Localization of Group V Phospholipase A₂ in Caveolin-enriched Granules in Activated P388D₁ Macrophage-like Cells*

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In murine P388D₁ macrophages, the generation of prostaglandin E₂ in response to long-term stimulation by lipopolysaccharide involves the action of Group V secreted phospholipase A₂ (sPLA₂), Group IV cytosolic PLA₂ (cPLA₂), and cyclooxygenase-2 (COX-2). There is an initial activation of cPLA₂ that induces expression of Group V PLA₂, which in turn induces both the expression of COX-2 and most of the arachidonic acid substrate for COX-2-dependent prostaglandin E₂ generation. Because Group V PLA₂ is a secreted enzyme, it has been assumed that after cellular stimulation, it must be released to the extracellular medium and re-associates with the outer membrane to release arachidonic acid from phospholipids. In the present study, confocal laser scanning microscopy experiments utilizing both immunofluorescence and green fluorescent protein-labeled Group V PLA₂ shows that chronic exposure of the macrophages to lipopolysaccharide results in Group V PLA₂ being associated with caveolin-2-containing granules close to the perinuclear region. Heparin, a cell-impermeable complex carbohydrate with high affinity for Group V PLA₂, blocks that association, suggesting that the granules are formed by internalization of the Group V sPLA₂ previously associated with the outer cellular surface. Localization of Group V PLA₂ in perinuclear granules is not observed if the cells are treated with the Group IV PLA₂ inhibitor methyl arachidonyl fluorophosphonate, confirming the important role for Group IV PLA₂ in the activation process. Cellular staining with antibodies against COX-2 reveals the presence of COX-2-rich granules in close proximity to those containing Group V PLA₂. Collectively, these results suggest that encapsulation of Group V PLA₂ into granules brings the enzyme to the perinuclear envelope during cell activation where it may be closer to Group IV PLA₂ and COX-2 for efficient prostaglandin synthesis.

The phospholipase A₂ (PLA₂) family includes a large group of enzymes that catalyze the hydrolysis of fatty acids

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| The abbreviations used are: PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secreted phospholipase A₂; AA, arachidonic acid; LPS, bacterial lipopolysaccharide; COX-2, cyclooxygenase-2; MAFP, methyl arachidonoyl fluorophosphonate; PG, prostaglandin; PGE₂, prostaglandin E₂; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GFP-GV, GFP-Group V sPLA₂. |
inhibition of Group V PLA₂, by antisense technology or by chemical inhibitors, abolishes COX-2 expression in the LPS-treated cells (8, 9). Thus Group V PLA₂ plays two separate roles in the process: on one hand, it provides the bulk of AA release; on the other, Group V PLA₂ controls the induction of the enzyme that metabolizes the free AA i.e. COX-2. It is interesting to note that, despite the marked differences between the two pathways of AA release involving LPS, both of them appear to utilize the same PLA₂ effectors, i.e. cPLA₂, and Group V sPLA₂. The cPLA₂ fundamentally plays a regulatory role, whereas the sPLA₂ plays an augmentative role by providing most of the AA metabolized by COX-2 (6, 8, 9).

It is has been clearly demonstrated that cPLA₂ primarily acts on perinuclear membranes (10), the precise site of action of secreted PLA₂ such as the Group V PLA₂, is still the subject of intense research. Recent work using transfected cell lines (11) or exogenously added enzymes (12–15) has revealed that some secreted PLA₂ can re-associate with the outer cellular surface and, subsequently, be re-internalized. Although some of these studies have suggested that this internalization may serve as a means to terminate signaling (i.e., the internalized protein is degraded) (12), other studies have suggested the possibility that the internalization process serves to bring the secreted PLA₂ in close proximity to COX-2 in the perinuclear area for efficient conversion of AA into PGs (11). In the current work we have sought to study the subcellular localization of the de novo Group V sPLA₂ in P388D₁ cells treated with LPS to release AA and generate PGs in a delayed phase. We demonstrate that Group V sPLA₂ localizes into perinuclear granules of the cell, which strongly correlates with the delayed onset of PG production (8).

