Role of secretory and cytosolic phospholipase A_2 enzymes in lysophosphatidylcholine-stimulated monocyte arachidonic acid release

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Received 29 August 2003; revised 17 October 2003; accepted 20 October 2003

First published online 4 November 2003

Edited by Felix Wieland

Abstract To determine if lysophosphatidylcholine (lysoPC) is able to induce proinflammatory changes in monocytes, its ability to stimulate arachidonic acid (AA) release, a product of phospholipase A2 (PLA_2) activity, has been analyzed. LysoPC increased AA release in THP-1 and Mono Mac6 cells in a time- and concentration-dependent manner. The monocytes expressed both secretory and cytosolic PLA_2 enzymes and AA release was strongly reduced by cellular pretreatment with different PLA_2 inhibitors and by pertussis toxin, an inhibitor of G_i-protein activation. This indicates that both cytosolic and secretory PLA_2 enzymes regulate specific lysoPC receptor-induced AA release, suggesting lysoPC participation in monocyte proinflammatory activation.

Key words: Phospholipase A2; Lysophosphatidylcholine; Atherosclerosis; Arachidonic acid; THP-1; Mono Mac6

1. Introduction

Phospholipase A2 (PLA_2) enzymes constitute a large family of lipolytic enzymes (for review see [1]) that catalyze hydrolysis of the fatty acid ester at the *sn*-2 position of phospholipids. This leads to production of lysophospholipids, including lysophosphatidylcholine (lysoPC), and free fatty acids. When the latter is arachidonic acid (AA), it is converted to potent proinflammatory mediators, prostaglandins, thromboxanes, leukotrienes, and lipoxins, collectively known as eicosanoids [2]. Lysophospholipids and eicosanoid lipid hormones are regulators of various physiologic processes and pathologic conditions [3]. Group IV cytosolic PLA_2 ($cPLA_2$), regulated by Ca^{2+}-dependent membrane translocation and phosphorylation, have a preference for AA in membrane phospholipids and play an essential role in agonist-induced AA release [4]. Secretory PLA_2s ($sPLA_2$) of group IIa and group V are also involved in AA release and subsequent eicosanoid production during inflammatory conditions [5].

LysoPC regulates a broad range of cellular processes such as monocyte chemotaxis, gene transcription, and monocyte proinflammatory cytokine secretion (for review see [6]). In human endothelial cells, extracellular lysoPC induces AA release through activation of $cPLA_2$ [7]. It has been indicated that lysoPC act through platelet-activating factor (PAF) receptor [8], however, G-protein-coupled receptors specific for lysoPC have been described [9,10].

Several lines of evidence have suggested that lysoPC may be responsible for many of the cellular effects of oxidized low density lipoprotein (oxLDL) in intima during early atherosclerosis development (for review see [11]). Group IIa $sPLA_2$ is present in human arteries [12], and can colocalize with LDL on proteoglycans [13]. This association of the enzyme with certain glucose aminoglycans increases the enzyme's LDL-modifying activity, rendering the LDL particle more proatherogenic [13,14]. Moreover, recently we showed that minimally oxLDL is able to induce expression of group IIa $sPLA_2$ in human macrophages [15], and that lipolytically modified LDL by $sPLA_2$ or SMase markedly increase monocyte AA mobilization (submitted).

It is likely that $sPLA_2$ may contribute to the pathogenesis of atherosclerosis by modifying LDL and releasing proinflammatory lipid mediators, such as lysoPC, at places of LDL retention in the arterial wall [14]. However, the biological consequence of local lysoPC accumulation for monocytes is uncertain. Our results suggest that lysoPC participates in monocyte proinflammatory activation due to specific dose- and time-dependent activation of AA release.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), fatty acid-free bovine serum albumin (BSA), insulin, oxalacetic acid, pyruvic acid, propranolol, pertussis toxin (PTX) and lysoPC (C16:0) were purchased from Sigma Chemical co. (St. Louis, MO, USA). L-glutamine, penicillin, streptomycin and non-essential amino acids were from Gibco BRL (Life technologies, Grand Island, NY, USA). SB203347, was a generous gift from James Winkler, Smith Kline Beedam (Pharmaceuticals, PA, USA). Methyl arachidonyl fluorophosphate (MAFP) and bromoenol lactone (BEL) were from Cayman Chemical (USA).

2.2. Cell culture

Human monocytic THP-1 cells and Mono Mac6 cells were main-

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Abbreviations: lysoPC, lysophosphatidylcholine; PLA_2, phospholipase A2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secretory PLA_2; AA, arachidonic acid; LDL, low density lipoprotein; oxLDL, oxidized LDL; PAF, platelet-activating factor; $iPLA_2$, calcium-independent PLA_2; PMA, 4β-phorbol 12-myristate 13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyl tetrazolium bromide; MAFP, methyl arachidonyl fluorophosphate; BEL, bromoenol lactone; OA, oleic acid; PTX, pertussis toxin; PAP, phosphatidic acid phosphohydrolase; BSA, fatty acid-free bovine serum albumin

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01242-0

tained as described by the Global Bioresource Center ATCC and [16], respectively. THP-1 cells were differentiated with 160 nM PMA.

