Group V Phospholipase A_2-derived Lysophosphatidylcholine Mediates Cyclooxygenase-2 Induction in Lipopolysaccharide-stimulated Macrophages[1]

Violeta Ruipérez, Javier Casas, María A. Balboa, and Jesús Balsinde[2]

Institute of Molecular Biology and Genetics,

Spanish National Research Council and University of Valladolid School of Medicine,

47003 Valladolid, Spain

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Abstract

Activation of macrophages and macrophage-cell lines by bacterial lipopolysaccharide (LPS) elicits a delayed phase of prostaglandin 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 paper 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 non-hydrolyzable 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.

Introduction

The release of arachidonic acid (AA)[3] from its phospholipid storage sites by the action of one or more phospholipase A_2s (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 more than 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 fifteen group types according to their primary sequence [5]. However, from a biochemical point of view, the PLA_2s are usually categorized into four broad families, namely the Ca^{2+}-dependent secreted enzymes ($sPLA_2$), the Ca^{2+}-dependent cytosolic enzymes ($cPLA_2$), the Ca^{2+}-independent cytosolic enzymes ($iPLA_2$), 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 cytosolic Group IV phospholipase $A_2\alpha$ ($cPLA_2\alpha$) 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], while $sPLA_2$-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], while 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 lipopolysaccharide (LPS)-treated $P388D_1$ macrophages [37] and, more recently, in antigen-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]. Here 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 Drs. Yasu Shirai and Ed Dennis (University of California at San Diego, La Jolla, CA). RPMI 1640 medium and fetal bovine serum were from Gibco Invitrogen (Carlsbad, CA). [5,6,8,9,11,12,14,14-^{3}H]AA (sp. act. 214 Ci/mmol) and [1-^{14}C]oleic acid (OA) (sp. act. 53 mCi/mmol) were from Amersham Ibérica (Madrid, Spain). LPS (E. coli 0111: B4) was from Sigma. Rabbit anti-mouse COX-2 antibody, methyl arachidonyl fluorophosphonate (MAFP), and bromoenol lactone (BEL) were from Cayman (Ann Arbor, MI). 12-Epi-scalaradial and manoalide were from Biomol (Plymouth Meeting, PA). 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (methyl-lysophosphatidyl-choline, methyl-lysoPC), and radicicol were from Calbiochem (La Jolla, CA). c-Rel antibody was from Santa Cruz. Pyrrophenone was a gift from Dr. Kohji Hanasaki (Shionogi, Osaka, Japan). LY311727 was provided by Dr. Edward Mihelich (Eli Lilly, Indianapolis, IN). Pure rat sPLA_2-V was provided by Dr. Anton Aarsman (Utrecht University). All other reagents were from Sigma.

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% fetal bovine serum, 2 mM glutamine, 50 units/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% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

siRNA Inhibition Studies — The siRNA directed against $sPLA_2$-V was from Ambion (Austin, TX) (sequence 5'-CAC GAC UCC UUC UGU CCA AdTdT-3'). The cells (3 x 10^5/ml) were transiently transfected with oligonucleotide (5-20 nM) in the presence of 10 μg/ml lipofectamine (Invitrogen, Carlsbad, CA) 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_3VO_4, 1 mM PMSF, and protease inhibitor cocktail (Sigma) at 4°C. Protein was quantified, and a 4-μg aliquot was analyzed by immunoblot exactly as described previously [46, 47], with antibodies 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 bovine serum albumin. After the 24-h incubation period, the supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Transcription Factor Activity Assay — Detection of transcription factor activity was performed with the BD Mercury TransFactor Profiling Kit, Inflammation 1 (Clontech BD Biosciences, Mountain View, CA), which uses an ELISA-based technique, following the manufacturer's instructions.

Other Methods —Protein concentration was determined utilizing the Bradford protein assay kit (BioRad) with bovine serum albumin as a standard. Data are presented as the mean ± S.E.M. of at least three different experiments.

Results

Inhibition of Group V sPLA$_2$ 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 utilized 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 utilized siRNA technology. P388D$_1$ cells were transfected with siRNA targeting sPLA$_2$-V, exposed to LPS and then assayed for COX-2 content. Since we have been unable to find reliable antibodies 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. 1A). However, as shown in Fig. 1B, 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. While 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.

