Coordinate Regulation of TLR-Mediated Arachidonic Acid Mobilization in Macrophages by Group IVA and Group V Phospholipase A_2s[1]

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Macrophages can be activated through TLRs for a variety of innate immune responses. In contrast with the wealth of data existing on TLR-dependent gene expression and resultant cytokine production, very little is known on the mechanisms governing TLR-mediated arachidonic acid (AA) mobilization and subsequent eicosanoid production. We have previously reported the involvement of both cytosolic group IVA phospholipase A_2 ($cPLA_2$) and secreted group V phospholipase A_2 ($sPLA_2$-V) in regulating the AA mobilization response of macrophages exposed to bacterial LPS, a TLR4 agonist. In the present study, we have used multiple TLR agonists to define the role of various PLA_2s in macrophage AA release via TLRs. Activation of $P388D_1$ and RAW2647.1 macrophage-like cells via TLR1/2, TLR2, TLR3, TLR4, TLR6/2, and TLR7, but not TLR5 or TLR9, resulted in AA mobilization that appears to involve the activation of both $cPLA_2$ and $sPLA_2$ but not of calcium-independent phospholipase A_2. Furthermore, inhibition of $sPLA_2$-V by RNA interference or by two cell-permeable compounds, namely scalaradial and manoalide, resulted in a marked reduction of the phosphorylation of ERK1/2 and $cPLA_2$ via TLR1/2, TLR2, TLR3, and TLR4, leading to attenuated AA mobilization. Collectively, the results suggest a model whereby $sPLA_2$-V contributes to the macrophage AA mobilization response via various TLRs by amplifying $cPLA_2$ activation through the ERK1/2 phosphorylation cascade. *The Journal of Immunology,* **2009, 182: 3877–3883.**

Phospholipase A_2 (PLA_2)[3] enzymes hydrolyze membrane phospholipids at the sn-2 position of the glycerol backbone, releasing a free fatty acid and a lysophospholipid. This reaction is particularly important when the fatty acid liberated is arachidonic acid (AA), which can be converted into biologically active compounds called the eicosanoids (1–5). In the latest update, the PLA_2 enzymes were classified into 15 group types, according to their primary sequence (1). Additionally, a 16th PLA_2 group has been reported very recently (6). However, a second classification of the PLA_2 enzymes, sometimes more useful, also exists that categorizes the enzymes into five major families attending to biochemical commonalities (2, 7). These families are the Ca^{2+}-dependent secreted enzymes, the Ca^{2+}-dependent cytosolic enzymes, the Ca^{2+}-independent cytosolic enzymes, the platelet-activating factor acetyl hydrolases, and the lysosomal PLA_2s. Of these families, the first two have been repeatedly implicated in AA mobilization in response to a variety of immunoinflammatory stimuli (1–5). Today, there is general consensus that the calcium-dependent cytosolic group IVA PLA_2 ($cPLA_2$) is the critical enzyme in AA release (8, 9) and that, depending on cell type and stimulation conditions, a secreted PLA_2, in particular that belonging to groups IIA, V and X, may also participate by creating an amplification loop (10–14).

TLRs are a family of structural proteins in mammals related to Toll receptors in *Drosophila*. TLRs play a central role in inflammation and innate immune defense by recognizing specific structures of pathogens (15, 16). To date, 13 members of the TLR family have been identified in mice. TLR4, the first mammalian TLR identified and the best characterized member of this family (15–18), has been established as an essential component in the recognition of Gram-negative bacterial LPS. Additionally, TLR4 recognizes several other ligands. TLR2 recognizes a variety of microbial components and cooperates with TLR1 or TLR6 to recognize triacyl and diacyl lipopeptides, respectively. TLR5 recognizes bacterial flagellin. TLR3 recognizes dsRNA, and TLR7 and TLR9 recognize single-stranded nucleic acids. (15–18).

In previous work we have extensively investigated the mechanism of PLA_2-mediated AA mobilization in $P388D_1$ macrophage-like cells stimulated via TLR4 by bacterial LPS (19–26). In the present work, we have extended these studies to other TLRs. Our studies highlight the differential contributions of $cPLA_2$ and secreted group V phospholipase A_2 ($sPLA_2$-V) to the AA mobilization response and, in addition, suggest a role for $sPLA_2$-V in regulating ERK-dependent $cPLA_2$ phosphorylation in response to stimulation of some, but not all, TLRs.

Materials and Methods

Reagents

$P388D_1$ macrophage-like cells (MAB clone) (19) were provided by Dr. Ed Dennis (University of California at San Diego, La Jolla, CA). RAW 264.7 macrophage-like cells were obtained from the American Type Culture Collection. RPMI 1640 medium and FBS were from Invitrogen.

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[3] Abbreviations used in this paper: PLA_2, phospholipase A_2; AA, arachidonic acid; $cPLA_2$, group IVA cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; $sPLA_2$-V, group V secreted phospholipase A_2; BEL, bromoenol lactone; Pam3SCK4, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-Cys-[S]-Ser-[S]-Lys(4) trihydrochloride; LTA, lipoteichoic acid from *Staphylococcus aureus*; FSL-1, *S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Ser-Phe; ODN1826, synthetic oligodeoxynucleotide 1826, 5′-TCCATGACGTTCCTGACGTT-3′; siRNA, small interfering RNA; $iPLA_2$, calcium-independent phospholipase A_2.

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[5,6,8,9,11,12,14,15-^{3}H]AA (sp. act. 214 Ci/mmol) was from GE Healthcare. LPS (*Escherichia coli* 0111: B4), poly(I:C), and bromoenol lactone (BEL), were from Sigma-Aldrich. 12-Epi-scalaradial and manoalide were from BIOMOL. The TLR agonists *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-Cys-[S]-Ser-[S]-Lys(4) trihydrochloride (Pam3SCK4), lipoteichoic acid from *Staphylococcus aureus* (LTA), flagellin from *Salmonella typhimurium*, *S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Ser-Phe (FSL-1), imiquimod, and synthetic oligodeoxynucleotide 1826 (5′-TCCATGACGTTCCTGACGTT-3′; ODN1826) were purchased from InvivoGen. Rabbit polyclonal Abs that detect activating phosphorylations of ERK1/2 (Thr^{202}/Tyr^{204}) and $cPLA_2$ (Ser^{505}) were from Cell Signaling Technology. All other reagents were from Sigma-Aldrich.

