Depletion of Human Monocyte 85-kDa Phospholipase A\textsubscript{2} Does Not Alter Leukotriene Formation*

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Human monocytes possess several acylhydrolase activities and are capable of producing both prostanoids (PG) and leukotriene (LT) products upon acute stimulation with calcium ionophore, A23187 or phagocytosis of zymosan particles. The cytosolic 85-kDa phospholipase (PLA\textsubscript{2}) A\textsubscript{2} co-exists with the 14-kDa PLA\textsubscript{2} in the human monocyte, but their respective roles in LT production are not well understood. Reduction in 85-kDa PLA\textsubscript{2} cellular protein levels by initiation site-directed antisense (SK 7111) or exposure to the 85-kDa PLA\textsubscript{2} inhibitor, arachidonyl trifluoromethyl ketone (AACOCF\textsubscript{3}), prevented A23187 or zymosan-stimulated monocyte prostanoid formation. In contrast, neither treatment altered stimulated LTC\textsubscript{4} production. This confirmed the important role of the 85-kDa PLA\textsubscript{2} in prostanoid formation but suggests that it has less of a role in LT biosynthesis. Alternatively, treatment of monocytes with the selective, active site-directed 14-kDa PLA\textsubscript{2} inhibitor, SB 203347, prior to stimulation had no effect on prostanoid formation at concentrations that totally inhibited LT formation. Addition of 20 \textmu M exogenous arachidonic acid to monocytes exposed to SK 7111 or SB 203347 did not alter A23187-induced PGE\textsubscript{2} or LTC\textsubscript{4} generation, respectively, indicating that these agents had no effect on downstream arachidonic acid-metabolizing enzymes in this setting. Taken together, these results provide evidence that the 85-kDa PLA\textsubscript{2} may play a more significant role in the formation of PG than LT. Further, utilization of SB 203347 provides intriguing data to form the hypothesis that a non-85-kDa PLA\textsubscript{2} of SB 203347 provides intriguing data to form the hypothesis that the 85-kDa PLA\textsubscript{2} may play a more significant role in prostanoid formation but suggests that it has less of a role in LT biosynthesis.

Much work has been directed toward understanding the liberation of arachidonic acid (AA)\textsuperscript{1} from human monocyte phospholipids (PL) and its subsequent metabolism to a number of cyclooxygenase (COX) and 5-lipoxygenase (5-LO) products (1–4). The first rate-limiting enzyme in eicosanoid formation is phospholipase A\textsubscript{2} (PLA\textsubscript{2}, EC 3.1.1.4), which liberates AA from the sn-2 position of cellular PL (5, 6). The two most studied mammalian forms are the type II 14-kDa PLA\textsubscript{2}\textsubscript{a}, known to exist as both an extracellular (7, 8) and cell-associated form (9–11) and the cytosolic 85-kDa-PLA\textsubscript{2} (12, 13). Although both enzymes have been extensively studied, the relative contribution of the two enzymes in stimulated eicosanoid production in a single cell system where they co-exist, as cell-associated forms, is poorly understood.

Correlative evidence exists for the participation of the 85-kDa PLA\textsubscript{2} in growth factor or cytokine-mediated AA liberation and prostanoid formation (14–17). More direct evidence for a role in prostanoid formation has been obtained through selective inhibitors of the 85-kDa PLA\textsubscript{2} activity or modulation of enzyme levels by antisense oligonucleotides (18–21). Arachidonyl trifluoromethyl ketone (AACOCF\textsubscript{3}), a slow, tight-binding inhibitor of 85-kDa PLA\textsubscript{2}, reduces stimulated AA release from platelets, U937 monocytes, and mesangial cells and subsequent platelet thromboxane (TXB\textsubscript{2}) and 12 hydroxyeicosatetraenoic acid biosynthesis (18–20). AACOCF\textsubscript{3} is selective with respect to other PLA\textsubscript{2} enzymes and does not have an effect on CoA-independent transacylase (18). It has, however, been reported to directly inhibit COX activity (20), and it must be used with caution in the evaluation of 85-kDa PLA\textsubscript{2} function in prostanoid biosynthesis. Its effects on the 5-LO pathway have not been reported. We have previously demonstrated the specific action of initiation site-directed 85-kDa PLA\textsubscript{2} antisense (SK 7111) in the reduction of endotoxin-stimulated human monocyte 85-kDa PLA\textsubscript{2} protein and enzyme activity levels without altering the cell-associated type II 14-kDa PLA\textsubscript{2} or COX II (21). This resulted in a concentration-dependent reduction in PGE\textsubscript{2} formation. Correlative evidence, such as coordinate enzyme expression (22–26) or subcellular localization (27, 28), has been reported, implicating the 85-kDa PLA\textsubscript{2} as a possible participant in 5-LO product formation; however, there are no reports directly linking the two.