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 prior to 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 $sPLA_2$s, has been widely utilized 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 utilizing membrane-based assays as described under Experimental Procedures. In these experiments, $cPLA_2$ activity was assayed in the presence of BEL 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. 2A 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. 2B). To further validate the specificity of the assay, experiments were also conducted utilizing pure $sPLA_2$-V. The amount of enzyme utilized was 50 ng/assay, which is in the physiological range [51]. Fig. 2C 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 (cf. Figs. 2B and 2C). The effect of scalaradial on COX-2 expression by LPS-treated macrophages is shown in Fig. 2D. 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. 2D). As shown in Fig. 3A, 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. 3B. 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. Since 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.

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. 4A).

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. While MAFP is a dual cPLA$_2$/iPLA$_2$ inhibitor [7, 53], pyrrophenone specifically inhibits the cPLA$_2\alpha$ [54, 55]. Fig. 4B shows that both inhibitors, utilized 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, where 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 hydrolyzes AA-containing membrane phospholipids, being able to significantly effect the release of other fatty acids such as oleic acid (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. 5A) AA release under these conditions was also inhibited by scalaradial, albeit to a lesser degree, i.e. 20-30% (Fig. 5B). This level of inhibition is consistent with the recent studies by Arm and associates with mice lacking sPLA$_2$-V, who estimated a contribution of Group V sPLA$_2$ to delayed eicosanoid production of about 35% [20]. 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 production. 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 utilized the stable lysoPC analog, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phospho-choline (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 scalaradial on LPS-induced COX-2 production. As shown in Fig. 6A, at a concentration of 8 μM, methyl-lysoPC overcame the scalaradial inhibition of COX-2 production by about 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. 6B). 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 (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 in the supernatants of LPS-treated cells *versus* 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 which 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 NFAT, all of which have been shown to act as positive regulatory elements for COX-2 transcription in different cell types [57, 58]. 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 ATF2, CREB-1, c-fos, c-Rel, NFκB p65, and NFκB p50 were measured in unstimulated or LPS-stimulated P388D$_1$ cells in the presence or absence of scalaradial for 6 h. NFκB 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 [59]. 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 here, 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. If so, that might help explain some apparently contrasting results that have appeared in the literature [60].

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 utilized inhibited $sPLA_2$-V only partially [37]. This adds complexity to the search for the $sPLA_2$-V-downstream metabolite involved, since 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 incomplete [37]. To circumvent this problem, in this paper 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 $sPLA_2$s 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 utilized [37]. Thus, we speculated that one reason for the failure of this inhibitor to produce quantitative effects on COX-2 induction could be due 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 utilized 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 $sPLA_2$s 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 re-internalized to exert an hydrolytic action at various cellular sites [16, 25, 33, 62, 63]. The ability of secreted $sPLA_2$-V to act on neighboring cells to induce phospholipid hydrolysis at physiological concentrations has been unambiguously demonstrated [29, 36, 62]. 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 effect 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 act 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 prior to or during secretion.

Although scalaradial is a selective inhibitor of $sPLA_2$, caution needs to be exercised when using this compound, as 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 non-selective 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 (i) 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 (ii) 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 cP$LA_2\alpha$-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_2s enzymes in regulating this process.

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Disclosures

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FOOTNOTES

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[2]Corresponding author: Instituto de Biología y Genética Molecular (IBGM-CSIC), Calle Sanz y Forés s/n, 47003 Valladolid, Spain. Phone: 34-983-423-062; FAX: 34-983-184-800; E-mail: jbalsinde@ibgm.uva.es

[3]The abbreviations used are: AA, arachidonic acid; PLA_2, phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; $cPLA_2\alpha$, Group IVA cytosolic phospholipase $A_2\alpha$; $sPLA_2$-V, Group V secreted phospholipase A_2; COX-2 cyclooxygenase-2; LPS, bacterial lipopolysaccharide; MAFP, methyl arachidonyl fluorophosphonate; BEL, bromoenol lactone; lysoPC, lysophosphatidylcholine; OA, oleic acid.

FIGURE LEGENDS

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 under "Experimental Procedures".