EXPERIMENTAL PROCEDURES
Materials—Iscove’s modified Dulbecco’s medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone Labs (Logan, UT). Non-essential amino acids were from Irvine Scientific (Santa Ana, CA). LPS (Escherichia coli 0111:B4) and heparin were from Sigma. Rabbit anti-Group V sPLA₂ polyclonal antibody was generously provided by Dr. Jonathan Arm (Harvard Medical School) (16). Anti-caveolin-2 and COX-2 mouse monoclonal antibodies were from Transduction Laboratories (Lexington, KY). FITC-conjugated anti-rabbit IgG and Red X-conjugated anti-mouse IgG were from Jackson Immunoresearch (West Grove, PA).

Cell Culture Conditions—P388D₁ cells (MAB clone) (8, 17) were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO₂ in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and non-essential amino acids. P388D₁ cells (0.8 × 10⁶ cells) were placed onto coverslips, allowed to adhere overnight, and used for experiments the following day. For stimulation, the cells were washed and placed in serum-free medium. Where indicated, the cells were incubated with 100 ng/ml LPS for the indicated periods of time. When heparin was used, it was added at 1 mg/ml. All experiments were conducted in serum-free medium.

Immunofluorescence—After each treatment, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Cells were then permeabilized with 0.3% Triton X-100/ phosphate-buffered saline for 5 min at room temperature and blocked for 30 min in 0.5% bovine serum albumin, 2% normal donkey serum, and 50 μM glycine in phosphate-buffered saline (blocking buffer). Incubations with primary and secondary antibodies were performed at a 1:500 dilution in working buffer (1:5 dilution of blocking buffer) for 1–2 h at room temperature. Secondary antibodies were FITC-conjugated anti-rabbit IgG for Group V sPLA₂ antibody and Red X-conjugated anti-mouse IgG for caveolin-2 and COX-2 antibodies. Extensive washes in between incubations with antibodies were performed in phosphate-buffered saline. Specimens were mounted in anti-fade medium and viewed with a Bio-Rad MRC-1024 laser-scanning confocal system coupled to a Zeiss Axiovert 35M microscope. The objective was a Zeiss Plan-Apochromat, 63×, 1.4 numerical aperture, oil immersion. The fluorescence of FITC was monitored at 488-nm argon excitation using a 522–535 nm band pass barrier filter whereas that of Red-X was monitored at 568-nm argon excitation using a 605–632 nm band pass barrier filter.

The specificity of the anti-Group V PLA₂ antibody was tested by using the antibody absorbed with the Group V PLA₂ peptide used to obtain the rabbit anti-Group V PLA₂ serum: ELKSMIEKVTGKNAFKNYG (16). To this end the Group V PLA₂ peptide or a control peptide (CMETTPLNSQKVLSE) were conjugated to an agarose column (Hitrap affinity column, Amersham Biosciences), and the pass-through fraction of the Group V antibody (1:500 final dilution) was used for immunostaining.

Construction of the Plasmid Encoding GFP—Group V sPLA₂ (GFPGV) and Production of Transfectants Stably Expressing GFP—A cDNA fragment of human Group V sPLA₂, having a native signal peptide at the N terminus was produced by PCR with HPLA2 and Group V sPLA₂ primers (CMETTPLNSQKVLSE) and sequenced. Approximately 5.5 μg of plasmids encoding GFP-GV or GFP (pEGFPN1) was mixed with 15 μl of TransIT™-LT1 (Panvera Co., Madison, WI) in 1000 μl of Opti-MEM for 10 min at room temperature and then added to the P388D₁ cells (0.8 × 10⁶ cells). After incubation at 37 °C for 4 h, the medium was replaced by the usual culture medium and incubated for an additional 12–18 h period. To obtain the GFP-GV or GFP stably expressing cells, the transfected cells were cloned by limiting dilution and kept in culture medium supplemented with 1 mg/ml Geneticin (Invitrogen) using 96-well plates. After 2 weeks, wells containing a single colony were chosen for further expansion, and the fluorescence of the cells was examined under an epifluorescence microscope. Among the cell clones exhibiting GFP fluorescence, stable-transfectants for GFP-GV or GFP were selected by immunoblotting and PLA₂ activity measurement. The stable cell lines were kept in culture medium supplemented with 1 mg/ml Geneticin.