Cell culture and labeling conditions

$P388D_1$ and RAW264.7 cells were maintained at 37°C in a humidified atmosphere at 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 5% and 10% FBS, respectively, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. 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 cells was achieved by including 0.25 μCi/ml during the overnight adherence period (20 h). Labeled fatty acid that had not been incorporated into cellular lipids was removed by washing the cells twice with serum-free medium containing 0.5 mg/ml albumin. Stimuli were added for 20 h at the following doses: LPS, 100 ng/ml; poly(I:C), 25 μg/ml; Pam3SCK4, 1 μg/ml; LTA, 1 μg/ml; flagellin, 50 ng/ml; FSL-1, 1 μg/ml; imiquimod, 5 μg/ml; ODN1826, 1 μM. Preliminary experiments indicated that at the above-specified concentrations, the inhibitors induced maximal responses. When inhibitors were used they were added 30 min before the stimuli. In phosphorylation assays, TLR agonists were added for 30 min.

Small interfering RNA (siRNA) inhibition studies

The siRNA directed against $sPLA_2$-V was from Eurofins MWG Operon (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) under serum-free conditions for 6 h. Afterward, 5% serum was added and the cells were maintained at normal culture conditions for 20 h. Afterward, the cells were used for experiments as described above. A scrambled siRNA was used as a negative control.

Immunoblot analyses

Cells were serum-starved and stimulated in the absence or presence 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, 10 mg/ml aprotinin, and 10 mg/ml leupeptin at 4°C. Protein was quantified, and a 50-μg aliquot for p-ERK1/2 and p-$cPLA_2$ was separated by SDS/PAGE (10% acrylamide) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked with 5% nonfat in PBS and then incubated with specific Abs. Bands were detected by the Amersham ECL system.

Measurement of extracellular fatty acid release

The cells were placed in serum-free medium for 1 h before the addition of TLR agonists in the absence or presence of inhibitors and in the presence of 0.5 mg/ml BSA to blunt fatty acid reacylation (27). After the 24-h incubation period, the supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

PLA_2 activity measurements

PLA_2 activity assays were conducted as previously described (28–35). Briefly, for calcium-independent PLA_2 ($iPLA_2$) activity, homogenates from cells treated with 10 μM BEL for 30 min were prepared, and 100-μg aliquots were incubated for 2 h at 37°C in 100 mM HEPES (pH 7.5) containing 5 mM EDTA and 100 μM labeled phospholipid substrate, 1-palmitoyl-2-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine, in a final volume of 150 μl. The phospholipid substrate was used in the form of sonicated micelles in buffer. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1:2). After lipid extraction, free [^{3}H]palmitic acid was separated by thin-layer chromatography using *n*-hexane/ethyl ether/acetic acid (70:30:1, v/v/v) as the mobile phase. For measuring $sPLA_2$ activity, the mammalian membrane assay described by Diez et al. (36) was used. Briefly, aliquots of U937 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, in a final volume of 0.15 ml. Before assay, the cell membrane substrate was heated at 57°C for 5 min to inactivate CoA-independent transacylase activity. The assay contained 1 μM pyrrophenone and 25 μM BEL to completely inhibit endogenous $cPLA_2$ and $iPLA_2$ activities (37, 38). After lipid extraction, free [^{3}H]AA was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1, v/v/v) as a mobile phase.

Table I. *TLR agonists used in this study*

Agonist	Description	TLR
Pam3CSK4	*N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-Cys-[S]-Ser-[S]-Lys(4)	1/2
LTA	Lipoteichoic acid from *Staphylococcus aureus*	2
Poly(I:C)	Polyinosinic-polycytidylic acid	3
LPS	LPS from *Escherichia coli* 0111:B4	4
Flagellin	Flagellin from *Salmonella typhimurium*	5
FSL-1	*S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Ser-Phe	6/2
Imiquimod	1-isobutyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine	7
ODN1826	5′-TCCATGACGTTCCTGACGTT-3′	9

Other methods

Protein concentration was determined utilizing the Bradford protein assay kit (Bio-Rad) with BSA as a standard. Densitometric quantitation of bands was analyzed by Quantity One program (Bio-Rad).

Data presentation

Unless otherwise stated, data are presented as the means ± SEM of at least three different experiments.

Results

TLR-mediated AA mobilization in $P388D_1$ cells

We have previously reported that both $cPLA_2$ and $sPLA_2$-V participate in the AA mobilization response of $P388D_1$ macrophage-like cells to LPS, a stimulus that signals through TLR4 (19–26). In the present work we have extended our studies to other TLRs, of which very little information is available with regard to AA mobilization and eicosanoid production. Thus, we began this study by analyzing the AA release response of $P388D_1$ macrophage-like cells to a variety of TLR agonists, which are listed in Table I. In keeping with our previous data (19–26), engagement of TLR4 receptors by LPS induced abundant AA mobilization. Additionally, stimulation via TLR1/2 ($Pam3CSK_4$), TLR2 (LTA), TLR3 (poly(I:C)), TLR6/2 (FSL-1), and TLR7 (imiquimod) also significantly enhanced AA release (Fig. 1*A*). However, stimulation of cells via TLR5 (flagellin) or TLR9 (ODN1826) had little or no effect on the response (Fig. 1*A*). To ensure that these results are physiologically relevant, we sought to discard unwanted cell-specific effects by conducting the same measurements on another murine macrophage-like cell line from a different genetic background. We used the cell line RAW264.7, which is derived from BALB/c mice. The $P388D_1$ cell line is derived from DBA/2 mice. Both cell lines are mature murine macrophage cell lines with similar phenotypic and functional characteristics (39–41). Furthermore, we have characterized by PCR the expression profile of PLA_2 isoforms potentially involved in AA release in these cell lines and found it to be comparable, that is, both cell lines express group V, group IVA, and group VIA PLA_2s, but not group IIA or group X PLA_2s (V. Ruipérez, Y. Sáez, and J. Balsinde, unpublished results). Compared with $P388D_1$ cells, activated RAW264.7 cells

