabolite in the process (results not shown).

Given the above results excluding the participation of free fatty acids and/or oxygenated derivatives in LPS-induced COX-2 production, we turned our eyes to the other product of sPLA$_2$-catalyzed phospholipid hydrolysis, i.e., lysophospholipid. Initial experiments indicated that, when added exogenously to the P388D$_1$ macrophage-like cells, lysophospholipids were rapidly metabolized by either acylation or hydrolysis reactions (results not shown). To circumvent this problem, we used the stable lysoPC analog, methyl-lysoPC, which, because of the ether bonds at the sn-1 and sn-2 positions, cannot be hydrolyzed to glycerophosphocholine, or acylated to form phosphatidylcholine. Using this compound, it was possible to greatly overcome the inhibitory actions of
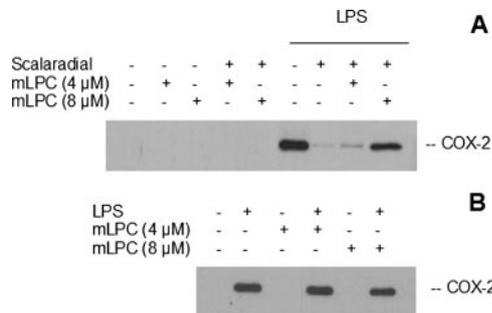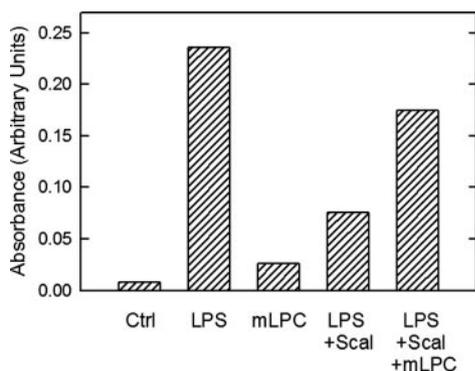
FIGURE 6. Methyl-lysoPC overcomes the inhibitory actions of scalaradial on LPS-stimulated COX-2 expression. *A*, The cells were pretreated for 30 min with 4 μM scalaradial. LPS (100 ng/ml) and the indicated concentrations of methyl-lysoPC (mLPC) were added, and the cells were cultured for an additional 20-h period. COX-2 production was determined by immunoblot. *B*, The cells were exposed to LPS (100 ng/ml) and the indicated concentrations of methyl-lysoPC (mLPC) for 20 h. Afterward, COX-2 expression was determined by immunoblot.

scalaradial on LPS-induced COX-2 production. As shown in Fig. 6*A*, at a concentration of 8 μM, methyl-lysoPC overcame the scalaradial inhibition of COX-2 production by ~80%. Importantly, methyl-lysoPC did not have any stimulatory effect by itself, nor did it enhance the LPS-induced COX-2 production in the absence of scalaradial (Fig. 6*B*). Collectively, these data suggest that methyl-lysoPC did not function through interaction with a surface receptor.

To study the specificity of action of methyl-lysoPC, the capacity of this compound to overcome the inhibitory effect of the cPLA$_2\alpha$ inhibitor pyrrophenone on LPS-induced COX-2 was assayed next. Unlike scalaradial, the inhibitory actions of pyrrophenone on COX-2 production were not overcome by methyl-lysoPC (data not shown). Thus, these results demonstrate that it is the lysoPC generated by sPLA$_2$-V, not the one produced by cPLA$_2\alpha$, that is specifically linked to COX-2 production.

To explore the possibility that sPLA$_2$-V might generate lysophospholipids by acting extracellularly, lysoPC levels were measured in the supernatants of LPS-stimulated cells in the presence or absence of scalaradial. To this end, cells labeled with 0.5 μCi/ml [^{3}H]choline for 2 days were used (56, 57). After the different treatments, lipids in the supernatants were extracted with ice-cold *n*-butanol and separated by thin-layer chromatography (56, 57). However, no significant amounts of [^{3}H]lysoPC could be detected

