Novel role of group VIB Ca^{2+}-independent phospholipase $A_2\gamma$ in leukocyte-endothelial cell interactions: An intravital microscopic study in rat mesentery

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BACKGROUND: Phospholipase A_2 (PLA_2) is associated with a variety of inflammatory processes related to polymorphonuclear neutrophil (PMN)–endothelial cell interactions. However, the cellular and molecular mechanisms underlying the interactions and the causative isoform(s) of PLA_2 remain elusive. In addition, we recently showed that calcium-independent $PLA_2\gamma$ ($iPLA_2\gamma$), but not cytosolic PLA_2 ($cPLA_2$), is responsible for the cytotoxic functions of human PMN including respiratory bursts, degranulation, and chemotaxis. We therefore hypothesized that $iPLA_2\gamma$ is a prerequisite for the PMN recruitment cascade into the site of inflammation. The aim of this study was to elucidate the roles of the three major phospholipases A_2, $iPLA_2$, $cPLA_2$ and secretory PLA_2, in leukocyte rolling and adherence and in the surface expression of β_2-integrins in vivo and in vitro in response to well-defined stimuli.

METHODS: Male Wistar rats were pretreated with PLA_2 inhibitors selective for $iPLA_2\beta$, $iPLA_2\gamma$, $cPLA_2$, or secretory PLA_2. Leukocyte rolling/adherence in the mesenteric venules superfused with platelet-activating factor (PAF) were quantified by intravital microscopy. Furthermore, isolated human PMNs or whole blood were incubated with each PLA_2 inhibitor and then activated with formyl-methionyl-leucyl-phenylalanine (fMLP) or PAF. PMN adherence was assessed by counting cells bound to purified fibrinogen, and the surface expression of lymphocyte function-associated antigen 1 and macrophage antigen 1 (Mac-1) was measured by flow cytometry.

RESULTS: The $iPLA_2\gamma$-specific inhibitor almost completely inhibited the fMLP/PAF-induced leukocyte adherence in vivo and in vitro and also decreased the fMLP/PAF-stimulated surface expression of Mac-1 by 60% and 95%, respectively. In contrast, the other inhibitors did not affect these cellular functions.

CONCLUSION: $iPLA_2\gamma$ seems to be involved in leukocyte/PMN adherence in vivo and in vitro as well as in the up-regulation of Mac-1 in vitro in response to PAF/fMLP. This enzyme is therefore likely to be a major regulator in the PMN recruitment cascade. (*J Trauma Acute Care Surg*. 2015;79: 782–789.

KEY WORDS: Calcium-independent phospholipase A_2; intravital microscopy; β_2-integrin; adherence; rats.

Polymorphonuclear neutrophils (PMNs) are recognized to play a key role in the innate host defense system. PMN–endothelial cell (EC) interactions, characterized by rolling along the vessel wall, adhesion to the vascular endothelium, and transmigration into the interstitial space, are indispensable to the sequestration of PMNs to infected/injured tissues.[1] However, excessive PMN infiltration can lead to inflammation-mediated organ injuries, such as adult respiratory distress syndrome and multiple-organ failure.[1] Although it has been demonstrated that the interruption of the PMN/EC interactions attenuates the tissue/organ damage associated with systemic inflammatory response syndrome, the cellular/molecular mechanisms behind the PMN recruitment cascade remain unclear.[2] A thorough investigation of these mechanisms will allow us to identify novel therapeutic targets and to develop pharmacologic therapies.

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyzes the acyl group from the *sn*-2 position of the glycerophospholipids, thereafter generating free fatty acids and lysophospholipids. The PLA_2 family is divided into three broad classes based on cellular disposition and calcium dependence: cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and calcium-independent PLA_2 ($iPLA_2$). The $cPLA_2$ enzyme plays an important role in the acute/chronic inflammatory processes through the selective production of arachidonic acid (AA).[3] Similarly, $sPLA_2$, which is secreted into the extracellular space in response to life-threatening insults, enzymatically reacts with the plasma membranes to produce lipid mediators or directly activates the immune cells by binding to the $sPLA_2$ receptor, consequently exaggerating the systemic inflammatory conditions.[3] However, the roles of $iPLA_2$ in acute/chronic inflammation are not well understood. Gilroy et al.[4] reported that $iPLA_2$ is expressed in immune cells earlier than $cPLA_2$/$sPLA_2$ and that the activation of the enzyme produces chemotactic agents to recruit a greater

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number of inflammatory cells into the site of pleurisy. Our basic research also suggested that iPLA$_2\gamma$ is responsible for respiratory bursts, degranulation, and chemotaxis in isolated human PMNs as well as the accumulation of inflammatory cells and increased vascular permeability in rat lungs exposed to trauma and hemorrhagic shock (T/HS).[5–7] Furthermore, previous studies have suggested that PLA$_2$ is involved in the surface expression of β_2-integrin and adherence to the endothelium of PMN, although the causative isoform(s) have not been identified.[8] According to these findings, we hypothesized that the iPLA$_2$ enzyme may be a potential regulator of PMN sequestration to the site of inflammation. Therefore, the purpose of the current study was to elucidate the roles of the three major PLA$_2$s (cPLA$_2$, sPLA$_2$, and iPLA$_2$) in the interaction between leukocytes/PMNs and ECs in vivo and in vitro.