Figure 2. *Scalaradial effects on PLA$_2$ activities and COX expression*. The cells were exposed to 100 ng/ml LPS in the basence 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 under "Experimental Procedures". The effect of scalaradial on the activity of pure rat sPLA$_2$-V (50 ng/assay) is shown in panel C.

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. Afterwards, COX-2 protein content was measured by immunoblot.

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 methyl

arachidonyl fluorophosphonate (MAFP) (B). Afterwards, COX-2 protein content was measured by immunoblot.

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 (open bars) or presence (black bars) 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.

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 and 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.

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 under "Experimental Procedures".

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). Panel B shows a densitometric quantitation of the bands in panel A (black bars, COX-2; gray bars, c-Rel). Panel C shows a plot of c-Rel content versus COX-2 content at each radicicol concentration.

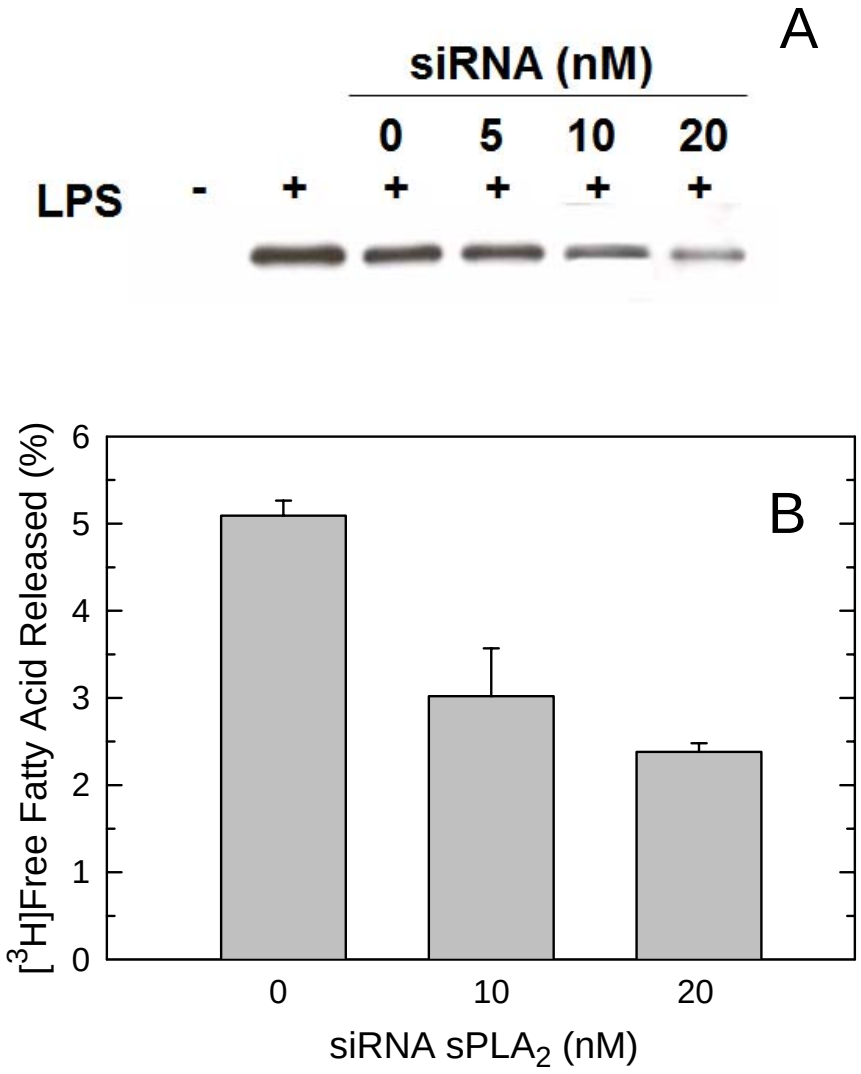
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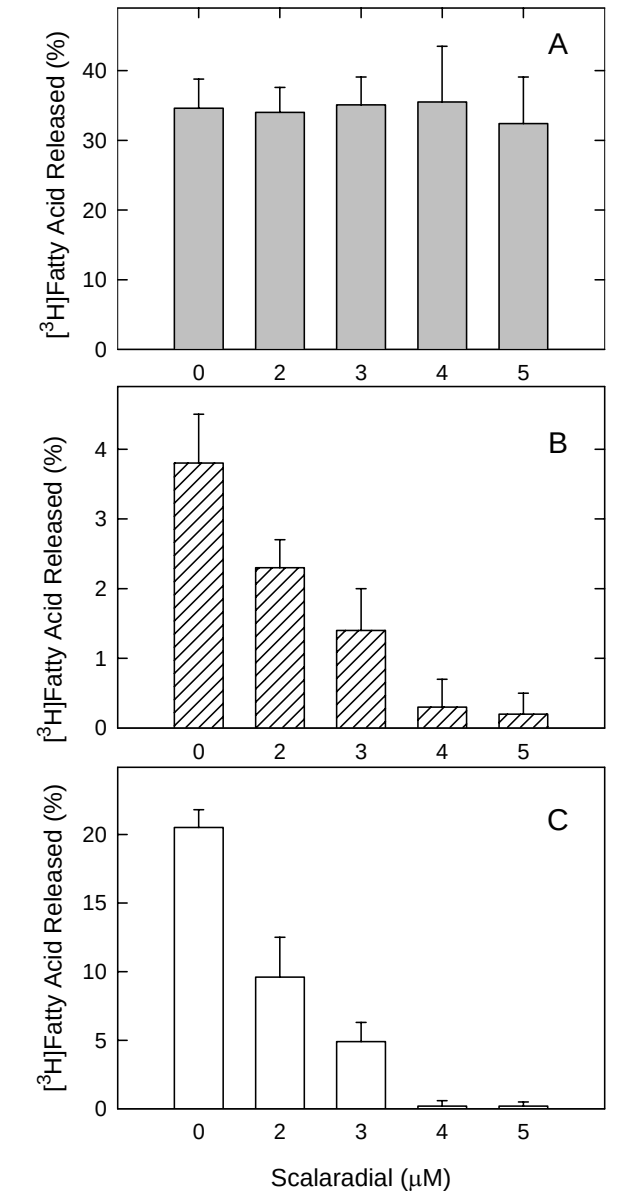
Figure 1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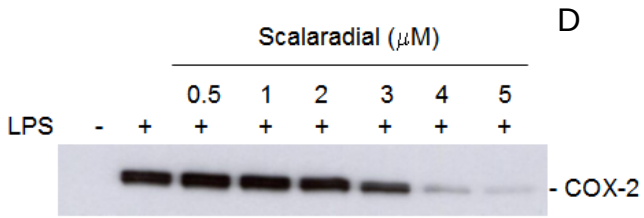
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Figure 2