FIGURE 7. Methyl-lysoPC overcomes the inhibitory effect of scalaradial on LPS-induced c-Rel transcriptional activity. The cells were pretreated for 30 min with 4 μM scalaradial. LPS (100 ng/ml) and 8 μM methyl-lysoPC (mLPC) were added as indicated, and the cells were cultured for an additional 6-h period. c-Rel transcriptional activity was measured with a commercial kit, as described in *Materials and Methods*.
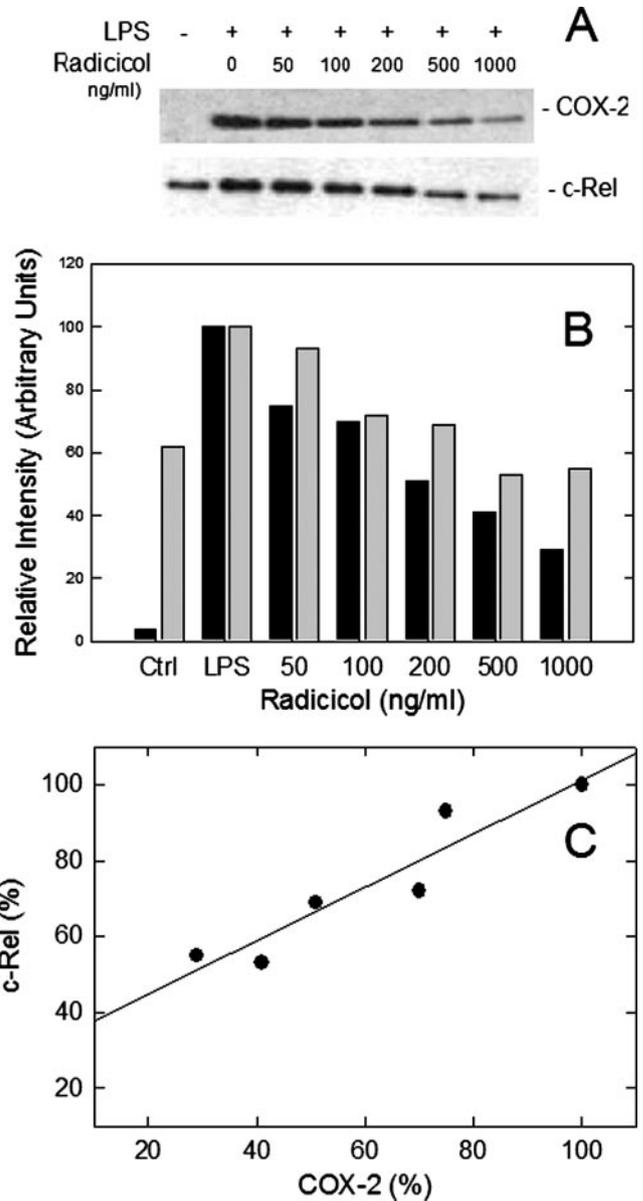
FIGURE 8. Effect of radicicol on c-Rel and COX-2 production by LPS-stimulated cells. The cells were treated with 100 ng/ml LPS for 6 h in the presence of the indicated concentrations of radicicol. Afterward, COX-2 and c-Rel content were measured by immunoblot (*A*). *B*, Shows a densitometric quantitation of the bands in *A* (■, COX-2; ▨, c-Rel). *C*, Shows a plot of c-Rel content vs COX-2 content at each radicicol concentration.

in the supernatants of LPS-treated cells vs control-untreated cells at any time, indicating that lysoPC does not accumulate extracellularly under these conditions.

Transcription factors involved in the sPLA$_2$-dependent expression of COX-2

Transcriptional regulation of mouse COX-2 expression is mediated by different regulatory elements that are distributed along the COX-2 promoter sequence. The COX-2 promoter contains a classical TATA box, an E-box, and binding sites for transcription factors such as NF-κB, C/EBP, CREB, AP-1, and NF-AT, all of which have been shown to act as positive regulatory elements for COX-2 transcription in different cell types (58, 59). To study which of these elements may lie downstream of sPLA$_2$-V during LPS stimulation of P388D$_1$ cells, we studied the inhibition of

transcription factor activity by scalaradial. In an ELISA-based transcription factor profiling assay, DNA-binding activities of the inflammation-related transcription factors activating transcription factor 2, CREB-1, c-*fos*, c-Rel, NF-κB p65, and NF-κB p50 were measured in unstimulated or LPS-stimulated P388D$_1$ cells in the presence or absence of scalaradial for 6 h. NF-κB p65, c-*fos*, and c-Rel were activated by LPS, but only the activation of c-Rel was significantly inhibited by scalaradial and restored by treating the cells with methyl-lysoPC (Fig. 7). Collectively, these results indicate that activation of c-Rel corresponds with COX-2 production in that both processes are inhibited by scalaradial, but inhibition can be overcome by methyl-lysoPC. To confirm that a link actually exists between these two proteins, we studied the effect of radicicol on COX-2 expression (Fig. 8). Radicicol is a fungal metabolite that has previously been demonstrated to inhibit c-Rel transcriptional activity in LPS-treated macrophages by reducing c-Rel expression (60). As shown in Fig. 8, radicicol inhibited in parallel the production of both c-Rel and COX-2 in LPS-treated cells, suggesting that both processes are related.