MATERIALS AND METHODS

The animal experiment was approved by the Animal Care and Use Committee of Shibaura Institute of Technology. All animals received humane care as defined under the institutional guidelines. The in vitro experiments were approved by the Medical Research Ethics Committee of Tokyo Medical and Dental University, and informed consent was obtained from all subjects.

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Heparin, Ficoll-Paque, and isoflurane were obtained from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan), GE Healthcare (Uppsala, Sweden), and DS Pharma Animal Health Co., Ltd (Osaka, Japan), respectively. The R- and S-enantiomers of the iPLA$_2$ inhibitor, (E)-6-(bromoethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL), and the cPLA$_2$ inhibitor, N-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide (pyrrophenone), were purchased from Cayman Chemicals (Ann Arbor, MI), and the sPLA$_2$ inhibitor, [[3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl]oxy] acetate (varespladib), was purchased from Selleck Chemicals LLC (Houston, TX). R-phycoerythrin (PE)–labeled monoclonal antibody against anti–human lymphocyte function-associated antigen 1 (LFA-1) (mouse IgG1, clone HI111) was obtained from BioLegend (San Diego, CA). PE-labeled anti–human macrophage antigen 1 (Mac-1) monoclonal antibody (mouse IgG1, clone ICRF44), isotype control (mouse IgG1κ, clone MOPC-21), Stain Buffer, and BD FACS Lysing Solution were purchased from Becton Dickinson & Co. (San Jose, CA).

Ca^{2+}-Independent PLA$_2$ Inhibitors

BEL, an irreversible and cell-permeable inhibitor of Ca^{2+}-independent PLA$_2$, exhibits 1,000-fold selectivity for iPLA$_2$ versus the cPLA$_2$ and sPLA$_2$ subclasses.[9] The R-BEL and S-BEL enantiomers further allow for the discrimination between the major isoforms of iPLA$_2$, as they are 10-fold selective for iPLA$_2\gamma$ and iPLA$_2\beta$, respectively.[9] The dosages of R-BEL used in the in vivo and in vitro experiments (0.4 mg/kg and 20 μM) almost completely inhibit iPLA$_2\gamma$ activity,[5,7] while the in vivo and in vitro concentrations of S-BEL (0.04 mg/kg and 5 μM) have been shown to inhibit the activity of iPLA$_2\beta$ by more than 90%.[5,9]

cPLA$_2$ Inhibitor

Pyrrophenone is a potent, reversible, and cell-permeable inhibitor, specific for cPLA$_2\alpha$. Its inhibitory effect on sPLA$_2$ and iPLA$_2$ is more than two orders of magnitude less potent than its effect on cPLA$_2\alpha$.[10,11] In the in vitro experiments, we selected the concentration (1 μM) that prevents activated human PMNs from producing AA in vitro.[10] In addition, the optimal dose (0.3 mg/kg) for the in vivo experiments was determined based on an assay of calcium ionophore–stimulated AA release from human whole blood.[12]

sPLA$_2$ Inhibitor

Varespladib is a potent, reversible, cell-impermeable inhibitor selective for group IIA-, V-, and X-sPLA$_2$; it does not affect cPLA$_2$, iPLA$_2$, or the COX pathway.[13,14] The in vitro concentration was determined based on a study by Furue et al.,[15] which showed that 100 nM of varespladib remarkably inhibits the release of sPLA$_2$-IIA–mediated free fatty acids and lysophospholipids. The in vivo optimal dosage was determined based on a report demonstrating that the intravenous administration of varespladib (1.0 mg/kg) markedly abolishes serum sPLA$_2$ activity.[13]

Intravital Microscopy in Rat Mesentery

Male Wistar rats (Nippon Bio-Supp. Center, Tokyo, Japan) were housed in cages in an environmentally controlled room at a temperature of 23°C to 25°C with a 12-hour light-dark cycle with ad libitum access to standard laboratory chow and tap water. The animals (185 ± 12 g) were anesthetized with inhaled isoflurane (1–2%), and their rectal temperature was maintained at 37°C during the experiment. After a midline laparotomy, the mesentery was gently exteriorized and spread over a pedestal with a glass window in a superfusion chamber. The chamber was continuously perfused with warmed Krebs-Ringer bicarbonate buffer (120-mM NaCl, 4.5-mM KCl, 0.5-mM $MgCl_2$, 10-mM D-glucose, 0.7-mM Na_2HPO_4, 1.5-mM NaH_2PO_4, 24-mM $NaHCO_3$) at pH 7.3 to 7.4, equilibrated with 5% CO_2 in nitrogen to maintain a physiologic pH. The microcirculation in the rat mesentery was transilluminated with a 150-W halogen light and observed through an intravital microscope (Olympus, Tokyo, Japan) equipped with a video camera (Sony, Tokyo, Japan). The microscopic image was displayed with a magnification of $\times$1,100 on a high-resolution television monitor and recorded for subsequent off-line analysis.[16]