RESULTS
Subcellular Distribution of Group V sPLA₂ in LPS-treated Cells—Arm and co-workers (16) have recently produced antibodies that specifically recognize Group V sPLA₂ and Group IIA sPLA₂. These antibodies make it possible now to conduct ultrastructural studies aimed at determining the cellular/subcellular site of action of the sPLA₂ during cell activation. In unstimulated cells, confocal laser scanning microscopy revealed a diffuse pattern of cytoplasmic staining with anti-Group V sPLA₂ antibody (Fig. 1). However, after exposure to LPS for 18 h, a treatment that results in the generation of free AA and PGs such as PGE₂ (8, 9), a dramatic change in the subcellular localization of the Group V sPLA₂ was observed in that a more granular staining was now readily seen (Fig. 1). The staining was ablated when the primary antibody was first absorbed out with the Group V PLA₂ peptide antigen (see “Experimental Procedures”) as shown in Fig. 2, but not with a control peptide. A magnification of those granules can be observed in Fig. 3, where the perinuclear localization is evident. Fig. 4 shows the kinetics of Group V sPLA₂ localization during exposure of the cells to LPS. An intense granular staining was already observed after 6 h of stimulation, increasing gradually with time. Staining was more prominent after 18 h of treatment (Fig. 4).
As an alternative approach to further elucidate the localization of Group V sPLA2 in P388D1 cells, these cells were stably transfected with C-terminally GFP-tagged human Group V sPLA2, and transfectants were examined by confocal laser microscopy (Fig. 5). The fusion protein was enzymatically active as judged by in vitro enzyme assay. Control unstimulated cells (0 h stimulation) showed diffuse fluorescence in the cytoplasm, whereas exposure to LPS increased staining of cytoplasmic granular structures (Fig. 5A). These observations fully agree with the results shown in previous figures utilizing immunofluorescence. Control cells transfected only with GFP did not show those structures but a pattern of fluorescence evenly distributed across the cells, and no changes were observed after activation (Fig. 5B).

To investigate the Group V sPLA2-containing granules in more detail, double antibody staining experiments were conducted in LPS-treated cells. Anti-Group V sPLA2 and anti-caveolin-2 antibodies revealed co-localization of both proteins in granules close to the nuclear envelope (Fig. 6), although it should be noted that not all of the Group V sPLA2 is associated with caveolin-2. In contrast to LPS-treated cells, unstimulated cells revealed a very poor staining with caveolin-2,2 a finding that suggests up-regulation of caveolin-2 during cell activation, as previously reported for caveolin-1 in other macrophage cell lines (19). No cross-reactivity was found between the secondary antibody anti-rabbit IgG and the monoclonal antibody bound to caveolin-2, or between the secondary antibody anti-mouse IgG against the polyclonal antibody bound to Group V PLA2. Lyso-Tracker or cathepsin D, markers for acidic granules, did not co-localize with Group V sPLA2, indicating that Group V-rich granules are not of lysosomal origin (data not shown).

The co-localization of Group V sPLA2 and caveolin-2 into perinuclear granules suggests the possibility that the sPLA2 present in these granules comes from the outside of the cell via a caveolae-mediated endocytotic event or potocytosis, as previously suggested by others (11). To test this possibility, cellular treatment with LPS was conducted in the presence of heparin in the extracellular medium. Heparin, a cell-impermeable poly-

2 Y. Shirai and E. A. Dennis, unpublished data.
cally reduced the appearance of Group V sPLA2-containing perinuclear granules in LPS-activated cells (Fig. 7), suggesting an extracellular origin for those structures. Also, a side-by-side comparison of the effects of heparin on the co-localization of Group V sPLA2 with caveolin-2 in LPS-treated cells is shown in Fig. 8. Because in the presence of heparin no perinuclear sPLA2-containing granules are observed, no colocalization between caveolin-2 and GV sPLA2 was detected.

In macrophages, PGE2 production in the delayed phase of LPS activation is due to the metabolism of AA by COX-2 (8, 9). It has been described that COX-2 localizes close to or by the nuclear envelope (23). We performed some experiments using antibodies against COX-2 to define the localization of this enzyme in LPS-treated cells. As shown in Fig. 9, COX-2 localizes in granules near the nucleus in close proximity to Group V PLA2.

As indicated above, long term stimulation by LPS promotes activation of cPLA2, which leads to an early increase of free AA. Blocking cPLA2 activity with MAFP prevents PGE2 synthesis in LPS-treated cells, due to inhibition of the induction of Group V PLA2. Confocal microscopy experiments were performed in the LPS-treated cells in the presence of MAFP. As shown in Fig. 10, no perinuclear Group V PLA2-enriched granules were found under those conditions (Fig. 10, C and D).