Discussion

Using the murine macrophage-like cell line P388D$_1$, we have shown that these cells exhibit a delayed eicosanoid generation response to LPS (11, 37). This response takes several hours to develop and strikingly involves the de novo synthesis of two of the regulatory enzymes involved, namely sPLA$_2$-V and COX-2 (11, 37). The elevated expression of these two effectors can be prevented by inhibiting the activity of cPLA$_2\alpha$, highlighting the key role that cPLA$_2\alpha$ plays as the primary regulator of the eicosanoid response in LPS-treated macrophages. Importantly, inhibition of COX-2 expression also occurs if sPLA$_2$-V activity is inhibited. Thus, sPLA$_2$-V may serve two different roles during the delayed eicosanoid generation response. sPLA$_2$-V may increase the free AA level that is derived to eicosanoid synthesis by directly hydrolyzing AA-containing phospholipids, and, alternatively, it may amplify the eicosanoid release response by directly regulating the induction of COX-2. In the studies reported in this work, the cell-permeable inhibitor scalaradial strongly inhibited COX-2 expression and OA release, but only modestly blunted AA release. These data may suggest that, under these settings, sPLA$_2$-V amplifies the LPS-mediated delayed eicosanoid response mainly through its effect on COX-2 expression. This would be consistent with recent data in mast cells implicating endogenous sPLA$_2$-V in COX-2 induction in murine mast cells from C57BL mice, but only modestly at best on provision of AA (20). Interestingly, in mast cells from another strain, BALB/c, no role for sPLA$_2$-V in either AA release or COX-2 induction could be found (20). These findings were made by using mice in which the gene encoding sPLA$_2$-V gene had been disrupted (20). Thus, they provide solid evidence that, depending on the genetic background, two different phenotypes may exist in cells regarding the involvement of sPLA$_2$ in eicosanoid generation. Whether these two phenotypes may also manifest in cells depending on culture conditions is unknown at present.

In our previous studies, pharmacologic and antisense oligonucleotide evidences were provided to indicate that the enzymatic activity of sPLA$_2$-V is required for COX-2 induction (37), suggesting that a sPLA$_2$-derived metabolite plays an instrumental role in the process. A weakness in our previous studies was that the strategies used inhibited sPLA$_2$-V only partially (37). This adds complexity to the search for the sPLA$_2$-V-downstream metabolite involved, because diminished levels of the metabolite in question could still produce biological effects, as suggested by the finding that reduction of COX-2 under these conditions was always in-

complete (37). To circumvent this problem, in this study we have tried new strategies to abrogate sPLA$_2$-V activity. Our attempts at using siRNA have failed to produce quantitative inhibition of sPLA$_2$-V. Thus, in our hands, siRNA technology produced no improvement over previous methods.

Recent work by the Gelb laboratory has raised the possibility that sPLA$_2$ may act inside the cells during secretion rather than outside the cells after secretion has occurred (30, 31). Thus, the authors suggest that the role of sPLA$_2$ in AA mobilization must be investigated before or during secretion of the enzyme (30, 31). In our previous studies, the cell-impermeable indole derivative LY311727 was used (37). Thus, we speculated that one reason for the failure of this inhibitor to produce quantitative effects on COX-2 induction could be that the inhibitor acted only on the fraction of sPLA$_2$-V already outside of the cell, but not on the enzyme acting inside, prior, or during secretion. To test this hypothesis, we used the cell-permeable inhibitor scalaradial. This compound, at concentrations as low as 4 μM, was able to completely block cellular sPLA$_2$ activity and induce a very strong inhibition of COX-2 expression (i.e., always over 90%). Collectively, these data are consistent with a role for intracellular sPLA$_2$-V in COX-2 induction.