At 30 minutes before laparotomy, R-BEL (0.4 mg/kg), S-BEL (0.04 mg/kg), pyrrophenone (0.3 mg/kg), varespladib (1.0 mg/kg), or dimethyl sulfoxide (DMSO) (0.25%) was intraperitoneally administered. After exteriorizing the rat mesentery, a single unbranched venule with a diameter ranging between 20 μm and 30 μm and a length greater than 100 μm was selected. The image of the mesenteric venule was recorded for 5 minutes to establish the baseline levels of leukocyte rolling and adhesion. The mesentery was superfused for 60 minutes with 200 nM of platelet-activating factor (PAF) in Krebs-Ringer bicarbonate buffer. Five-minute recordings were taken again at 60 minutes after initiating the superfusion. The number of rolling and adherent leukocytes was counted by off-line analysis reproducing video images on the television monitor. Rolling leukocytes were defined as leukocytes moving at a slower velocity

than the erythrocytes in the same venule and were expressed as the number of cells per minute.[17] Adherent leukocytes were defined as if they remained stationary for 30 seconds or longer and were expressed as the number of leukocytes per 100-μm length of venule in a period of 5 minutes.[17] In addition, the centerline red blood cell velocity (V_{RBC}) was measured with a commercially available microcirculation image analysis software program (CapiScope, KK Technology, Devon, United Kingdom) and verified by a frame-by-frame analysis using plasma pocket progression (correlation coefficient, 0.82; $p < 0.05$; n = 18).[18,19] The venular blood flow was calculated from the mean red blood cell velocity ($V_{mean} = V_{RBC} / 1.6$), and the wall shear rate (γ) was determined by the Newtonian definition: $\gamma = 8$ (V_{mean} / venular diameter).[17] These hemodynamic parameters were not affected by the superfusion with PAF or the administration of the PLA_2 inhibitors over the time course of the experiments (Table 1).

Isolation of Human Neutrophils

Human PMNs from healthy donors were purified by dextran sedimentation with 0.9% NaCl solution containing 3.0% dextran. Ficoll-Paque gradient centrifugation and hypotonic lysis were performed as previously described.[20] After washing, the cells were resuspended in Krebs-Ringer phosphate with dextrose buffer (KRPD buffer; 12.5-mM NaH_2PO_4, 3.0-mM $NaH_2PO_4H_2O$, 4.8-mM KCl, 120-mM NaCl, 1.3-mM $CaCl_2$, 1.2-mM $MgSO_47H_2O$, and 0.2% dextrose) at a pH of 7.35 to a final concentration of 1.0×10^7 cells/mL. The degree of cell viability, as estimated based on trypan blue exclusion, was more than 98%.

Neutrophil Adhesion Assay

The adherence of the isolated human PMNs to fibrinogen, a ligand of the Mac-1 adhesion molecule, was determined in 48-well microplate.[20] Before the adhesion assay, the microplates were coated at room temperature for 24 hours with purified fibrinogen (0.1 mg/mL) in phosphate-buffered saline (PBS) and then washed twice with PBS. The cells (2×10^6 cells) were pretreated in KRPD buffer for 10 minutes at 37°C with R-BEL (0.1–20 μM), S-BEL (5 μM), pyrrophenone (1 μM), varespladib (100 nM) or DMSO in a shaking water bath and then activated for 10 minutes at 37°C with 1 μM of formyl-methionyl-leucyl-phenylalanine (fMLP), 2 μM of PAF, or vehicle (Cont.). After removing the nonadherent PMNs by inverted centrifugation at 250 G for 5 minutes, the number of the cells left in each well (cells/μL) was counted using a hemocytometer.

Flow Cytometric Analysis of the Surface Expression of β_2-Integrins

The surface expression on human PMNs of β_2-integrins, LFA-1 and Mac-1, was quantified by flow cytometry. Briefly, heparinized whole blood was obtained from healthy donors. The samples were pretreated for 15 minutes at 37°C with R-BEL (0.2–20 μM), S-BEL (5 μM), pyrrophenone (1 μM), varespladib (100 nM), or DMSO in a shaking water bath and then activated for 10 minutes at 37°C with 1 μM of fMLP, 200 nM of PAF, or vehicle (Cont.). After stimulation, the cells were stained with PE-labeled anti–human LFA-1 antibody, PE-labeled anti–human MAC-1 antibody, or isotype antibody for 30 minutes at 4°C in the dark. The erythrocytes were then lysed using FACS Lysing Solution. The fluorescence intensity of the cells was measured using a flow cytometer (Becton-Dickinson Biosciences). The data were expressed as the mean fluorescence intensity of the cells.

Statistical Analysis

The data are expressed as the mean ± SD. Analysis of variance was performed with a post hoc analysis using the Tukey-Kramer test. p values less than 0.05 were considered to be statistically significant.

RESULTS

Leukocyte Rolling and Adherence In Vivo

The number of rolling and adherent leukocytes was similar among all groups before PAF superfusion (Figs. 1*A* and 2*A*). At 60 minutes after superfusion, the numbers of rolling leukocytes increased in all groups, except for the sham group. However, there were no significant differences between these groups (Fig. 1*B*). In contrast, treatment with R-BEL inhibited leukocyte adherence by 84% after PAF superfusion (Fig. 2*B*). The other inhibitors did not inhibit the PAF-induced leukocyte adherence to any degree (Fig. 2*B*).

Figure 3 shows representative images of leukocytes (*white arrowheads*) adhering to the venular walls in the sham-, DMSO-, and R-BEL–treated groups at 60 minutes after PAF superfusion. The R-BEL treatment (Fig. 3*C*) resulted in a marked reduction of leukocyte adherence in comparison with the DMSO treatment (Fig. 3*B*).