The role of the cell-associated 14-kDa PLA\textsubscript{2} has been studied employing structurally distinct 14-kDa PLA\textsubscript{2} inhibitors, e.g. scalaradial (29, 30), BMS-181162 (31), WAY-125984 (32), and the active site-directed inhibitor, SB 203347 (33). Use of these agents indicates that the type II 14-kDa PLA\textsubscript{2} does participate in cellular AA metabolism, as exemplified by their ability to inhibit stimulated neutrophil AA release and both leukotriene (LT) B\textsubscript{4} and platelet-activating factor biosynthesis. Since neutrophils do not produce prostaglandins (PG), neither 85- nor 14-kDa PLA\textsubscript{2} inhibitors can be assessed for their effects on prostanoid biosynthesis in this system. When studied in cells, such as monocytes, which produce both LT and PG, 14-kDa PLA\textsubscript{2} inhibitors such as scalaradial have been shown to inhibit stimulated LTC\textsubscript{4} production but have no effect on PGE\textsubscript{2} production (29). Failure of 14-kDa PLA\textsubscript{2} inhibitors to reduce prostanoid synthesis has also been reported in cell systems that produce predominantly prostanoids and little or no 5-LO products, e.g. peritoneal guinea pig macrophage PGE\textsubscript{2} (34), human keratinocyte PGD\textsubscript{2} (16), or endotoxin-induced human monocyte...
PGE$_2$ production (21). Interestingly, exceptions to this exist. Antisense designed against the murine type II 14-kDa PLA$_2$ reduced PGE$_2$ production by an activated murine macrophage cell line, which may indicate species or cell line differences (35). Further, there are cell systems where both the 85-kDa and the type II 14-kDa PLA$_2$ are implicated in prostanoid formation, e.g. stimulated mesangial cells (36, 37), mast cells (38), or endothelial cells (39). In these models, the 14-kDa PLA$_2$ has been studied primarily as an extracellular enzyme. Application of the 14-kDa PLA$_2$ inhibitor, CGP 43182, in the stimulated rat mesangial cell system resulted in marked attenuation of PGE$_2$ production (36), illustrating alternative functional roles for secreted 14-kDa PLA$_2$. This could be due, in part, to neutralization of the extracellular function of this enzyme (85–90% of the 14-kDa PLA$_2$ is secreted in this system) and possible interference with its interaction on a cell surface receptor. This in turn could prevent activation of cellular 85-kDa PLA$_2$, which has been hypothesized to occur in the mast cell models (40). Taken together, it appears that both the cell-associated and secreted forms of the type II 14-kDa PLA$_2$ are important in cellular AA metabolism but may act through distinct pathways.

The bulk of the studies described above have been performed in cell systems where only prostanoids or leukotrienes are generated. As such the participation of the two distinct, cell-associated sn-2 acylhydrolases may not be fully appreciated. The monocyte/macrophage possess several acylhydrolase activities, including the 14- and 85-kDa PLA$_2$ enzymes (11, 12, 41–43). They offer an optimal system for studying the respective roles of the two enzymes on eicosanoid synthesis, because they simultaneously produce both LT and PG products upon stimulation with soluble or receptor-mediated stimuli (4).

In addition, we have reported previously that monocytes do not secrete the 14-kDa PLA$_2$, even with endotoxin treatment (21), and therefore the cellular form can be exclusively evaluated in these acute activating systems. Here we report the utilization of 85-kDa PLA$_2$ initiation site antisense (SK 7111), AACOCF$_3$ the 85-kDa PLA$_2$ inhibitor, and the selective 14-kDa PLA$_2$ inhibitor, SB 203347 to provide data to suggest that the 85-kDa PLA$_2$ and the 14-kDa PLA$_2$ both provide AA substrate for secreted 14-kDa PLA$_2$, even with endotoxin treatment (21), and that the cellular form can be exclusively evaluated in these acute activating systems. Here we report the utilization of 85-kDa PLA$_2$ initiation site antisense (SK 7111), AACOCF$_3$, the 85-kDa PLA$_2$ inhibitor, and the selective 14-kDa PLA$_2$ inhibitor, SB 203347 to provide data to suggest that the 85-kDa PLA$_2$ and the 14-kDa PLA$_2$ both provide AA substrate for secreted human monocyte eicosanoid biosynthesis, but possibly for distinct metabolizing pathways.