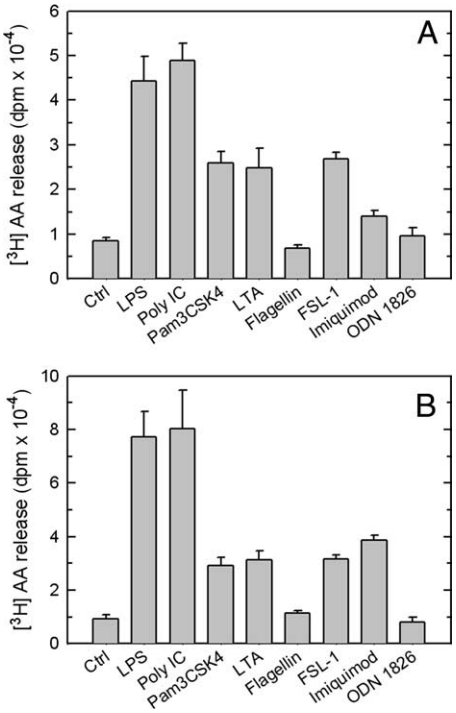

FIGURE 1. TLR-mediated AA mobilization in macrophage-like cell lines. $P388D_1$ (*A*) or RAW 264.7 (*B*) cells, labeled with [^{3}H]AA, were incubated with various TLR agonists for 20 h at the concentrations indicated in *Materials and Methods*. [^{3}H]AA in the extracellular media was estimated by scintillation counting.

generally released higher amounts of AA to the extracellular medium. However, qualitatively, the results were very similar in both cell lines, that is, significant AA release in response to agonists of TLR1/2, TLR2, TLR3, TLR4, TLR6/2, and TLR7, and no effect to agonists of TLR5 and TLR9 (Fig. 1*B*).

PLA_2 forms potentially capable of catalyzing AA mobilization are usually categorized into three major families, namely the Ca^{2+}-dependent $cPLA_2$s, the $sPLA_2$s, and the $iPLA_2$s (2, 7). Macrophage cells are known to express all three classes of PLA_2 (37). Thus, a useful way to initially distinguish among the PLA_2s is the use of chemical inhibitors that selectively inhibit each of the forms. To this end, we utilized pyrrophenone ($cPLA_2$ inhibitor), scalaradial ($sPLA_2$ inhibitor), and BEL ($iPLA_2$ inhibitor) (38). In previous work we have extensively characterized the effect of these inhibitors on cells and demonstrated that, at the concentrations employed in this work, none of the inhibitors interferes with PLA_2 activities distinct from those against which the inhibitors are directed (26, 28–35). Table II shows that 1 μM pyrrophenone almost

Table II. *Effect of PLA_2 inhibitors on TLR-mediated AA release in $P388D_1$ cells*[a]

Agonist	Pyrrophenone	Scalaradial	BEL
Pam3CSK4	1 ± 1	52 ± 1	100 ± 6
LTA	2 ± 1	49 ± 3	100 ± 4
Poly(I:C)	6 ± 1	76 ± 1	89 ± 4
LPS	7 ± 2	77 ± 2	92 ± 6
FSL-1	2 ± 1	50 ± 1	85 ± 4
Imiquimod	5 ± 1	62 ± 3	100 ± 1

[a] The cells, labeled with [^{3}H]AA, were treated with 1 μM pyrrophenone, 4 μM scalaradial, or 10 μM BEL, as indicated, for 30 min. Afterward the cells were incubated with the indicated TLR agonists for 20 h. AA in the supernatants was measured by scintillation counting. The results are expressed as a percentage of the response observed to each stimulus in the absence of inhibitor.

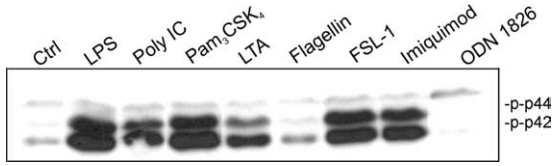

FIGURE 2. ERK1/2 phosphorylation in response to TLR agonists. $P388D_1$ cells were treated with TLR agonists for 30 min at the concentrations indicated in *Materials and Methods*, and ERK1/2 phosphorylation was determined by immunoblot.

completely ablated the AA release response to all TLR agonists used, pointing to $cPLA_2$ as the key regulatory enzyme. Interestingly, the $sPLA_2$ inhibitor scalaradial also produced significant inhibitory responses, albeit much lower than those elicited by pyrrophenone (Table II). On the other hand, the $iPLA_2$ inhibitor BEL exerted no significant effects on any of the agonists tested (Table II), suggesting no role for $iPLA_2$ in TLR-mediated AA mobilization. Since the BEL experiments produced negative results, and to assess whether the inhibitor was indeed acting on the $iPLA_2$ under these conditions, we measured as a control the $iPLA_2$ activity of homogenates from BEL-treated cells. We found a 70 ± 6% reduction of endogenous $iPLA_2$ activity in BEL-treated homogenates with respect to that measured in homogenates from untreated cells. Thus, our inability to detect any effect of $iPLA_2$ inhibition on TLR-mediated AA release under conditions where cellular $iPLA_2$ activity is effectively blunted demonstrates the lack of involvement of $iPLA_2$ in the response under study.