Human PMN Adherence In Vitro

Both fMLP and PAF caused a significant increase in PMN adhesion. It is noteworthy that the R-BEL treatment almost completely inhibited the fMLP/PAF-induced adherence, whereas

TABLE 1. Hemodynamic Parameters

Parameters	Sham	DMSO	R-BEL	S-BEL	Pyrrophenone	Varespladib
Diameter, μm	28.8 ± 2.0	26.8 ± 1.1	25.8 ± 2.3	28.2 ± 1.4	27.2 ± 2.4	26.8 ± 2.5
Baseline						
V_{RBC}, mm/s	2.43 ± 0.27	2.56 ± 0.57	2.45 ± 0.46	2.38 ± 0.18	2.36 ± 0.66	2.64 ± 0.54
Share rate, s^{-1}	699 ± 133	701 ± 55	670 ± 88	651 ± 69	681 ± 196	649 ± 90
60 min after superfusion						
V_{RBC}, mm/s	2.30 ± 0.34	2.46 ± 0.23	2.31 ± 0.77	2.18 ± 0.38	2.30 ± 0.66	2.32 ± 0.63
Share rate, s^{-1}	664 ± 146	664 ± 47	659 ± 143	649 ± 93	662 ± 199	623 ± 51

Data are expressed as the mean ± SD (each group n ≥ 4).

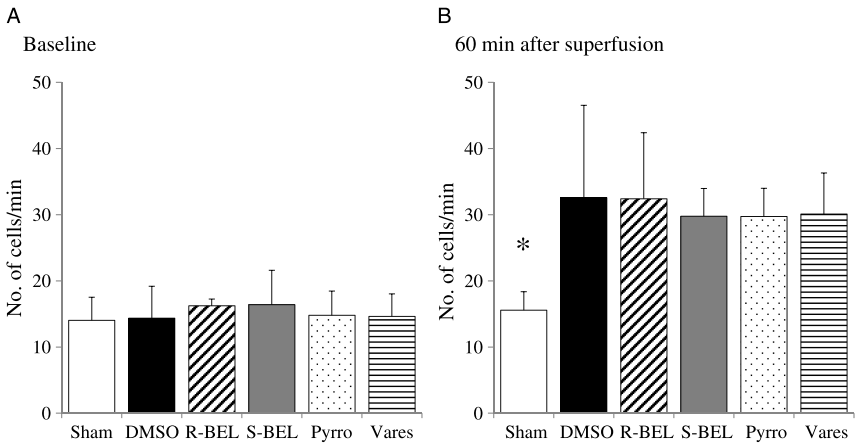

Figure 1. Leukocyte rolling in the mesenteric postcapillary venules. Animals were pretreated with DMSO or the specific inhibitors R-BEL, S-BEL, pyrrophenone (Pyrro), varespladib (Vares) 30 minutes before laparotomy followed by superfusion of the rat mesentery with 200 nM of PAF in Krebs-Ringer bicarbonate buffer except the sham group. Images of mesenteric postcapillary venules were recorded at baseline (*A*) and 60 minutes after superfusion (*B*). The number of rolling leukocytes (cells per minute) was determined by offline video analysis. The data are expressed as the mean ± SD (n ≥ 6 in each group). *$p < 0.05$ in comparison with the DMSO group at 60 minutes after superfusion.

the other PLA_2 inhibitors exhibited no inhibitory effects (Fig. 4*A* and *B*). In the dose-dependent experiments, R-BEL attenuated the fMLP/PAF-stimulated adherence in a dose-dependent manner (Fig. 4*C* and *D*).

Surface Expression of LFA-1 and Mac-1 Adhesion Molecules

Stimulation with fMLP/PAF in the presence or absence of each PLA_2 inhibitor did not change the surface expression of LFA-1 (data not shown). In contrast, the presence of the $iPLA_2\gamma$ inhibitor resulted in the significant reduction of the fMLP- and PAF-stimulated surface expression of Mac-1 (60% and 95%, respectively; Fig. 5*A* and *B*). Furthermore, treatment with R-BEL was found to inhibit the Mac-1 surface expression in a dose-dependent manner (Fig. 5*C* and *D*). In contrast, treatment with S-BEL, pyrrophenone, or varespladib did not abrogate the Mac-1 up-regulation in response to fMLP/PAF stimulation (Fig. 5*A* and *B*).

DISCUSSION

The intravital microscopic experiments demonstrated that R-BEL is the only PLA_2 inhibitor to significantly inhibit the PAF-induced leukocyte adherence at 60 minutes after PAF superfusion, while none of the PLA_2 inhibitors attenuated leukocyte rolling. Intravital microscopy is an elegant in vivo technique for observing the behavior of leukocytes in microvessels under physiologic/pathophysiologic conditions. To our knowledge, there are few in vivo studies investigating the relevance of PLA_2s in the interactions between leukocytes and ECs. We