EXPERIMENTAL PROCEDURES

Measurement of Stimuli-induced Eicosanoid Release—Monocytes (5 × 10$^6$/ml) isolated as described previously (21) were incubated in RPMI 1640 medium (Life Technologies, Inc.) containing the treatment and/or the relevant vehicle after which the stimulus was added. The amount of stimulus was chosen from the linear portion of a concentration versus product curve usually representing 40–70% maximal stimulation as described previously (21). After 10 min (1 µM (7–15 min) or pepsinogen zymosan, 5 mg/ml (2 h)) (21, 29). Both prostanoids and leukotriene production in response to the respective stimuli were submaximal, representing the linear portion of a product versus time curve.

In antiserum studies, monocytes were exposed to phosphorothioate oligonucleotides SK7111 (‘-TACAGTAAATACTAGGAATG-5’), directed against the initiation site (lane 1), GATCCTTAC-3’ (lane 2), MS 203347 (‘-ATGTCATTTATA-', lane 4) or Lipofectin vehicle, alone (5 µg/ml) or pepsinogen zymosan, 5 mg/ml (2 h)) (21, 29). Both prostanoids and leukotriene production in response to the respective stimuli was submaximal, representing the linear portion of a product versus time curve.

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In antiserum studies, monocytes were exposed to phosphorothioate oligonucleotides SK7111 and MS 203347, designated periods in order to override the need for cell-associated endogenous deacylation and AA liberation. At the end of the incubation, cell-free medium was collected and stored at −20°C until analyzed.

Prostaglandin E$_2$, PGD$_2$, TXA$_2$, 6-keto-PGF$_{1\alpha}$, LTB$_4$, or LTC$_4$ were directly measured in cell-free medium using enzyme immunoassay kits purchased from Cayman Chemical Co. as described previously. Data were expressed as picograms or nanograms/ml of sample.

Mouse Mast Cells—Mast cells were obtained from bone marrow cells from BALB/c mice (Jackson Laboratories, Bar Harbor, ME) as described previously (42). Briefly, cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 50 µM 2-mercaptoethanol, 2 mM glutamine, and 10–20% of the WEHI-3 cells (Collaborative Biomedical, Bedford, MA) conditioned medium. Cells were grown for 3–4 weeks in an incubator with a humidified 5% CO$_2$ atmosphere at 37°C. For stimulation, mast cells were passively sensitized overnight by incubation with 20 µg/ml mouse anti-dinitrophenol IgE and then stimulated with antigen bovine serum albumin-dinitrophenol (2 µg/ml) for 15 min. Following stimulation, the cells were pelleted by brief centrifugation and the supernatant fluids collected as described previously (43). LTC$_4$, and PGD$_2$ in the supernatants were determined by enzyme immunoassay kits (Cayman Chemical Co.) as described above.

Phospholipase A$_2$ Activity Analysis of Monocyte Subcellular Fractions—Human recombinant 85-kDa PLA$_2$ and type II 14-kDa PLA$_2$ were prepared and assayed with and without inhibitors as described previously (21). Monocyte subcellular fractions were prepared from 3–4 human donors and 100,000 × g supernatant (cytosol, containing 85-kDa PLA$_2$) and particulate (micromes, containing 14-kDa PLA$_2$) were used to evaluate inhibitory action of compounds on monocyte PLA$_2$ activity as described previously (21). Cytosol was treated with dithiothreitol (30 min at 37°C) to inactive contaminating 14-kDa PLA$_2$ activity (44). 85-kDa PLA$_2$ activity in cytosol fractions was assayed using the 85-kDa PLA$_2$ preferred substrate, 1-palmitoyl-2-[C$^{14}$]AA phosphatidylylcholine (100 µg phosphatidylcholine, 50 µCi/mmol; DuPont NEN) vesicles (5, 21, 45). The 100,000 × g particulate fraction was assayed using [H]lA-Enricheria coli (0.5 µCi/5 µmol phosphorus phospholipid (PL), DuPont NEN) substrate as described previously (11, 21). All cell fractions were maintained at equal protein concentrations (29–30 µg of protein/assay) for comparative purposes. Fractions were preincubated with various concentrations of AACOCF$_3$, SB 203347, or Me$_2$SO vehicle (<1%) for 10 min prior to addition of the respective substrate for 30–120 min at 37°C. Reactions were processed as reported previously (21) and results expressed as percent free fatty acid hydrolyzed/reaction time or specific activity (picomoles of free fatty acid hydrolyzed/mg/h).

Drug results were calculated by comparison with untreated Me$_2$SO vehicle control hydrolysis and expressed as percent control.