$sPLA_2$ modulates ERK and $cPLA_2$ phosphorylation during stimulation via TLRs

In the next series of experiments, we directed our attention toward the enzymes that activate $cPLA_2$. Phosphorylation of $cPLA_2$ on Ser^{505} results in an ~2–2.5-fold increase in its specific activity (42). Many of the data currently available in the literature suggest that $cPLA_2$ is phosphorylated and activated by either the ERK1/2 (p44/p42) or p38 isoforms of MAPK; the activation mechanism may vary with the cell type and activation regimen (42). In this regard, we have previously shown that phosphorylation activation of the AA-releasing $cPLA_2$ at Ser^{505} occurs during stimulation of $P388D_1$ macrophage-like cells by various agonists, including the TLR4 agonist LPS, and that this phosphorylation is effected by the ERKs p42 (ERK1) and p44 (ERK2) (43, 44). Fig. 2 shows that these two kinases are activated in $P388D_1$ cells stimulated not only via TLR4, but also via TLR1/2, TLR2, TLR3, TLR6/2, and TLR7. In our hands, agonists of TLR5 and TLR9 did not have any effect. Note that this pattern of ERK phosphorylation activation by TLR agonists is identical to that observed previously for AA mobilization (Fig. 1). Interestingly, when the ERK phosphorylation measurements were conducted in cells incubated with 4 μM scalaradial to block $sPLA_2$ activity, a clear inhibition of the phosphorylation of the ERKs was detected in response to stimulation via TLR1/2, TLR2, TLR3, and TLR4 (Fig. 3*A*). Intriguingly, phosphorylation activation of the ERKs via TLR6/2 and TLR7 was not appreciably changed by the presence of scalaradial (Fig. 3*A*), which highlights a selectivity of action of the drug. Thus, depending on the agonist, there appear to be scalaradial-dependent and -independent pathways for ERK phosphorylation within the same cell type. More importantly, the phosphorylation of $cPLA_2$ was also found to be inhibited in scalaradial-treated cells stimulated via TLR1/2, TLR2, TLR3, and TLR4 (Fig. 3*A*).

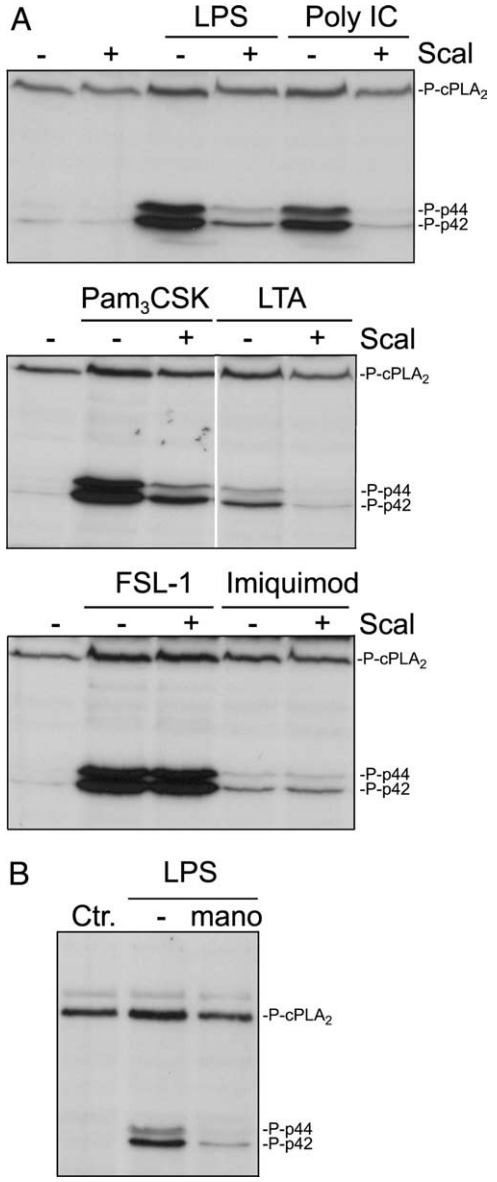

FIGURE 3. Effect of $sPLA_2$ inhibition on ERK1/2 and $cPLA_2$ phosphorylation. *A*, $P388D_1$ cells were treated with (+) or without (−) 4 μM scalaradial (Scal) for 30 min, as indicated. Afterward the cells were exposed to the TLR agonists for 30 min at the concentrations indicated in *Materials and Methods*, and the phosphorylation of ERK1/2 and $cPLA_2$ was determined by immunoblot. *B*, $P388D_1$ cells were treated with 4 μM manoalide, exposed to LPS for 30 min, and the phosphorylation of ERK1/2 and $cPLA_2$ was determined by immunoblot.

The effect of another $sPLA_2$ inhibitor, manoalide (26), on TLR-dependent phosphorylation of ERKs and $cPLA_2$ 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 (26), also promoted a marked inhibition of the phosphorylation reactions. Because manoalide is structurally unrelated to scalaradial, these data provide additional independent evidence for the role of cellular $sPLA_2$ in regulating the phosphorylation state of $cPLA_2$.

Collectively, the results shown in Fig. 3, obtained with two structurally unrelated $sPLA_2$ inhibitors, are suggestive of $sPLA_2$ activity regulating ERK activation by TLRs and, in turn, $cPLA_2$ phosphorylation and enhanced AA release. However, studies using chemical inhibitors are limited in their ability to identify a particular group of $sPLA_2$. To confirm the molecular identity of the $sPLA_2$ and, in turn, to obtain more definite evidence for the role of $sPLA_2$ in regulating $cPLA_2$, we utilized siRNA technology to selectively knock down the expression of $sPLA_2$-V, the enzyme that accounts for most of the $sPLA_2$ activity of $P388D_1$ cells (45), and also the form that has been implicated by us and others as being involved in AA mobilization in cells relevant to innate immunity, such as murine macrophages and mast cells (46, 47). Since we have been unable to find reliable Abs against murine $sPLA_2$-V, the efficiency of siRNA knockdown was judged by enzyme activity assay in cell homogenates. As shown in Fig. 4, homogenates from cells deficient in $sPLA_2$-V showed a marked reduction in $sPLA_2$ activity as compared with negative controls. Importantly, when the effect of scalaradial was studied in homogenates from $sPLA_2$-V-deficient cells, no further inhibition of activity was detected, providing experimental support to the idea that $sPLA_2$-V contributes to most of the $sPLA_2$ activity of $P388D_1$ cells (44). In turn, the data of Fig. 4 provide additional evidence of the selectivity of action of scalaradial on $sPLA_2$-V in $P388D_1$ macrophage-like cells.