**DISCUSSION**

Ongoing studies in our laboratory for several years have delineated two pathways for AA release and metabolism in LPS-treated macrophages. The first one, referred to as the “primed immediate pathway” takes place in minutes and is elicited by the Ca2+-mobilizing agonist platelet-activating factor, but requires the cells to be exposed first to LPS for 1 h (4–7, 16). The second route, or “delayed pathway,” is elicited by LPS for periods of time spanning several hours (8, 9). Interestingly, both pathways utilize the same effectors, namely Group IVA cPLA2, Group V sPLA2, and COX-2, although the molecular mechanisms involved dramatically differ. In both of these...
routes the cPLA2, appears to behave primarily as an initiator of the response, whereas Group V sPLA2 plays an augmentative role by providing most of the AA to be converted to prostaglandins via COX-2 (24). Although in the immediate pathway all the enzymes implicated in PGE2 production are already present in the cell, in the long term pathway both Group V PLA2 and COX-2 are up-regulated, and these events are triggered after the cPLA2 has become activated. Expression of COX-2 is also dependent on the activation of Group V PLA2 (8, 9).

The importance of Group V sPLA2 in AA mobilization and prostaglandin production by major immunoinflammatory cells has been clearly recognized (24, 25). Group V sPLA2 is rapidly secreted by the activated cells to the extracellular medium. The enzyme has traditionally been thought to re-associate with the outer leaflet of the plasma membrane to hydrolyze phospholipids and release AA and, in this manner, amplify the response already initiated by Group IVA PLA2 in the interior of the cell. According to this view, it was assumed that part of the AA released by Group V sPLA2 at the plasma membrane would travel to the nuclear envelope and endoplasmic reticulum, either by passive diffusion or active transport mechanisms, for metabolism by 5-lipoxygenase or COX isoforms (5–7, 21, 26, 27).

Structurally, Group V sPLA2 is remarkably similar to other sPLA2 family members present in mammalian cells (1, 3), most of which seem to have limited or no role in AA mobilization and attendant eicosanoid production (2, 28). This has made it difficult to establish the exact site(s) of action of this enzyme within the cell. In fact, most of the anti-sPLA2 antibodies that have been used in subcellular localization studies have later been found not to distinguish among the different sPLA2 forms. Recently however, Arm and co-workers (see Ref. 16 and “Experimental Procedures”) have described a polyclonal antibody directed against a unique 19-mer peptide sequence in the N-terminal end of the molecule. Utilizing this antibody, Bingham et al. (16) have studied the subcellular localization of Group V sPLA2 in resting mast cells and compared it with the distribution of Group IIA sPLA2 in these cells. Both sPLA2 types were found to localize in a distinct manner. Although Group IIA sPLA2 was found in secretory granules, Group V sPLA2 was found on the plasma membrane but also on cytoplasmic membranes, particularly those of the Golgi and the nuclear envelope (16). Unfortunately, Bingham et al. (16) did not extend their studies to stimulated cells and thus the significance of intracellularly located Group V sPLA2 was not ascertained.

In the present study we have carried out studies with the same antibody employed in the studies of Bingham et al. (16) to determine the localization of Group V PLA2 in stimulated cells. In our cellular model, the P388D1 macrophage cell line Group V sPLA2 has a diffuse distribution across the cell during resting conditions (Fig. 1A). After treatment with LPS for periods of time longer than 6 h, cell-associated Group V sPLA2 is found to be present in cytoplasmic granules. These large size granules are located in the perinuclear region and contain caveolin-2 together with Group V sPLA2, although some of the Group V sPLA2 is not associated with these large granules. Given the presence of caveolin-2 in those granules, and the fact that treatment with heparin blocks the Group V sPLA2 present therein (Figs. 7 and 8), it seems logical to suggest that these granules are formed by internalization of the Group V sPLA2 that has associated with the cellular surface after being secreted to the incubation medium, via a caveolae-mediated endocytotic event (Fig. 11). A similar process was suggested by Murakami et al. (11) utilizing transfected enzyme.