Immunoblot Analysis—Monocyte cell fractions were prepared as described above. Cytosol protein or rh 85-kDa PLA$_2$ protein standard were analyzed by SDS-PAGE (10–20% gradient gels; Integrated Separation Systems). Cytosol protein or 85-kDa PLA$_2$ protein standard were expressed as picograms of 14-kDa PLA2/mg of cytosolic protein of monocytes or nanograms of 85-kDa PLA$_2$ protein/mg of cell-free medium using enzyme immunoassay kits purchased from Cayman Chemical Co. as described previously.

Quantitation of Type II 14-kDa PLA$_2$—To quantitate 14-kDa PLA$_2$, human monocyte 100,000 × g particulates from 4 donors were treated with 0.36 N H$_2$SO$_4$ for 1 h at 4°C, then brought back to pH 7.4 by addition of 2 M Tris buffer. This sample was directly assayed for 14-kDa PLA$_2$ mass using an enzyme immunoassay kit developed for rh type II 14-kDa PLA$_2$ as described previously (21, 48). Data were expressed as picograms of 14-kDa PLA$_2$/µg of particulate protein.

Measurement of Intracellular Ca$^{2+}$ Mobilization—A23187-induced Ca$^{2+}$ mobilization was determined as described previously using the calcium fluorescent probe fura 2 (49). Isolated human monocytes were suspended at 5 × 10$^6$/ml and incubated with fura 2 (2 µM) 45 min at 37°C.
Human monocytes from 3–5 donors were treated with Lipofectin (5 μg/ml) or 3 μM SK 7111 for 18 h as described under “Experimental Procedures,” prior to challenge with either vehicle, A23187 (1 μM, 7 min, 37 °C) or opsonized zymosan (5 mg/ml, 2 h, 37 °C). LTC₄ and PGE₂ measured in the cell supernatants is expressed as ng/ml, mean ± S.D. (n = 3), *, significantly different from control at p < 0.01 using ANOVA.

**TABLE I**
The effect of initiation site-directed 85-kDa PLA₂ antisense on stimulated monocyte LTC₄ and PGE₂ production

| Donor | Lipofectin PGE₂ (ng/ml) | SK 7111 (3 μM) | % change | Lipofectin LTC₄ (ng/ml) | SK 7111 (3 μM) | % change |
|-------|-------------------------|----------------|----------|-------------------------|----------------|----------|
|       | Unstimulated Stimulated | Unstimulated Stimulated |           | Unstimulated Stimulated | Unstimulated Stimulated |           |
| A     | 1.1 ± 0.1 5.1 ± 1.2 5.1 ± 0.1 | 0.1 ± 0.1 8.6 ± 1.1 8.6 ± 0.5 | –75* | 0.1 ± 0.1 8.6 ± 1.1 8.6 ± 0.5 | +1 |
| B     | 0.4 ± 0.1 3.9 ± 0.3 3.9 ± 0.1 | 1.0 ± 0.0 11.8 ± 1.0 11.8 ± 0.8 | –77* | 0.1 ± 0.0 11.8 ± 1.0 11.8 ± 0.8 | –28 |
| C     | 0.1 ± 0.0 4.2 ± 0.1 4.2 ± 0.0 | 0.1 ± 0.0 6.3 ± 0.9 6.3 ± 0.7 | –89* | 0.0 ± 0.0 6.3 ± 0.9 6.3 ± 0.7 | –20 |
| D     | 1.3 ± 0.1 3.4 ± 0.8 3.4 ± 0.7 | 0.1 ± 0.0 8.6 ± 1.0 8.6 ± 0.8 | –74* | 0.1 ± 0.0 8.6 ± 1.0 8.6 ± 0.8 | –28 |
| E     | 0.5 ± 0.2 6.0 ± 1.4 6.0 ± 1.2 | 0.1 ± 0.0 6.2 ± 0.5 6.2 ± 0.3 | –90* | 0.1 ± 0.0 6.2 ± 0.5 6.2 ± 0.3 | –20 |

37 °C. The effect of inhibitor on fluorescence of fura 2 was assessed on cells exposed to cytochalasin B (5 μg/ml) for 2.5 min. The [Ca²⁺], was calculated as described previously (49).

**Statistical Analysis—** All studies were performed using 2–6 human donors. Data were expressed as mean ± S.D. (n = 3 determinations) and analyzed where indicated using analysis of variance and Duncan’s multiple range test (p > 0.05).