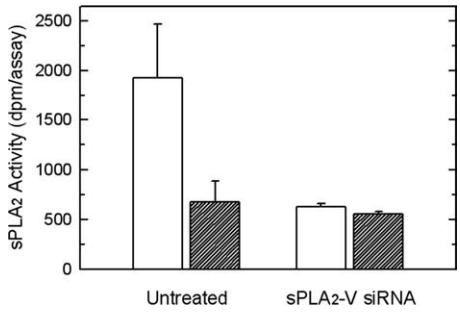

FIGURE 4. Effect of siRNA targeting $sPLA_2$-V on $sPLA_2$ activity. $P388D_1$ cells were treated or not with siRNA targeting $sPLA_2$-V, as indicated, and $sPLA_2$ activity was measured in cell homogenates from LPS-treated cells in the absence (open bars) or presence (dashed bars) of 4 μM scalaradial.

Blockade of $sPLA_2$-V expression by siRNA resulted in the inhibition of the phosphorylation of the ERKs in response to stimulation via TLR4 (Fig. 5), and also in the phosphorylation of $cPLA_2$ (Fig. 5). Collectively, these results suggest that, in addition to any direct effect of $sPLA_2$-V on cellular phospholipids,

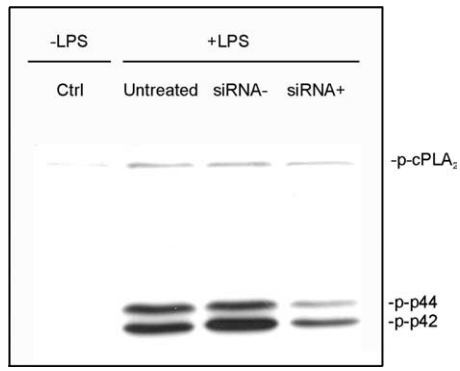

FIGURE 5. Inhibition of phosphorylation of ERK1/2 and $cPLA_2$ by siRNA targeting $sPLA_2$-V in TLR4-stimulated cells. Cells were either untreated or transfected with a siRNA negative control ($siRNA^-$) or siRNA targeting $sPLA_2$-V ($siRNA^+$). Afterward, the cells were incubated with or without 100 ng/ml LPS for 30 min, as indicated, and the phosphorylation of ERK1/2 and $cPLA_2$ was determined by immunoblot.

$sPLA_2$-V may participate in TLR-mediated AA mobilization by modulating the phosphorylation state of $cPLA_2$.

Discussion

TLRs are a class of receptors on the surface of immune cells that recognize structurally conserved molecules derived from pathogens (15–18). TLRs interact with different combinations of adapter proteins and activate transcription factors leading to specific immune responses (15–18). In contrast to the wealth of data that exist on TLR-dependent gene expression and resultant cytokine expression, very little is known on the mechanisms governing TLR-mediated AA mobilization and eicosanoid production. Most of what is known in this regard has been obtained from studies using the TLR4 agonist LPS as a model stimulus (48).

Aside from TLR4, there are very limited data on the AA mobilization mechanisms triggered by engagement of other TLRs. Here we have comparatively investigated the effectors involved in the AA release response triggered by various TLRs in murine macrophages. An unexpected finding is that not all TLRs couple to stimulation of AA mobilization. Stimulation of the cells via TLR5 and TLR9 fail to promote significant AA mobilization, an observation that was made utilizing two different cell lines, namely $P388D_1$ and RAW264.7. It seems likely that cellular stimulation of the cells by TLR5 or TLR9 fails to recruit an adapter/regulatory protein that is crucial for eliciting the AA release response. Studies are ongoing in our laboratory to identify regulatory components of TLR signaling that are not recruited after TLR5/TLR9 occupancy but may be involved in signaling via other TLRs.

Our inability to detect significant AA mobilization via TLR9 in two macrophage cell lines is at variance with a very recent study by Lee et al. (49), who reported significant AA release via TLR9 in RAW264.7 cells. At present we have no explanation for this discrepancy, and we speculate that it may be due to differences in the experimental conditions utilized. It has been demonstrated that IFN-γ can sensitize murine macrophages to produce various cytokines in response to TLR9 stimulation (50–52). This raises the intriguing possibility that a "priming step" could be necessary for the cells to mobilize AA in response to TLR9 agonists. Regarding the lack of effect of TLR5 agonists on AA mobilization in our hands, another recent report by Buczynski et al. (53) also failed to detect eicosanoid production via stimulation of this receptor by flagellin in RAW264.7 macrophage-like cells.

On the other hand, stimulation of TLR1/2, TLR2, TLR3, TLR4, TLR6/2, and TLR7 by the appropriate stimuli results in significant AA mobilization, although some stimuli behave as more potent than others. In particular, stimulation via TLR3 and TLR4 yields very robust responses. A pharmacological analysis of PLA_2 effectors potentially involved in the response clearly reflected the participation of $cPLA_2$ and $sPLA_2$ but not of $iPLA_2$. It is striking that the pharmacological profile was identical for all the TLRs, that is, inhibition of $cPLA_2$ by pyrrophenone almost ablates the AA responses to all agonists, inhibition of $sPLA_2$ by scalaradial partially blunts it, and inhibition of $iPLA_2$ by BEL has no significant effect. Our data are fully consistent with recent data in murine peritoneal macrophages and mast cells showing that disruption of the $cPLA_2$ gene nearly abrogates eicosanoid biosynthesis, while disruption of the $sPLA_2$-V gene leads to a 35–50% reduction of the response (54–56). Thus, our data are in line with a scenario whereby $cPLA_2$ acts as the main effector of AA release, and $sPLA_2$ acts to amplify the $cPLA_2$-mediated response (57, 58).