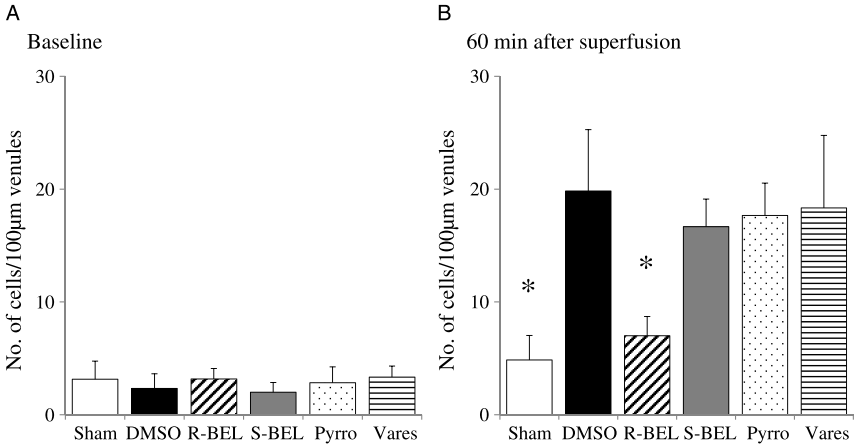

Figure 2. Leukocyte adherence to the mesenteric postcapillary venules. Animals were pretreated with DMSO or the specific inhibitors R-BEL, S-BEL, pyrrophenone (Pyrro), varespladib (Vares) 30 minutes before laparotomy followed by superfusion of the rat mesentery with 200 nM of PAF in Krebs-Ringer bicarbonate buffer except the sham group. Images of the mesenteric postcapillary venules were recorded at baseline (*A*) and 60 minutes after superfusion (*B*). The number of adherent leukocytes (cells per 100-μm length of venule) was determined by offline video analysis. The data are expressed as the mean ± SD (n ≥ 6 in each group). *$p < 0.05$ in comparison with the DMSO group at 60 minutes after superfusion.

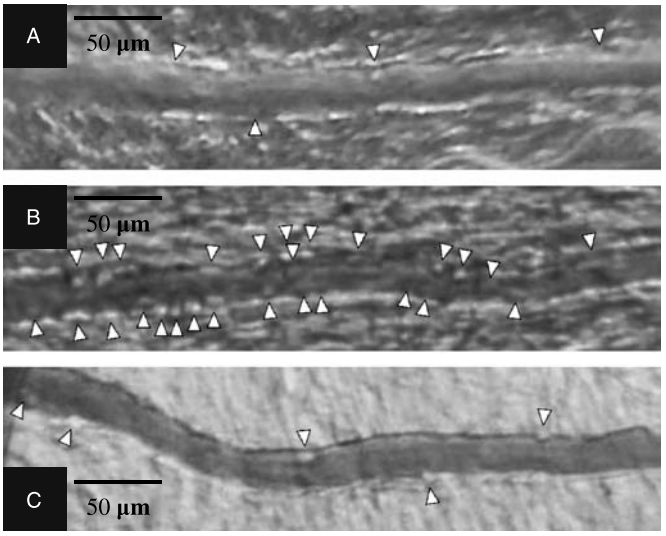

Figure 3. Photographs of the mesenteric postcapillary venules at 60 minutes after superfusion. A small number of adherent leukocytes (*white arrowheads*) were seen in the postcapillary venule of sham animals superfused without PAF (*A*). *B*, a representative image demonstrating that superfusion with 200 nM of PAF provokes numerous adherent leukocytes. In contrast, the image taken from the R-BEL–treated rat shows a few leukocytes adhering to a venular wall (*C*).

therefore performed in vivo experiments to distinguish the roles of the three major PLA_2s in PAF-mediated leukocyte rolling and adherence. Regarding the mechanisms of the leukocyte adherence cascade, β_2-integrin adhesion molecules are recognized as requisite elements.[21,22] A previous study reported that varespladib, a highly specific inhibitor for $sPLA_2$, had no impact on the chemokine-stimulated surface expression of CD11b/CD18 in isolated human PMNs.[23] In our in vitro experiments, varespladib failed to inhibit the Mac-1 surface expression and adherence in response to PAF/fMLP. With respect to the $cPLA_2$ enzyme, Amandi-Burgermeister et al.[24] demonstrated that arachidonyl trifluoromethyl ketone ($AACOCF_3$), an inhibitor of $cPLA_2$, prevents the calcium ionophore–induced surface expression of Mac-1 on human PMNs. Moreover, an in vitro study by Meliton et al.[25] showed that trifluoromethyl ketone, another inhibitor of $cPLA_2$, inhibits the binding of leukotriene D_4-stimulated human PMNs to intercellular adhesion molecule 1 (ICAM-1). These findings suggested that $cPLA_2$ is possibly associated with signaling pathways in the PMN adherence cascade; nevertheless, these $cPLA_2$ inhibitors also block the enzymatic activity of $iPLA_2$.[11] In contrast, our in vitro experiments showed that pyrrophenone, a $cPLA_2\alpha$ inhibitor more specific than $AACOCF_3$ and trifluoromethyl ketone, does not decrease CD11b/CD18 surface expression or PMN adherence in response to PAF/fMLP. Notably, Rubin et al.[26] indicated that the genetic ablation of $cPLA_2\alpha$ does not affect the surface expression of CD11b on fMLP/PMA-activated murine PMNs. Therefore, $sPLA_2$ and $cPLA_2$ may not be relevant to the leukocyte adherence in mesenteric microvessels superfused with PAF. The roles of $iPLA_2$ isoforms in the leukocyte recruitment cascade, however, remain entirely unclarified. Our recent study suggested that $iPLA_2\gamma$, but not $iPLA_2\beta$, is obviously involved in the PAF-induced PMN chemotaxis in the Boyden chamber system, which mimics PAF superfusion in vivo.[5] According to the current study, the $iPLA_2\gamma$ enzyme seems to regulate the adherence to EC and fibrinogen as well as the surface expression of CD11b/CD18. In addition, White et al.[27] demonstrated that $iPLA_2$ in vascular ECs may be related to the surface expression of P-selectin and PMN adherence to tryptase/thrombin-stimulated ECs. Thus, it is likely that the $iPLA_2\gamma$ enzyme in both PMNs and ECs is crucial for the molecular mechanisms that drive PMN sequestration into the sites of injury and infection. Finally, our in vivo experiments have some limitations, including pretreatment with PLA_2 inhibitors and the application of a single inflammatory mediator (PAF). Further studies using systemic inflammatory models (sepsis, T/HS, etc.) with posttreatment of the inhibitors will be required to translate these findings into clinical settings.