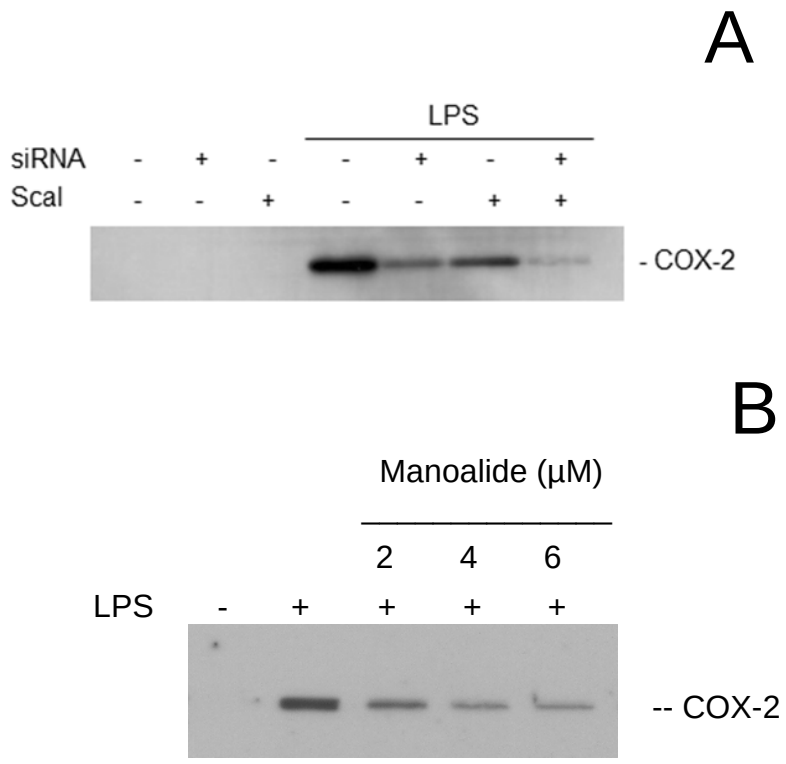

Figure 3

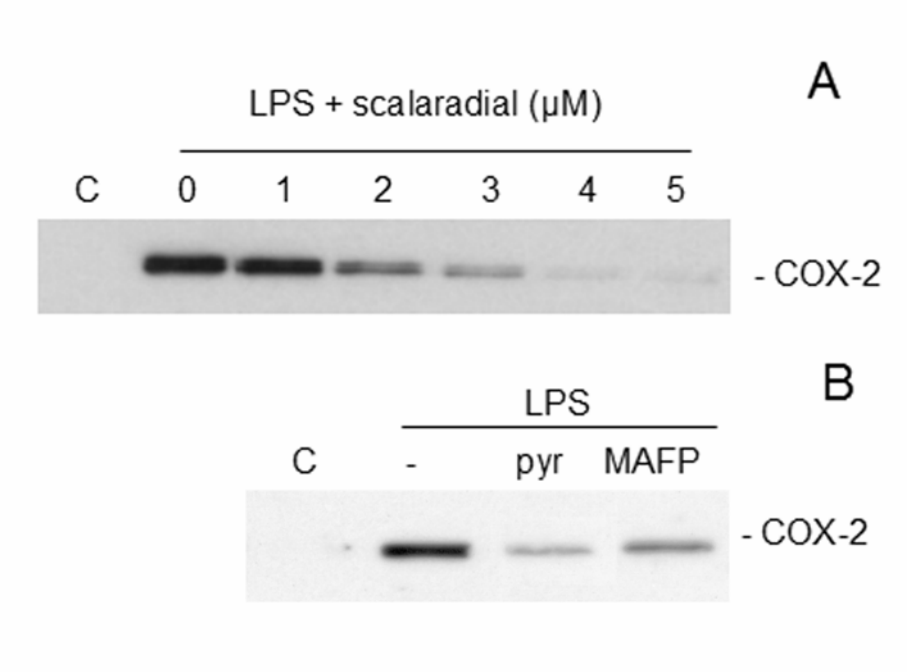

Figure 4

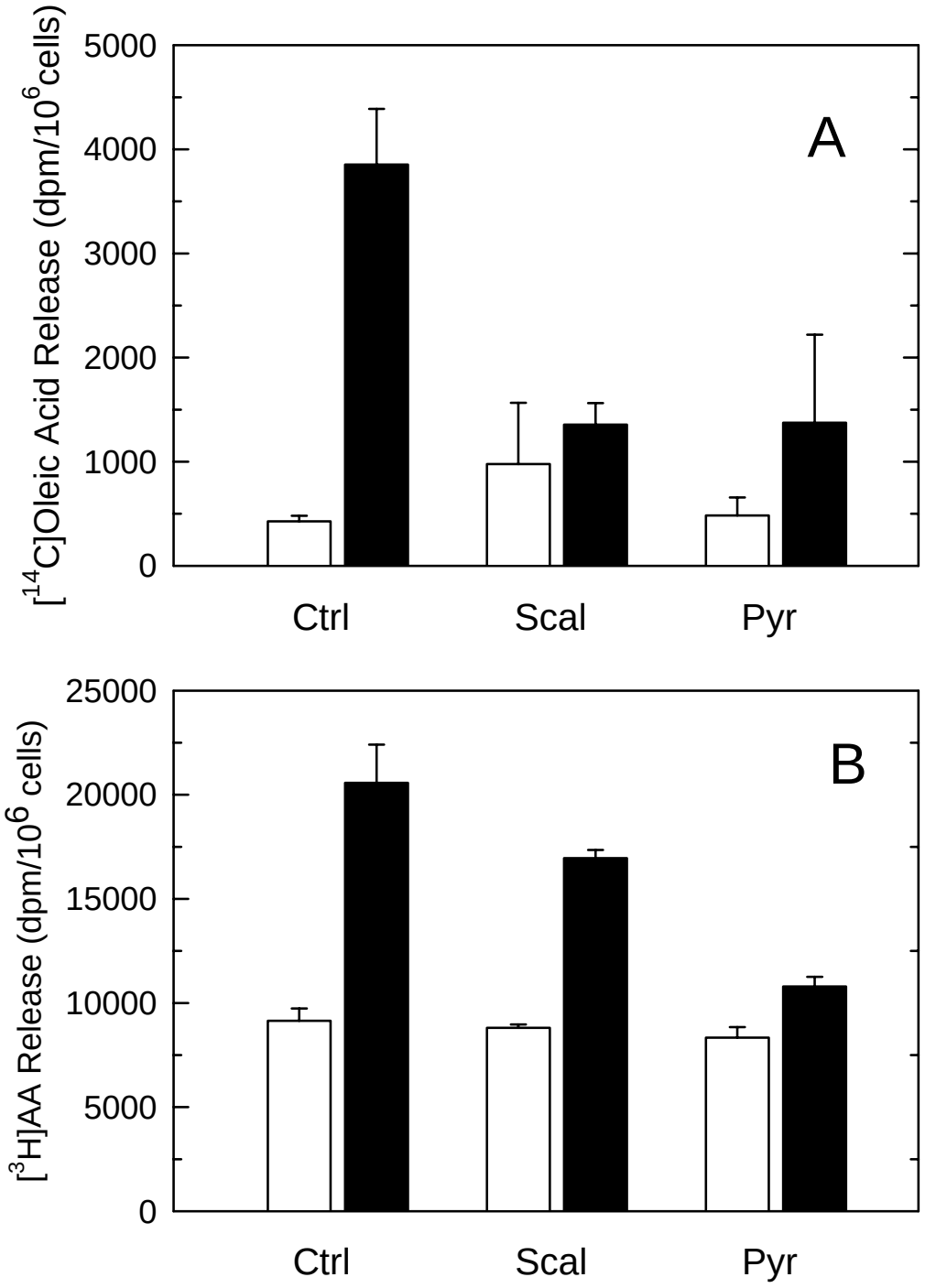