The involvement of two different kinds of PLA_2, namely $sPLA_2$ and $cPLA_2$, raises the question of whether there is coordinated action between the two enzymes in regulating AA release via engagement of TLR receptors. A major mechanism for the regulation of $cPLA_2$ is the phosphorylation of the enzyme at Ser^{505}, which results in an increased ability of the enzyme to hydrolyze membrane phospholipids (8, 42). Depending on cell type and/or stimulus, various members of the MAPK family may catalyze $cPLA_2$ phosphorylation (8, 42). In the $P388D_1$ macrophage-like cell system we previously showed that phosphorylation of $cPLA_2$ at Ser^{505} occurs during stimulation of the cells via TLR4, and that this phosphorylation is mediated by the ERKs p42 (ERK1) and p44 (ERK2) (43). We demonstrate in this work that inhibition of $sPLA_2$-V expression and activity by either siRNA or chemical inhibitors results in reduced phosphorylation of the ERKs and $cPLA_2$ in response to some but not all TLRs. Thus, our results suggest that $sPLA_2$-V participates in the AA mobilization response of various TLRs, namely TLR1/2, TLR2, TLR3, and TLR4, by modulating the phosphorylation state of $cPLA_2$ and, hence, its enzymatic activity. This is a relevant observation, since cross-talk between $sPLA_2$ and $cPLA_2$ enzymes in eliciting AA release has been repeatedly suggested in many instances, but a clear picture of this interaction is yet to emerge (5).

There is ample evidence that exogenous $sPLA_2$s and/or $sPLA_2$s overexpressed in various cell types are capable of amplifying the essential role of $cPLA_2$ in AA mobilization and subsequent eicosanoid synthesis (11, 14, 25, 59–61). $sPLA_2$-V could potentially be involved in $cPLA_2$-dependent AA mobilization through three pathways, one involving reinternalization via caveolin-rich domains (24, 62), the second involving direct interaction with phosphatidylcholine-rich outer membrane domains (62–64), and the third one involving an undefined intracellular action prior to secretion of the enzyme (65, 66). On the other hand, very limited evidence is available on the role of "endogenous", preexisting $sPLA_2$. In this regard, our results provide a molecular mechanism whereby $sPLA_2$-V modulates the activity of $cPLA_2$ by regulating its phosphorylation via ERK, which is in agreement with recent data in murine mast cells (58). Thus, in addition to any direct effect on phospholipid hydrolysis per se, endogenous $sPLA_2$-V may participate in the AA mobilization responses mediated by some TLRs by regulating the sequential phosphorylation of ERK1/2 and $cPLA_2$.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Schaloske, R., and E. A. Dennis. 2006. The phospholipase A_2 superfamily and its group numbering system. *Biochim. Biophys. Acta* 1761: 1246–1259.
2. Balsinde, J., M. V. Winstead, and E. A. Dennis. 2002. Phospholipase A_2 regulation of arachidonic acid mobilization. *FEBS Lett.* 531: 2–6.
3. Kudo, I., and M. Murakami. 2002. Phospholipase A_2 enzymes. *Prostaglandins Other Lipid Mediat.* 68–69: 3–58.
4. Balsinde, J., and M. A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells. *Cell. Signal.* 17: 1052–1062.
5. Balboa, M. A., and J. Balsinde. 2006. Oxidative stress and arachidonic acid mobilization. *Biochim. Biophys. Acta* 1761: 385–391.