AA is thought to be a potent lipid mediator in the initiation of the PMN adhesion cascade, as evidenced by previous data, which showed that exogenously added AA promotes PMNs to adhere to ECs and to express the CD11b/CD18 adhesion molecule independent of its derivatives via the cyclooxygenase or lipoxygenase pathways.[8,28] Marshall et al.[29] demonstrated that $cPLA_2$ and $sPLA_2$ are possibly implicated in the release of AA from the fMLP-stimulated PMNs, while the inhibition of $iPLA_2$ has no effect on the AA liberation in response to stimuli. In contrast, Jacobson and Schrier[8] reported that manoalide and scalaradial, nonspecific PLA_2 inhibitors markedly abolish the fMLP-induced PMN adherence/Mac-1 up-regulation in a concentration-dependent manner, suggesting that PLA_2 modulates PMN adhesion in the site of acute inflammation. Thus, $cPLA_2$ and $sPLA_2$ are predicted to play a central role in the PMN/EC interactions, presumably through the mediation of AA; however, our findings showed that treatment with an inhibitor specific to $iPLA_2\gamma$ (but not to $cPLA_2$ or $sPLA_2$) entirely inhibits the PAF/fMLP-induced adherence. Taken together, the ineffectiveness of the $cPLA_2$- and $sPLA_2$-specific inhibitors suggests that $iPLA_2\gamma$-mediated second messenger(s) other than AA may exist in the PMN adherence cascade.

The surface expression of LFA-1 and Mac-1 on leukocytes is primarily involved in the sophisticated interactions with ECs such as slow rolling/firm adhesion, crawling to endothelial junctions and transmigration across the vessel wall.[22] In the intravital microscopic experiments, PAF superfusion elicited a fourfold increase in leukocyte adherence (vs. the sham group), while only treatment with R-BEL dramatically reduced the number of adherent cells, but not rolling cells. Nevertheless, stimulation with PAF/fMLP did not up-regulate the LFA-1 adhesion molecule, which was constitutively expressed on PMNs (data not shown). This finding is in line with the findings of a previous study by Kuijpers et al.[30] Similarly, none of PLA_2 inhibitors induced any significant changes of the surface expression of LFA-1. Integrin-mediated adherence relies on the avidity, which consists of affinity (the conformational changes of integrins) and valency (the density of integrin expression on plasma membrane). The engagement of selectin with its ligands during the rolling phase sends multiple signals toward the inside of the cell (outside-in signaling), which subsequently execute conformational changes of the integrins (inside-out signaling), resulting in their increased affinity to their ligands, ICAM-1/