**RESULTS**

The Effect of 85-kDa PLA₂ Initiation Site Antisense on Monocyte-stimulated Eicosanoid Synthesis

**Western Analysis of the Effect of Antisense on Unstimulated Monocytes—** Human monocytes (5 × 10⁶/ml) from one donor were treated over 18 h with 3 μM 85-kDa PLA₂ initiation site-directed antisense, SK7111, or sense oligonucleotide, SK9030 (3 μM) and cytosols were evaluated for 85-kDa PLA₂ protein. Pixel values measured from scanning densitometry of the Westerns are as follows: Lipofectin control (lane 2), 87; 3 μM SK7111 (lane 3), 22; and 3 μM SK9030 (lane 4), 158, showing that in this donor a 75% reduction in the levels of cytosolic 85-kDa PLA₂ protein was induced by antisense but not sense oligonucleotide treatment (Fig. 1). This agrees with previous reports in the monocyte (21) and human synovial fibroblast (50), illustrating the reduction of 85-kDa PLA₂ protein by antisense but not sense oligonucleotide. In both cases this directly corresponds with reduced cytosolic sn-2 acylhydroltic activity (21, 50).

**The Effect of Antisense on Stimulated Monocyte Eicosanoid Formation—** Antisense SK7111 (3 μM) treatment significantly reduced (75–90% with A23187 induction or 58–77% with zymosan phagocytosis) stimulated PGE₂ formation when compared to stimulated Lipofectin controls. In some cases the 3 μM SK7111-treated A23187-stimulated cells produced PGE₂ levels that were equal to or below the basal PGE₂ levels measured in the media of unstimulated cells (Table I, Fig. 2). Furthermore, the inhibition of PGE₂ production was concentration-dependent over 0.1–3 μM (Fig. 2). A23187-induced LTC₄ production was approximately equivalent or in some cases 2–3-fold higher than PGE₂ levels formed over the same time periods. Up to 3 μM antisense oligonucleotide SK7111 had no significant effect on LTC₄ formation (Table I, Fig. 2). Similarly, SK7111 had no effect on zymosan-induced LT formation (Table I).

Antisense inhibition of prostanoids was not restricted to PGE₂ only, as prostacyclin formation, measured as 6-keto-PGF₂α was also reduced in A23187-treated cells. A23187-induced (1 μM, 7 min, 37 °C) 6-keto-PGF₂α produced by monocytes of 2 individuals was reduced 86–88% by antisense but not sense treatment (donor 1; Lipofectin control, 0.3 ± 0.03; SK7111 (1 μM), 0.42 ± 0.01; SK9030 (1 μM), 0.5 ± 0.06 ng of 6-keto-PGF₂α/5 × 10⁵ (mean ± S.D.; n = 3) and donor 2; Lipofectin control, 0.5 ± 0.09; SK7111 (1 μM), 0.66 ± 0.009; SK9030 (1 μM), 0.5 ± 0.05 ng of 6-keto-PGF₂α/5 × 10⁵ (mean ± S.D.; n = 3)).

In a separate study, addition of AA (20 μM) to SK7111-treated cells during stimulation with A23187 (1 μM, 7 min, panel A) or opsonized zymosan (5 mg/ml, 2 h, panel B) PGE₂ and LTC₄ data are expressed as mean ± S.D. (n = 3), * indicates significantly different from control at p < 0.05 using ANOVA and Duncan’s multiple range analysis.
Assessment of Human Monocytes for Type II 14-kDa PLA₂

Analysis of 100,000 x g monocyte particulate fractions from 4 donors by enzyme-linked immunosorbent assay (as described under “Experimental Procedures”) demonstrated the presence of 2.1 ± 0.5 pg of 14-kDa PLA₂/μg particulate protein (mean ± S.D.) and confirmed the findings of previous reports of the existence of a biochemically identical 14-kDa PLA₂ in monocyte/macrophage particulate fractions (11, 21, 34).

The Effect of 85- or 14-kDa PLA₂ Inhibition on Monocyte PLA₂ Enzyme Activities

SB 203347 and AACOCF₃ were assessed for their effects on human monocyte subcellular fraction 85-kDa PLA₂ or 14-kDa PLA₂ enzyme activities as described under “Experimental Procedures.” These results were compared to the effects of these compounds on purified rh PLA₂ enzymes. SB 203347 demonstrated potent inhibition of rh type II 14-kDa PLA₂ (IC₅₀, 0.5 μM (Fig. 3, panel B) and 40-fold less inhibition of the rh 85-kDa PLA₂ (IC₅₀, 20 μM (panel B). In contrast, AACOCF₃ selectively inhibited rh 85-kDa PLA₂ (IC₅₀, 0.1 μM) with a 300-fold greater potency than against rh type II 14-kDa PLA₂ (IC₅₀, 91 μM (Fig. 3, panel A).