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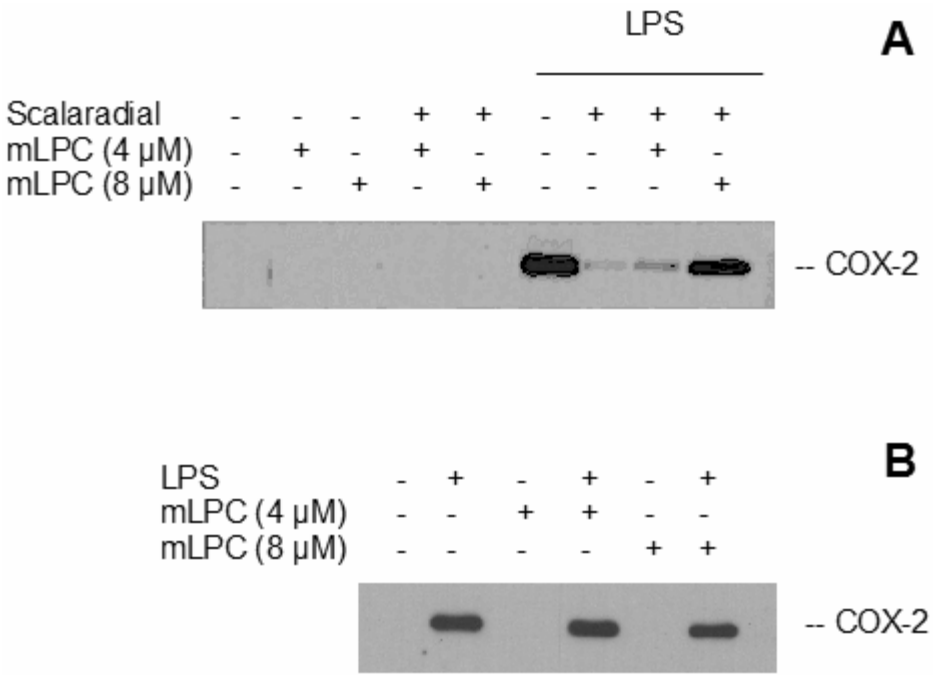
Figure 5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mLPC (8 µM) - - - - + +
-- COX-2