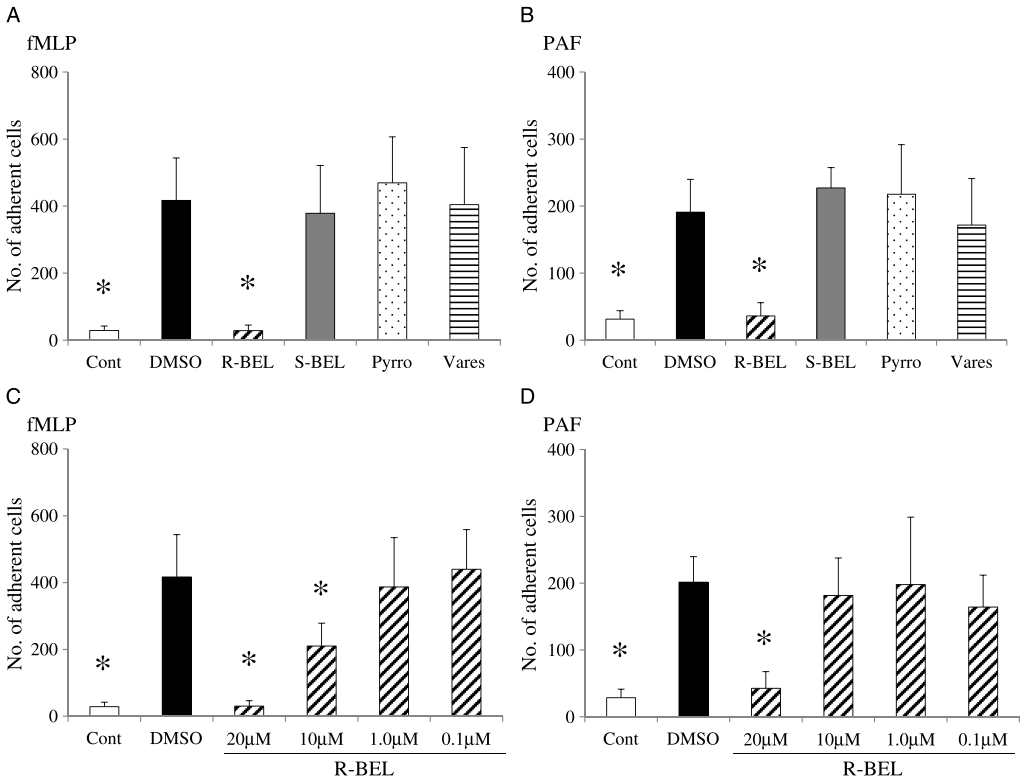

Figure 4. PMN adherence to fibrinogen. The adherence of human PMNs to purified fibrinogen (0.1 mg/mL in PBS) in response to fMLP or PAF was evaluated using a modified PMN adhesion assay. The isolated PMNs were incubated for 10 minutes at 37°C with R-BEL (20 μM), S-BEL (5 μM), pyrrophenone (Pyrro) (1 μM), varespladib (Vares) (100 nM), or DMSO (*A* and *B*). In the dose-dependent experiments with R-BEL, the cells were incubated for 10 minutes at 37°C with R-BEL (0.1–20 μM) (*C* and *D*). The pretreated cells (2.0×10^6 cells) were stimulated for 10 minutes at 37°C with fMLP (1 μM, *A* and *C*) or PAF (2 μM, *B* and *D*). Nonadherent PMNs were removed by inverted centrifugation. The number of adherent cells left in each well (cells per microliter) was counted using a hemocytometer. The data are expressed as the mean ± SD (n = 5 in each group). *$p < 0.05$ in comparison with the DMSO group.

ICAM-2.[21] However, the exact mechanisms of integrin activation remain to be elucidated. Thus, the $iPLA_2\gamma$ enzyme may be associated with the activation of LFA-1/Mac-1 through outside-in/inside-out signaling pathway. With respect to the surface expression of Mac-1, the binding of cytokines/chemokines and lipid mediators to G-protein–coupled receptors prompts PMNs to express the Mac-1 adhesion molecule, which is stored in the intracellular granules (including specific granules, gelatinase granules, and secretory vesicles) in unstimulated PMNs, thus suggesting a close relationship between the regulatory mechanisms of degranulation and the Mac-1 surface expression.[31] Previous studies using nonspecific inhibitors demonstrated that PLA_2 activation possibly induces CD11b/CD18 surface expression on human PMNs; however, it is not clear which of the PLA_2 isoform(s) are responsible.[8,26] The current study has shown that the $iPLA_2\gamma$ enzyme may be essential for the surface expression of Mac-1 on human PMNs in response to PAF/fMLP. In contrast, several lines of evidence advocate that PLA_2 serves as a critical modulator in the release of a variety of granules from PMNs.[8,32] In particular, a study by Balboa et al.[33] demonstrated that the inhibition of $iPLA_2$, but not $cPLA_2$, diminishes lysozyme secretion in activated promonocytes. Furthermore, we recently showed that the specific inhibitor for $iPLA_2\gamma$ achieves the complete inhibition of degranulation from fMLP/PMA-stimulated PMNs, whereas the $cPLA_2$ inhibitor is ineffective.[5] Collectively, $iPLA_2\gamma$ is likely to be a dominant enzyme in the intracellular signaling mechanisms of CD11b/CD18 surface expression.

The $iPLA_2\gamma$ enzyme is a membrane-binding enzyme that was originally identified from a search of a nucleic acid database as being homologous to $iPLA_2\beta$.[34] This enzyme is widely expressed in mammalian tissues including those in skeletal muscle, the heart, and the brain as well as the peripheral blood leukocytes and resides.[34] The enzyme resides primarily in endoplasmic reticulum, mitochondria, and peroxisomes.[3,34] Since the genetic ablation of $iPLA_2\gamma$ leads to multiple bioenergetic dysfunctions, including growth retardation and muscle atrophy in mice, the enzymatic functions of $iPLA_2\gamma$ have been thought to be associated with energy production.[3,35] However, the roles of this enzyme in acute/chronic inflammation have not been well understood. There is compelling evidence to suggest that the activation of $iPLA_2\gamma$ results in the elaboration of AA, $sPLA_2$-IIA, chemokine, and so on, supporting the involvement of this enzyme in the inflammatory processes.[36,37] Furthermore, $iPLA_2\gamma$ seems to regulate human PMN cytotoxicity such as respiratory bursts, degranulation, and chemotaxis.[5] Our preliminary study using a severe T/HS model (25–30 mm Hg × 60 minutes)