6. Duncan, R. E., E. Sarkadi-Nagy, K. Jaworski, M. Ahmadian, and H. S. Sul. 2008. Identification and functional characterization of adipose-specific phospholipase A_2 (AdPLA). *J. Biol. Chem.* 283: 25428–25436.
7. Balsinde, J., R. Pérez, and M. A. Balboa. 2006. Calcium-independent phospholipase A_2 and apoptosis. *Biochim. Biophys. Acta* 1761: 1344–1350.
8. Ghosh, M., D. E. Tucker, S. A. Burchett, and C. C. Leslie. 2006. Properties of the group IV phospholipase A_2 family. *Prog. Lipid Res.* 45: 487–510.
9. Kita, Y., T. Ohto, N. Uozumi, and T. Shimizu. 2006. Biochemical properties and pathophysiological roles of cytosolic phospholipase A_2s. *Biochim. Biophys. Acta* 1761: 1317–1322.
10. Balsinde, J., S. E. Barbour, I. D. Bianco, and E. A. Dennis. 1994. Arachidonic acid mobilization in $P388D_1$ macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. *Proc. Natl. Acad. Sci. USA* 91: 11060–11064.
11. Murakami, M., S. Shimbara, T. Kambe, H. Kuwata, M. V. Winstead, J. A. Tischfield, and I. Kudo. 1998. The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release: type IIA and type V secretory phospholipase A_2s are functionally redundant and act in concert with cytosolic phospholipase A_2. *J. Biol. Chem.* 273: 14411–14423.
12. Balsinde, J., M. A. Balboa, and E. A. Dennis. 1998. Functional coupling between secretory phospholipase A_2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A_2. *Proc. Natl. Acad. Sci. USA* 95: 7951–7956.
13. Fujishima, H., R. Sánchez-Mejía, C. O. Bingham, B. K. Lam, A. Sapirstein, J. V. Bonventre, K. F. Austen, and J. P. Arm. 1999. Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proc. Natl. Acad. Sci. USA* 96: 4803–4807.
14. Han, W. K., A. Sapirstein, C. C. Hung, A. Alessandrini, and J. V. Bonventre. 2003. Cross-talk between cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and secretory phospholipase A_2 ($sPLA_2$) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: $sPLA_2$ regulates $cPLA_2\alpha$ activity that is responsible for arachidonic acid release. *J. Biol. Chem.* 278: 24153–24163.
15. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
16. Underhill, D. M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* 14: 103–110.
17. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449: 819–826.
18. Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* 24: 353–389.
19. Shinohara, H., M. A. Balboa, C. A. Johnson, J. Balsinde, and E. A. Dennis. 1999. Regulation of delayed prostaglandin production in activated $P388D_1$ macrophages by group IV cytosolic and group V secretory phospholipase A_2s. *J. Biol. Chem.* 274: 12263–12268.
20. Balsinde, J., H. Shinohara, L. J. Lefkowitz, C. A. Johnson, M. A. Balboa, and E. A. Dennis. 1999. Group V phospholipase A_2 dependent induction of cyclooxygenase-2 in murine $P388D_1$ macrophages. *J. Biol. Chem.* 274: 25967–25970.
21. Balsinde, J., M. A. Balboa, S. Yedgar, and E. A. Dennis. 2000. Group V phospholipase A_2-mediated oleic acid mobilization in lipopolysaccharide-stimulated $P388D_1$ macrophages. *J. Biol. Chem.* 275: 4783–4786.
22. Balsinde, J., M. A. Balboa, and E. A. Dennis. 2000. Identification of a third pathway for arachidonic acid mobilization and prostaglandin production in activated $P388D_1$ macrophage-like cells. *J. Biol. Chem.* 275: 22544–22549.
23. Balsinde, J., M. A. Balboa, W. H. Li, J. Llopis, and E. A. Dennis. 2000. Cellular regulation of cytosolic group IV phospholipase A_2 by phosphatidylinositol bisphosphate levels. *J. Immunol.* 164: 5398–5402.
24. Balboa, M. A., Y. Shirai, G. Gaietta, M. E. Ellisman, J. Balsinde, and E. A. Dennis. 2003. Localization of group V phospholipase A_2 in caveolin-enriched granules in activated $P388D_1$ macrophage-like cells. *J. Biol. Chem.* 278: 48059–48065.
25. Shirai, Y., J. Balsinde, and E. A. Dennis. 2005. Localization and functional interrelationships among cytosolic group IV, secreted group V, and Ca^{2+}-independent group VI phospholipase A_2s in $P388D_1$ macrophages using GFP/RFP constructs. *Biochim. Biophys. Acta* 1735: 119–129.
26. Ruipérez, V., J. Casas, M. A. Balboa, and J. Balsinde. 2007. Group V phospholipase A_2-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. *J. Immunol.* 179: 631–638.
27. Dieter, P., H. Krause, and A. Schulze-Specking. 1990. Arachidonate metabolism in macrophages is affected by albumin. *Eicosanoids* 3: 45–51.
28. Balsinde, J. 2002. Roles of various phospholipases A_2 in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. *Biochem. J.* 364: 695–702.
29. Balboa, M. A., and J. Balsinde. 2002. Involvement of calcium-independent phospholipase A_2 in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J. Biol. Chem.* 277: 40384–40389.
30. Fuentes, L., R. Pérez, M. L. Nieto, J. Balsinde, and M. A. Balboa. 2003. Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A_2. *J. Biol. Chem.* 278: 44683–44690.
31. Pérez, R., R. Melero, M. A. Balboa, and J. Balsinde. 2004. Role of group VIA calcium-independent phospholipase A_2 in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. *J. Biol. Chem.* 279: 40385–40391.
32. Balboa, M. A., R. Pérez, and J. Balsinde. 2003. Amplification mechanisms of inflammation: paracrine stimulation of arachidonic acid mobilization by secreted phospholipase A_2 is regulated by cytosolic phospholipase A_2-derived hydroperoxyeicosatetraenoic acid. *J. Immunol.* 171: 989–994.
33. Balboa, M. A., Y. Sáez, and J. Balsinde. 2003. Calcium-independent phospholipase A_2 is required for lysozyme secretion in U937 promonocytes. *J. Immunol.* 170: 5276–5280.
34. Pérez, R., X. Matabosch, A. Llebaria, M. A. Balboa, and J. Balsinde. 2006. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J. Lipid Res.* 47: 484–491.
35. Balboa, M. A., R. Pérez, and J. Balsinde. 2008. Calcium-independent phospholipase A_2 mediates proliferation of human promonocytic U937 cells. *FEBS J.* 275: 1915–1924.
36. Diez, E., J. Balsinde, M. Aracil, and A. Schüller. 1987. Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim. Biophys. Acta* 921: 82–89.
37. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase A_2 enzymes present in $P388D_1$ macrophages. *J. Biol. Chem.* 271: 6758–6765.
38. Balsinde, J., M. A. Balboa, P. A. Insel, and E. A. Dennis. 1999. Regulation and inhibition of phospholipase A_2. *Annu. Rev. Pharmacol. Toxicol.* 39: 175–189.
39. Koren, H. S., B. S. Handwerger, and J. R. Wunderlich. 1975. Identification of macrophage-like characteristics in cultured murine tumor line. *J. Immunol.* 114: 894–897.
40. Ralph, P., and I. Nakoinz. 1977. Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. *J. Immunol.* 119: 950–954.
41. Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz. 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* 15: 261–267.
42. Leslie, C. C. 1997. Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.* 272: 16709–16712.
43. Balboa, M. A., J. Balsinde, and E. A. Dennis. 2000. Phosphorylation of cytosolic group IV phospholipase A_2 is necessary but not sufficient for arachidonic acid release in $P388D_1$ macrophages. *Biochem. Biophys. Res. Commun.* 267: 145–148.
44. Balboa, M. A., J. Balsinde, D. A. Dillon, G. M. Carman, and E. A. Dennis. 1999. Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *J. Biol. Chem.* 274: 522–526.
45. Schaloske, R. H., J. W. Provins, U. A. Kessen, and E. A. Dennis. 2005. Molecular characterization of the lipopolysaccharide/platelet activating factor- and zymosan-induced pathways leading to prostaglandin production in $P388D_1$ macrophages. *Biochim. Biophys. Acta* 1687: 64–75.
46. Balboa, M. A., J. Balsinde, M. V. Winstead, J. A. Tischfield, and E. A. Dennis. 1996. Novel group V phospholipase A_2 involved in arachidonic acid mobilization in murine $P388D_1$ macrophages. *J. Biol. Chem.* 271: 32381–32384.
47. Balestrieri, B., and J. P. Arm. 2006. Group V $sPLA_2$: classical and novel functions. *Biochim. Biophys. Acta* 1761: 1280–1288.
48. Qi, H. Y., and J. H. Shelhamer. 2005. Toll-like receptor 4 signaling regulates cytosolic phospholipase A_2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. *J. Biol. Chem.* 280: 38969–38975.
49. Lee, J. G., S. H. Lee, D. W. Park, S. H. Lee, H. S. Yoon, B. R. Chin, J. H. Kim, and S. H. Baek. 2008. Toll-like receptor 9-stimulated monocyte chemoattractant protein-1 is mediated via JNK-cytosolic phospholipase A_2-ROS signaling. *Cell. Signal.* 20: 105–111.
50. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
51. Ahmad-Nejad, P., H. Häcker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* 32: 1958–1968.
52. Bauer, S., C. J. Kirschning, H. Häcker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98: 9237–9242.
53. Buczynski, M. W., D. L. Stephens, R. C. Bowers-Gentry, A. Grkovich, R. A. Deems, and E. A. Dennis. 2007. TLR4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *J. Biol. Chem.* 282: 22834–22847.
54. Satake, Y., B. L. Diaz, B. Balestrieri, B. K. Lam, Y. Kanaoka, M. J. Grusby, and J. P. Arm. 2004. Role of group V phospholipase A_2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* 279: 16488–16494.
55. Balestrieri, B., V. W. Hsu, H. Gilbert, C. C. Leslie, W. K. Han, J. V. Bonventre, and J. P. Arm. 2006. Group V secretory phospholipase A_2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. *J. Biol. Chem.* 281: 6691–6698.
56. Diaz, B. L., Y. Satake, E. Kikawada, B. Balestrieri, and J. P. Arm. 2006. Group V secretory phospholipase A_2 amplifies the induction of cyclooxygenase 2 and delayed prostaglandin D_2 generation in mouse bone marrow culture-derived mast cells in a strain-dependent manner. *Biochim. Biophys. Acta* 1761: 1489–1497.
57. Fitzpatrick, M. A., and R. Soberman. 2001. Regulated formation of eicosanoids. *J. Clin. Invest.* 107: 1347–1351.
58. Kikawada, E., J. V. Bonventre, and J. P. Arm. 2007. Group V secretory PLA_2 regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK and $cPLA_2\alpha$ activation. *Blood* 110: 561–567.
59. Balsinde, J., M. A. Balboa, and E. A. Dennis. 1997. Inflammatory activation of arachidonic acid signaling in murine $P388D_1$ macrophages via sphingomyelin synthesis. *J. Biol. Chem.* 272: 20373–20377.