Figure 6

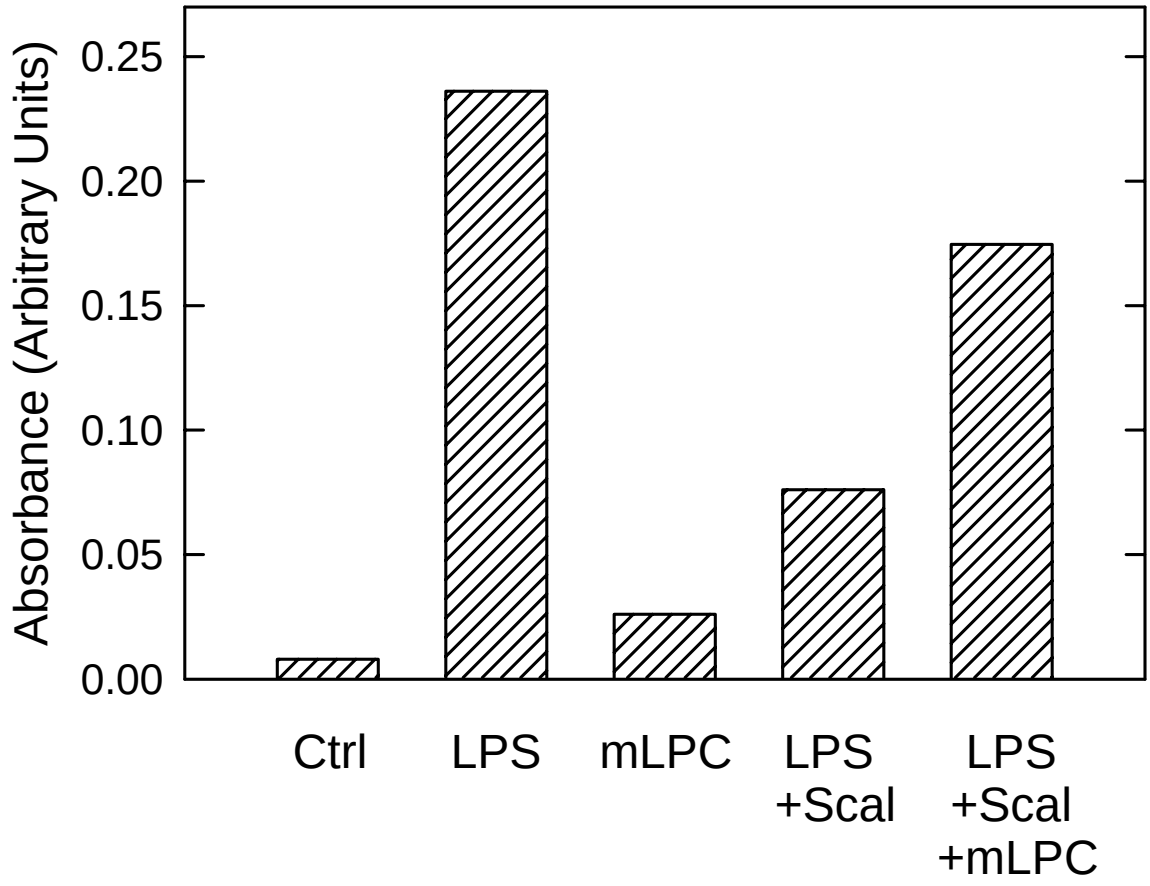
Absorbance (Arbitrary Units)
0.25
0.20
0.15
0.10
0.05
0.00
Ctrl
LPS
mLPC
LPS +Scal
LPS +Scal +mLPC