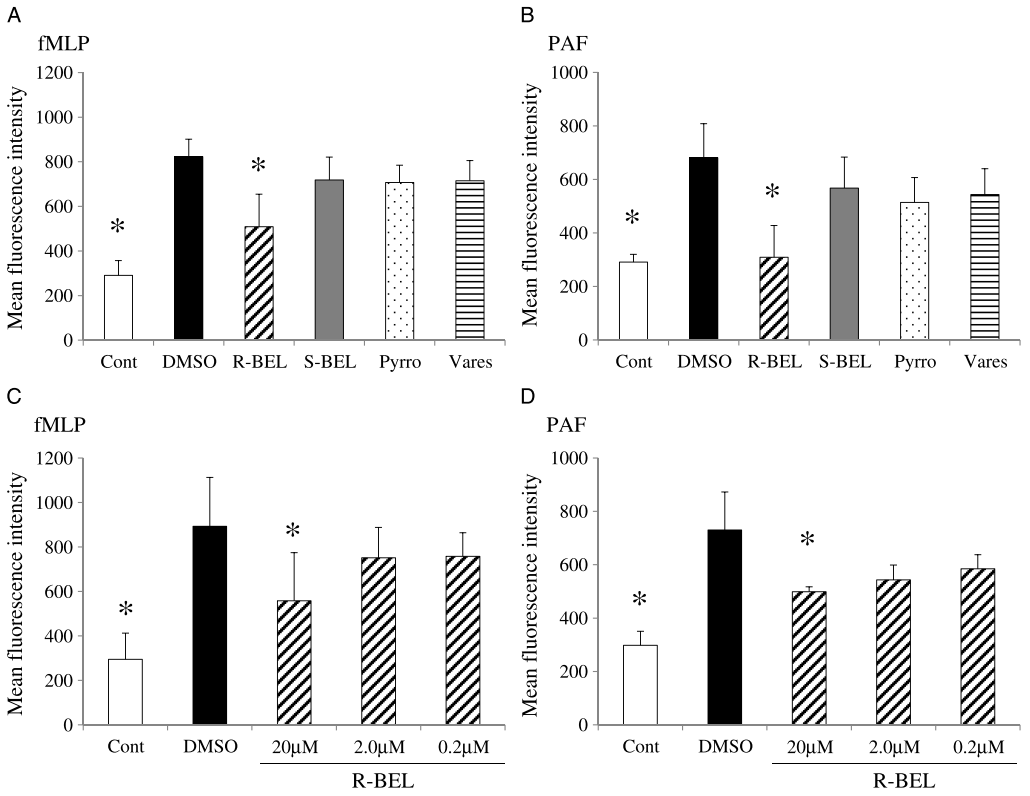

Figure 5. Surface expression of Mac-1 on human PMNs. Flow cytometry was used to quantify the surface expression of the Mac-1 adhesion molecule on human PMNs. Heparinized whole blood (n = 5 in each group) was preincubated for 15 minutes at 37°C with R-BEL (20 μM), S-BEL (5 μM), pyrrophenone (Pyrro) (1 μM), varespladib (Vares) (100 nM), or DMSO (*A* and *B*). In the dose-dependent experiments with R-BEL, samples (n ≥ 3 in each group) were incubated for 10 minutes at 37°C with R-BEL (0.2–20 μM) followed by stimulation at 37°C for 10 minutes with fMLP or PAF (1 μM and 200 nM, respectively; *C* and *D*). After stimulation, the pretreated blood was stained for 30 minutes at 4°C with PE-labeled anti–human MAC-1 antibody or isotype antibody. Erythrocytes were lysed, and then, mean fluorescence intensity was measured by flow cytometry. The data are expressed as the mean ± SD. *$p < 0.05$ in comparison with the DMSO group.

indicated that the iPLA$_2$γ-specific inhibitor attenuates the infiltration of inflammatory cells into multiple organs including the lung, gut, kidney, and liver (data not shown); hence, it is likely that the iPLA$_2$γ enzyme is of major importance to the diverse biologic functions of PMN in acute inflammation. In addition, the current study pointed out the possibility of new signaling pathway(s) related to cytotoxicity of PMN. The iPLA$_2$γ enzyme possesses unique substrate specificity, namely, its exertion of either PLA$_1$ or PLA$_2$ activity depending on its substrates. This enzyme catalyzes the cleavage at the *sn*-1 position of the phosphatidylcholine molecular species with polyunsaturated fatty acids, which are esterified to the *sn*-2 position, subsequently yielding free saturated fatty acids (SFAs) and polyunsaturated fatty acid–containing lysophosphatidylcholine (LPC) species such as 2-AA-LPC.[38] 2-AA-LPC can be metabolized into 2-AA-glycerol and 2-AA-lysophosphatidic acid, well-known biologically active mediators.[39] Moreover, the SFAs (the other metabolites of iPLA$_2$γ) stimulate PMNs to provoke the surface expression of adhesion molecules, adherence to EC, and the production of oxygen radicals.[40,41] Consequently, 2-AA-LPC and SFAs may be candidates for intracellular signaling molecules in the leukocyte recruitment cascade.

In conclusion, the current study showed that iPLA$_2$γ, but not iPLA$_2$β, cPLA$_2$, or sPLA$_2$, seems to be involved in PAF-induced leukocyte-EC interactions as well as in PMN adherence to fibrinogen and the surface expression of CD11b/CD18 in vitro. Thus, this enzyme is likely to be a key regulator of PMN sequestration into the inflammatory site. Considering the pharmacologic limitations of the PLA$_2$ inhibitors, further research using PLA$_2$ gene-manipulated animals is warranted to elucidate how the iPLA$_2$γ enzyme is incorporated into the molecular mechanisms of the leukocyte recruitment cascade.

AUTHORSHIP

M.K., J.A., and M.S. performed the surgical procedures and data collection/analysis of the in vivo experiments. M.K. and J.A. performed the data collection/analysis of the in vitro experiments. M.K., J.A., M.S., T.K., and Y.O. drafted the manuscript. All authors read and approved the final manuscript.

DISCLOSURE

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