As recently discussed elsewhere (31), investigators have usually assumed that sPLA$_2$ functions outside of the cells. This assumption is inferred mainly from earlier findings showing high sPLA$_2$ levels in inflammatory exudates and the biochemical properties of the protein, in particular its high disulfide content. Circulating phagocytes secrete significant amounts of sPLA$_2$ (51, 61), which, eventually, could associate with the cell membranes of the originating or neighboring cells, and/or be reinternalized to exert an hydrolytic action at various cellular sites (16, 25, 33, 62, 63). The ability of sPLA$_2$-V to act on neighboring cells to induce phospholipid hydrolysis at physiological concentrations has been unambiguously demonstrated (29, 36, 64). Importantly, extracellular sPLA$_2$-V may promote AA mobilization from cells lacking cPLA$_2\alpha$, which indicates that, regardless of any secondary effect on cPLA$_2\alpha$, sPLA$_2$-V does indeed have the capacity to affect phospholipid hydrolysis and produce lysoPC on its own (35). These findings, together with the aforementioned results demonstrating intracellular sPLA$_2$ actions before secretion (30, 31), raise the key concept that multiple sites and modes of action may exist for sPLA$_2$-V in cells. Thus, whether sPLA$_2$-V functions transcellularly by a paracrine mechanism or acts on its originating cells by an autocrine mechanism, or simply functions intracellularly before secretion under physiological conditions may ultimately depend on cell type and nature of the stimulus. In this regard, we recently found that in the LPS-treated P388D$_1$ macrophages, sPLA$_2$-V localized in large cytoplasmic granules containing caveolin (16). Localization of sPLA$_2$-V in these granules correlates with the appearance of COX-2 protein, suggesting a cause-effect relationship (11, 16). In our studies, the localization of sPLA$_2$-V in cytoplasmic granules could be partially prevented by treating the cells with heparin, a polysaccharide that sequesters some sPLA$_2$ enzymes in the culture medium, and in this manner, blunts their effects on the cells (16). These data would be compatible with an autocrine role for sPLA$_2$-V in P388D$_1$ cells in that the enzyme, after being secreted to the extracellular medium, would be internalized back to the cells to exert its biological role in an intracellular compartment. However, given that heparin is pleiotropic in its effects, we do not rule out that sPLA$_2$-V exerts its effects on COX-2 before or during secretion.

Although scalaradial is a selective inhibitor of sPLA$_2$, caution needs to be exercised when using this compound, because other enzyme activities could be inhibited as well. At concentrations

slightly higher than those required to inhibit $sPLA_2$, scalaradial may also affect the activity of $cPLA_2\alpha$ (49, 50). This nonselective action does not appear to participate in the inhibitory effects of scalaradial on LPS-induced COX expression, because scalaradial, at concentrations that inhibit both $sPLA_2$ activity and COX-2 induction (<5 μM), does not affect cellular $cPLA_2$ activity, as measured in an in vitro assay. Additional evidence to support that inhibition of $sPLA_2$-V by scalaradial is responsible for inhibition of COX-2 expression includes the following: 1) the finding that a low concentration of scalaradial that exerts little effect on COX-2 expression in normal cells is able to quantitatively block COX-2 expression in cells expressing lower $sPLA_2$-V levels by siRNA treatment, and 2) the ability of methyl-lysoPC, an analog of the $sPLA_2$-V product lysoPC, to almost completely overcome the inhibitory actions of scalaradial on LPS-induced COX-2 expression. Importantly, methyl-lysoPC does not restore COX-2 expression in pyrrophenone-treated cells, suggesting that it is the lysoPC produced by $sPLA_2$-V that is specifically implicated in COX-2 expression.

The intracellular signaling mechanism by which $sPLA_2$-V-derived lysoPC mediates COX-2 expression is associated with the activation of the transcription factor c-Rel. Using an ELISA-based transcription activity assay, we simultaneously measured the effect of $sPLA_2$-V inhibition on six different transcription factors. The activation of c-*fos* and c-Rel is inhibited by scalaradial, and of these, only the scalaradial-sensitive c-Rel activation, but not that of c-*fos* is significantly overcome by treating the cells with methyl-lysoPC. In addition, we show that inhibition of c-Rel expression by radicicol in LPS-treated cells results in a corresponding inhibition of COX-2. Our results, coupled with previous data (11, 37), suggest a model for COX-2 induction in stimulated P388D$_1$ cells whereby LPS first induces $sPLA_2$-V expression in a $cPLA_2\alpha$-dependent manner. $sPLA_2$-V, acting intracellularly either prior/during secretion or in an autocrine manner on the cells that originated it, catalyzes phospholipid hydrolysis leading to lysoPC accumulation, which in turn would be implicated in c-Rel activation leading to COX-2 induction. Collectively, our findings highlight the complexity of the macrophage response to LPS regarding COX-2 expression and suggest a pivotal role for intracellular PLA$_2$ enzymes in regulating this process.

Acknowledgments

Disclosures

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