Figure 7

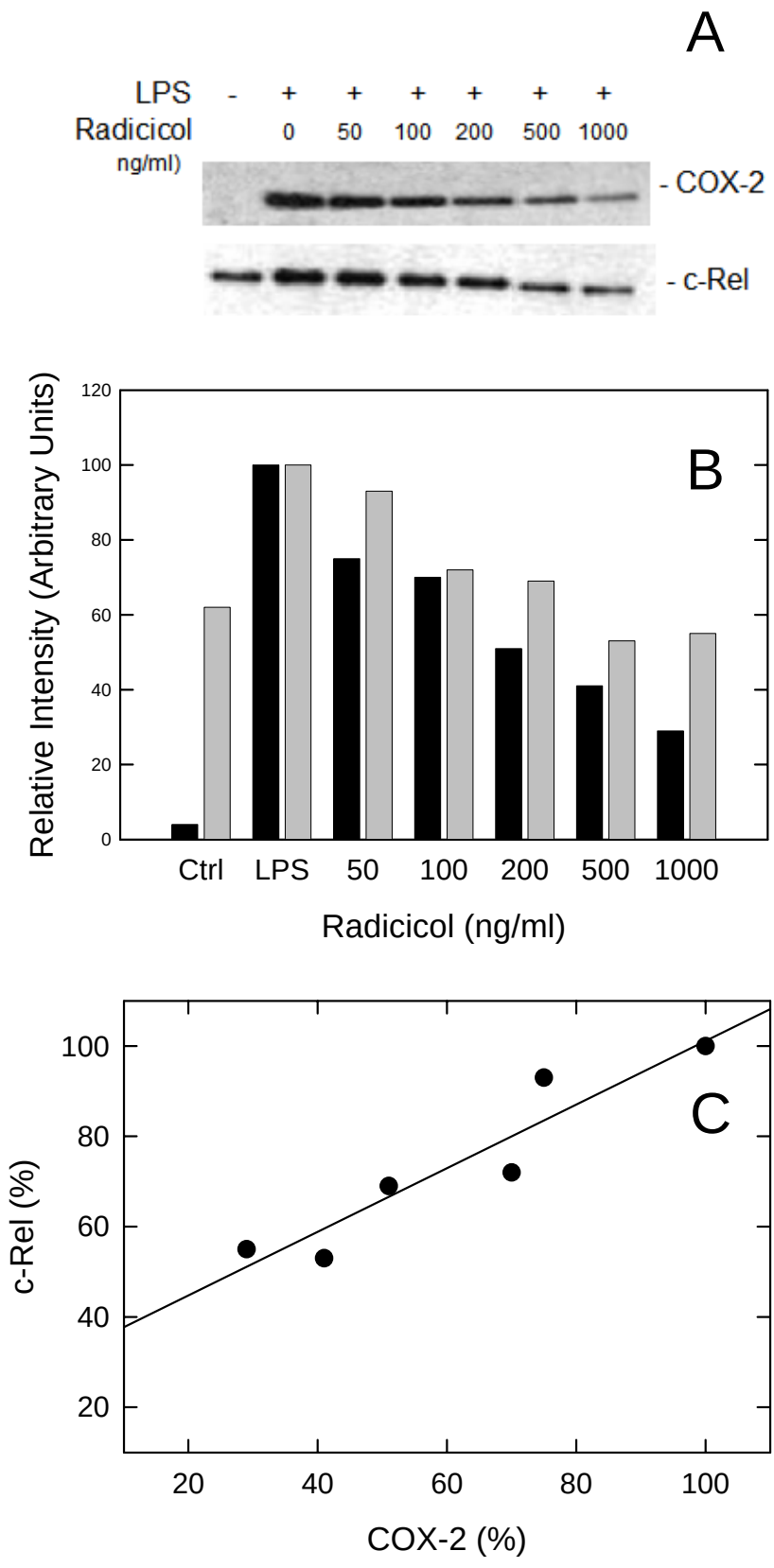
A
LPS - + + + + + +
Radicicol 0 50 100 200 500 1000
ng/ml)
- COX-2
- c-Rel
B
Relative Intensity (Arbitrary Units)
120
100
80
60
40
20
0
Ctrl LPS 50 100 200 500 1000
Radicicol (ng/ml)
C
c-Rel (%)
100
80
60
40
20
20 40 60 80 100
COX-2 (%)

Figure 8