Consistent with the results observed using the recombinant enzymes, AACOCF₃ inhibited monocyte cytosolic 85-kDa PLA₂ in a concentration-dependent fashion (IC₅₀, 0.17 μM; Fig. 3, panel C). Furthermore, AACOCF₃ was ~350-fold more potent against the 85-kDa PLA₂ than against monocyte particulate 14-kDa PLA₂ activity (IC₅₀, 64 μM). Alternatively, SB 203347 inhibited monocyte 14-kDa PLA₂ activity of particulate fractions in a concentration-dependent manner (IC₅₀, 4.5 μM), which was ~20-fold more potent then its action against the 85-kDa PLA₂ activity of cytosolic fractions (IC₅₀, 93 μM, panel D).

The Effect of 85- or 14-kDa PLA₂ Inhibition on Stimulated Monocyte Eicosanoid Formation

Effect of SB 203347 or AACOCF₃ on Monocyte Eicosanoid Formation—To further delineate the contribution of the respective PLA₂ enzymes in monocyte AA metabolism, the effect of SB 203347 or AACOCF₃ on stimulated monocyte eicosanoid formation was assessed in monocytes from 2 donors as described under “Experimental Procedures.” A concentration curve was generated for AACOCF₃, an 85-kDa PLA₂ inhibitor, exposing human monocytes from one donor to 0.03, 0.1, 0.3, 1, or 3 μM AACOCF₃ prior to activation with 1 μM A23187 (7 min, 37°C). The concentration where PGE₂ was inhibited 50% (IC₅₀) was 0.3 μM (confidence limits, 0.25–0.41 μM) with total inhibition at 3 μM (vehicle-stimulated control, 4.0 ± 0.3 versus 3 μM AACOCF₃, 0.3 ± 0.05, PGE₂/5 × 10⁶ (mean ± S.D.; n = 3), where LT formation remained unchanged (vehicle-stimulated control, 6.8 ± 0.1 versus 3 μM AACOCF₃, 8.5 ± 1.2, LTC₄/5 × 10⁶ (mean ± S.D.; n = 3).

Fig. 4 shows that SB 203347 (0.003–100 μM) treatment resulted in concentration-dependent inhibition of A23187-induced LTC₄ formation (IC₅₀, 0.1 μM, donor 1; 0.4 μM, donor 2 (Fig. 4, panel A). Alternatively, PGE₂ production of monocytes from donor 1 was not affected by as much as 100 μM SB 203347, while A23187-induced PGE₂, from donor 2 (Fig. 4, panel A) was inhibited at 100 μM SB 203347, only. This was possibly due to the initiation of nonspecific inhibition of 85-kDa PLA₂ activity. SB 203347 exhibited a similar effect on the eicosanoid profile of monocytes activated by phagocytosis of opsonized zymosan.
(Fig. 4, panel B). LTC4 was inhibited (IC50, 0.33 and 0.49 μM, donor 1 and 2, respectively) by SB 203347 and had no effect on PGE2 produced by cells from either donor, at concentrations as high as 100 μM.

To assess the effect of SB 203347 on other prostanoids produced, A23187-stimulated TXB2 levels were measured (7.9 ± 1.0 ng of TXB2/mL, n = 3). Treatment with as high as 30 μM SB 203347 (9.0 ± 0.7 ng of TXB2/mL, n = 3) did not alter TXB2 production. Alternatively, LT inhibition was not specific to LTC4. LTC4 measured in 2 different donors was inhibited 79 ± 2.2% or 61 ± 15%, respectively, by 10 μM SB 203347 with control levels of donor 1 being 19.4 ± 1.4 or donor 2 being 15.8 ± 0.6 ng of LTB4/5 × 106 cells (mean ± S.D., n = 3).

To assess the possibility that the effect of SB 203347 was cell-specific, mouse bone marrow-derived mast cells which produce both PGD2 and LTC4 were evaluated. Mouse bone marrow-derived mast cells were exposed to increasing concentrations (0.3–10 μM) of SB 203347 (–10 min, 25 °C) prior to addition of IgE-complex-antigen stimulation as described under “Experimental Procedures.” Untreated control mast cells produced 707 ± 47 LTC4 ng/ml, mean ± S.D. and 78 ± 13 PGD2 ng/ml, mean ± S.D. (n = 3) upon stimulation. SB 203347 inhibited mast cell LTC4 in a concentration-dependent fashion (IC50 3 μM) and had no significant effect on PGD2 up to 10 μM (–12% and +2%, respectively for two studies).