Caveolae are known to form a unique endocytic and exocytic compartment at the surface of most cells, capable of importing...
molecules from the exterior of the cells and delivering them to specific locations within the cell (29). Moreover, caveolae are sites of Ca\(^{2+}\) storage and entry into the cell (29). This is important because Group V sPLA\(_2\) absolutely requires millimolar levels of calcium for activity (1). Moreover, encapsulation of the Group V sPLA\(_2\) in the caveolin-2-containing granules would protect the enzyme from the reducing cytosolic environment that would rapidly denature the enzyme. Thus, Group V sPLA\(_2\) inside caveolin-2 granules may well retain enzyme activity after internalization and translocation to the perinuclear membrane. Because the perinuclear membrane is precisely the site where upstream (cPLA\(_2\)) (10) and downstream (5-lipoxygenase and cyclooxygenase-2) (23) eicosanoid-metabolic enzymes reside, such a mechanism would result in an extremely efficient utilization of the free AA liberated by the Group V sPLA\(_2\) at this location (Fig. 11). This is an important consideration because prostaglandin formation during the delayed phase of AA mobilization in macrophages normally occurs at levels of free AA that are much lower than those available during the immediate pathway of AA mobilization. For instance, analyses of the \(^{3}\text{H}\)-radioactive material released during chronic exposure of the \(^{3}\text{H}\)-labeled cells to LPS reveals that free unmetabolized AA represents less than 2% of total. This is in stark contrast with the primed immediate pathway, where free AA constitutes more than 95% of the released \(^{3}\text{H}\)-radioactive material.

Recent data by Cho and co-workers (12) utilizing exogenous Group V sPLA\(_2\) addition to human neutrophils showed the internalization of the enzyme only after all the AA release was completed. Such an internalization resulted in the enzyme being slowly degraded in the interior of the cell, which prompted the authors to suggest that Group V sPLA\(_2\) internalization would serve as a mechanism to modulate the extent of membrane hydrolysis of cell surface bound Group V sPLA\(_2\) (12). The results by Cho and co-workers (12) directly relate to signal termination and thus are both experimentally and phenomelogically unrelated to the internalization event herein described for Group V sPLA\(_2\) during chronic exposure of the cells to LPS. We have failed to co-localize Group V sPLA\(_2\) with the lysosomal markers Lysotracker and cathepsin D, which suggests that caveolin-2 granules containing Group V sPLA\(_2\) do not fuse with lysosomes after internalization. Interestingly as well, we have found that even after 36 h following LPS addition to the cells, a time at which fatty acid release and prostaglandin release have long ceased, a large number of Group V sPLA\(_2\)-containing granules are still observable in the perinuclear area, as judged by both immunofluorescence and GFP fluorescence. Interestingly, other recent reports also from Cho’s group described the internalization and apparent action of Group V sPLA\(_2\) on perinuclear membranes of HEK cells, human neutrophils, and human eosinophils (13–15), a view that is consistent with the results of this study.

The studies by Kim et al. and Muñoz et al. (13–15) were conducted with exogenous, and in some cases, mutated enzymes and not with endogenous native enzyme, as performed in the current study. Nevertheless, as a whole, these studies clearly indicate that Group V PLA\(_2\) may have different modes of action in different cells. This view is also highlighted by the recent studies of Murakami et al. (11). These investigators have proposed a “glypican-shuttling mechanism” of action on adherent cells, like fibroblasts or HEK cells, where secreted PLA\(_2\)g that bind heparin (Groups IIA, IID, and V) would directly bind to heparan sulfate chains of glypicans inside secretory vesicles prior to being released to the extracellular space. Then, those enzymes would be redirected to caveole-rich domains and reinternalized by potocytosis. They have proposed also a “glypicanc-independent” model that would be prevalent on mast cells and other hematopoietic cells poor in caveolae and/or glypican (11), where enzymes like Group V and Group X PLA\(_2\)s would act on the phosphatidylcholine present at the plasma membrane.

Our current work fits well with the glypican-shuttling mechanism proposed by Murakami et al. (11) on the basis of the following evidence: (i) P388D1 cells do express caveolin and caveole, (ii) Group V PLA\(_2\) colocalizes with caveolin-2 in the same intracellular granules, and (iii) PGE\(_2\) production depends on COX-2, in contrast to the COX-1-dependent PGE\(_2\) production postulated for the glypican-independent model. Importantly, however, we have performed Group V PLA\(_2\) staining experiments during the primed immediate phase of AA release in activated P388D1 macrophages and found that under those conditions the enzyme is relocated in some patches in the plasma membrane of the cells, but it does not localize in intracellular granules. These results would fit better with the glypican-independent mechanism proposed by Murakami et al. (11). Thus, it seems possible that the two mechanisms of action proposed by Murakami et al. (11) may occur within the same cell, and that the stimulation conditions dictate which mechanism takes place.

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