60. Kim, Y. J., S. P. Kim, S. K. Han, N. M. Muñoz, X. Zhu, A. Sano, A. R. Leff, and W. Cho. 2002. Group V phospholipase A_2 induces leukotriene biosynthesis in human neutrophils through the activation of group IVA phospholipase A_2. *J. Biol. Chem.* 277: 36479–36488.
61. Huwiler, A., G. Staudt, R. M. Kramer, and J. Pfeilschifter. 1997. Cross-talk between secretory phospholipase A_2 and cytosolic phospholipase A_2 in rat renal mesangial cells. *Biochim. Biophys. Acta* 1348: 257–272.
62. Murakami, M., R. S. Koduri, A. Enomoto, S. Shimbara, M. Seki, K. Yoshihara, A. Singer, E. Valentin, F. Ghomashchi, G. Lambeau, M. H. Gelb, and I. Kudo. 2001. Distinct arachidonate-releasing functions of mammalian secreted phospholipase A_2s in human embryonic kidney 293 and rat mastocytoma RBL-2H3 cells through heparan sulfate shuttling and external plasma membrane mechanisms. *J. Biol. Chem.* 276: 10083–10096.
63. Han, S. K., K. P. Kim, R. Koduri, L. Bittova, M. M. Muñoz, A. R. Leff, D. C. Wilton, M. H. Gelb, and W. Cho. 1999. Roles of Trp^{31} in high membrane binding and proinflammatory activity of human group V phospholipase A_2. *J. Biol. Chem.* 274: 11881–11888.
64. Kim, Y. J., K. P. Kim, H. J. Rhee, S. Das, J. D. Rafter, Y. S. Oh, and W. Cho. 2002. Internalized group V secretory phospholipase A_2 acts on the perinuclear membranes. *J. Biol. Chem.* 277: 9358–9365.
65. Mounier, C., F. Ghomashchi, M. R. Lindsay, S. James, A. G. Singer, R. G. Parton, and M. H. Gelb. 2004. Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A_2 occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase $A_2\alpha$. *J. Biol. Chem.* 279: 25024–25038.
66. Ni, Z., N. M. Okeley, B. P. Smart, and M. H. Gelb. 2006. Intracellular actions of group IIA secreted phospholipase A_2 and group IVA cytosolic phospholipase A_2 contribute to arachidonic acid release and prostaglandin production in rat gastric mucosal cells and transfected human embryonic kidney cells. *J. Biol. Chem.* 281: 16245–16255.