Evaluation of Potential Non-selective Inhibitor Effects—To assess the possibility that SB 203347 may be acting through alteration of A23187 signal transduction, its effect on A23187-mediated Ca2+ mobilization in human monocytes was examined. A23187 at 1 μM induced Ca2+ saturation indicating that the Ca2+ concentration was ≥2 μM. This response was unaffected by SB 203347 at 3.3 μM, a concentration 10-fold greater than its LTC4 50% inhibitory concentration (0.3 μM). Comparisons where also made using submaximal A23187 concentrations (5 or 10 nM). In these cases exposure to SB 203347 (0.05–10 μM) had no effect on Ca2+ mobilization generated by vehicle alone (data not shown). This indicates that SB 203347 inhibition of LT formation is not through interference of stimulated intracellular Ca2+ flux.

SB 203347 and AACOCF3 were compared for their effects on monocyte-A23187-stimulated eicosanoid formation with and without exogenous AA (20 μM) to reveal possible effects on downstream AA-metabolizing enzymes. Concentrations that induced ~70% or greater inhibitory effects in previous studies were chosen. As shown previously, SB 203347 (1 μM) inhibited LTC4 (~86%) and had no effect on PGE2 production upon A23187 stimulation (Fig. 5, panel A). Addition of 20 μM AA produced a 240-fold and 700-fold increase in stimulated LTC4 and PGE2 production, respectively (Fig. 5, panel B), clearly demonstrating that exogenous AA was available for conversion by the respective intracellular downstream metabolizing enzymes. In the presence of AA, SB 203347 had no significant effect on either LTC4 or PGE2 formation, indicating a lack of action on enzymes in either the 5-LO or COX pathways. Alternatively, the selective 5-LO inhibitor, zileuton, inhibited LTC4 production 75% and 60% (compared to the stimulated control), at 1 μM, without and in the presence of AA, respectively.

Fig 5 (panel A) shows that AACOCF3 (3 μM) inhibited PGE2 formation 73% in this donor. However, consistent with its having some inhibitory action against COX, PGE2 was again reduced when in the presence of 20 μM AA, to a lesser extent (44%, Fig. 5, panel B). This suggested PGE2 inhibition by AACOCF3 in the absence of exogenous AA, was due to its ability to inhibit both the 85-kDa PLA2 and the COX enzyme. Indomethacin (1 μM) inhibited PGE2 formation 98% in the absence of exogenous AA and inhibited the enhanced PGE2 levels 80%, in the presence of 20 μM AA. AACOCF3 had no effect on LTC4 formation when A23187-stimulated monocytes were incubated with or without 20 μM AA. This indicated that at a concentration where AACOCF3 clearly had prostanoid inhibitory effects, it had no effect on downstream 5-LO AA-metabolizing enzymes.

**DISCUSSION**

We have provided further support for the co-existence of both the 85-kDa PLA2 and the type II 14-kDa PLA2 in human monocyte as cell-associated enzymes. Monocytes can be induced to co-produce a number of eicosanoid classes by a variety of stimuli (4, 29) and therefore offer an ideal system for the simultaneous study of the two enzymes. Early studies indicated that cell-associated type II 14-kDa PLA2 participated in stimulated AA release and subsequent eicosanoid formation. With the discovery of 85-kDa PLA2, many laboratories turned their focus toward this enzyme, as it had the characteristics that one would expect for an enzyme responsible for cellular AA metabolism, i.e. regulation by intracellular (nanomolar) Ca2+ levels, phosphorylation, up-regulation by growth factors or inflammatory cytokines and a selectivity for AA in the sn-2 position of substrate phospholipid (6, 17). However, there are no convincing studies that would discount the type II 14-kDa PLA2 as a relevant intracellular enzyme, as it also responds to nM levels of intracellular Ca2+ (42, 35) and readily hydrolyzes...
AA from the sn-2 position of substrate phospholipid, such as the AA-rich phosphatidylethanolamine, despite the lack of fatty acid specificity noted in vitro (45, 51). We utilized a variety of tools to directly assess the role of the respective cell-associated PLA2 enzymes in eicosanoid formation in response to A23187 or phagocytosis of zymosan particles.

Product formation is the best measure of the COX or 5-LO activity at the whole cell level. In our studies, production of both LT and prostaglandin products were well within the linear portion of a product versus time curve. The substrate requirements for the purified or recombinant cyclooxygenase or 5-LO enzymes, in solution, have been broadly reported, the substrate affinities are reasonably similar ($K_m$, 5–50 μM) and both require 1 mol of AA to produce 1 mol of respective product (52–54). SK7111, antisense treatment, significantly reduced human monocyte 85-kDa PLA2 enzyme protein, which corresponded with comparable reductions in the ability to form prostaglandins induced by either A23187 or phagocytosis of zymosan. The enzyme reduced most likely represents the constitutive form, as we did not stimulate induction of enzyme. A23187-stimulated monocytes treated with 3 μM SK7111 antisense produced 75–90% less PGE2 compared to that produced by the stimulated control monocytes. These levels were near or, in some cases below, the amount measured in the unstimulated controls, suggesting that the majority of the enzyme was depleted. Complete reversal of the PGE2 inhibition by addition of exogenous AA indicated that antisense did not alter the AA metabolism through the COX or 5-LO pathways. Under these conditions the stimulated LT formation was not altered, leading one to conclude that the 85-kDa PLA2 provides little or no AA for LT formation. The possibility that the basal levels of 85-kDa PLA2 enzyme remaining after antisense treatment could support full 5-LO metabolism is possible, but seems remote in light of the identical results with the 85-kDa PLA2 inhibitor and the data generated using a selective 14-kDa PLA2 inhibitor discussed below. Finally, zymosan-stimulated eicosanoid production responded to antisense treatment in an identical fashion, with no significant alteration in LT generation, suggests that this response is not unique to the ionophore stimulating system.

Utilization of the two PLA2 inhibitors provides further support for a lack of a role of the 85-kDa PLA2 in LT formation and preliminary evidence to hypothesize that a non-85-kDa PLA2 activity, possibly the type II 14-kDa PLA2, may also provide substrate for LT formation. Both inhibitors demonstrated the appropriate selective actions on the respective monocyte cytosolic 85-kDa PLA2 and particulate fraction 14-kDa PLA2 activities. Consistent with this, we have shown both to inhibit A23187-induced monocyte AA liberation, as assessed by mass, in a concentration-dependent fashion (data not shown). When evaluated in the whole cell system, the 85-kDa PLA2 inhibitor, AACOCF3, effectively reduced PGE2 formation (IC50, 0.3 μM), but at 10-fold greater concentrations had no effect on A23187-induced human monocyte LTC4 formation. Alternatively, concentration-dependent inhibition of 14-kDa PLA2 by SB 203347 prevented both A23187 (IC50, 0.4 μM) or phagocytosis-activated (IC50, 0.3 μM) LTC4 formation but had no effect on prostanoid generation at 10–100-fold greater concentrations. These observations were not restricted to PGE2 or LTC4 alone but were true of other LT or PG family members as well. SB 203347 inhibition of LT was not due to blockade of A23187-induced intracellular Ca2+ flux. Furthermore, overriding the need for deacylation of AA substrate by evaluation of SB 203347 in the presence of exogenous AA, prevented SB 203347 inhibition of LT formation. This supports the lack of SB 203347 direct action on AA conversion to LT via the 5-LO system. Alternatively, AACOCF3 inhibited the augmented PGE2 formation in the presence of exogenous AA, confirming reports that it also has actions on the COX pathway (20). The specific LT inhibitory actions of SB 203347 in the human monocyte corroborate its actions reported in neutrophil LT formation (33) and concur with the identical LT inhibitory actions of a variety of structurally diverse 14-kDa PLA2 inhibitors (29–32). The similar inhibitory effect of SB 203347 on antigen-induced mast cell LTC4 with no change in PGD2 production by SB203347 provide preliminary data to suggest that the lack of a role of 14-kDa PLA2 in prostaglandin biosynthesis is not restricted to the monocyte. Taken together, these studies provide intriguing observations to support the hypothesis that cell-associated 14-kDa PLA2 could be important in stimulated LT formation. More definitive studies are indicated to verify its participation.

In conclusion, the data provide additional evidence that the 85-kDa PLA2 primarily supports prostaglandin formation. The data indicate that this is the case in both acute stimuli systems as well as the ligand-activated cell systems previously reported. Alternatively, neither 75–90% reduction in 85-kDa PLA2 by antisense nor specific inhibition of its activity with AACOCF3 altered LT formation. Inhibition of cell-associated 14-kDa PLA2 with SB 203347 produced the reverse stimulated eicosanoid profile, i.e. inhibition of LT while prostaglandins were spared. The concept that two distinct enzymes might hydrolyze AA from different pools and/or supply distinct AA-metabolizing systems in a single-cell system is not new (4, 55). Taken together, the results provide a basis for the hypothesis that monocyte LT formation could be supported by substrate AA liberated by an sn-2 acylhydrolase distinct from the 85-kDa PLA2.

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