2.3. RNA extraction

Total cellular RNA was isolated by Trizol extraction in accordance with the manufacturer's instructions (Gibco BRL, Life Technologies Inc., Grand Island, NY).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) detection of different PLA_2 isoforms

cDNA was synthesized from total RNA and cDNA templates were used in PCR amplification with primers for different groups of human PLA_2s as previously described [15,17]. Two new primers were designed [18,19]: group IId $sPLA_2$ fwd 5′-GTTCCTCAGCATGGAGCTC-3′, rev 5′-TCCGAGACTATATTGGAGG-3′; group VI PLA_2 fwd 5′-TGACAATTCTCAGGTGCTGC-3′, rev 5′-TCTTTCCAGGGAGAAGGGAT-3′. The conditions for the PCR reactions were denaturation at 95°C for 50 s, annealing at 58°C for 50 s and elongation at 72°C for 40 s, repeated 20–45×. For group VI (Ca^{2+}-independent PLA_2, $iPLA_2$) and X PLA_2 the annealing temperature was 65°C. PCR products were electrophoresed as previously described [17]. Fragment length after polymerization: group IIa $sPLA_2$: 247 bp, group IId $sPLA_2$: 339 bp, group IV $cPLA_2$: 465 bp, group V $sPLA_2$: 381 bp, group VI $iPLA_2$: 727 bp, group X $sPLA_2$: 417 bp.

2.5. Measurement of [^{3}H]AA and [^{14}C]oleic acid (OA) release

THP-1 or Mono Mac6 cells were labelled with [^{3}H]AA and [^{14}C]OA as previously described [20]. LysoPC was added to the cell cultures in prescribed concentrations and time periods in RPMI 1640 medium containing 1 mg ml^{-1} BSA. PLA_2 inhibitors were added at indicated concentrations 60–120 min prior to stimulation, propranolol 30 min prior to stimulation and PTX 3 h before stimulation.

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-difenyl tetrazolium bromide (MTT) assay

LysoPC and different chemical inhibitors were tested for cytotoxicity by MTT assay as previously described [21].

2.7. $cPLA_2$ enzyme activity assay

THP-1 cells were seeded (2×10^6 cells per well) and starved (0,5% fetal calf serum) 18 h before stimulation. Thereafter, $cPLA_2$ enzyme activity was measured as described previously [20].

2.8. Data presentation

The data were compared by Kruskal–Wallis test for random samples, and data with $P < 0{,}05$ were considered significant. The data are shown as means ± S.D. Data in Fig. 1 are given as disintegrations per minute (DPM), while data in Figs. 2 and 3 are given as percentage of [^{3}H]AA or [^{14}C]OA release with respect to total cellular radioactivity setting the control value to zero. Figs. 5 and 7 are shown as percent activity compared to lysoPC-stimulated cells. Fig. 6 is shown as percent $cPLA_2$ activity relative to non-stimulated cells. Each set of experiments was repeated three times.

3. Results

3.1. LysoPC stimulates [^{3}H]AA release in THP-1 and Mono Mac6 cells

In order to determine lysoPC-induced proinflammatory activation of monocytes, this activation was assessed as [^{3}H]AA release in THP-1 and Mono Mac6 cells. Compared to untreated cells, lysoPC gave an eight-fold increase in [^{3}H]AA release in THP-1 cells and a three-fold increase in Mono Mac6 cells (Fig. 1). However, when THP-1 cells were differentiated with PMA for 72 h and then stimulated with lysoPC the [^{3}H]AA release was similar to the release observed in Mono Mac6 cells (data not shown). Hence, our results suggest that lysoPC stimulates AA release in monocytes, and that the magnitude of AA release is differentiation dependent.

LysoPC elicited a dose-dependent increase of [^{3}H]AA release (Fig. 2), as well as a time-dependent increase with a maximum after 10 and 5 min in THP-1 and Mono Mac6, respectively (Fig. 3). Measurement of [^{14}C]OA release was performed in parallel with [^{3}H]AA release, and showed a similar pattern to [^{3}H]AA release, except that [^{14}C]OA release was approximately 2/3 of [^{3}H]AA release (Figs. 2 and 3). Cell viability was determined by MTT assay and both cell lines were 100% viable for optimal concentrations (40 and 30 μM) for 1 h after lysoPC exposure (results not shown). Based on these results, 10 min stimulation with 40 μM lysoPC was applied for THP-1, while 5 min stimulation with 30 μM lysoPC was used for Mono Mac6 cells in subsequent experiments.

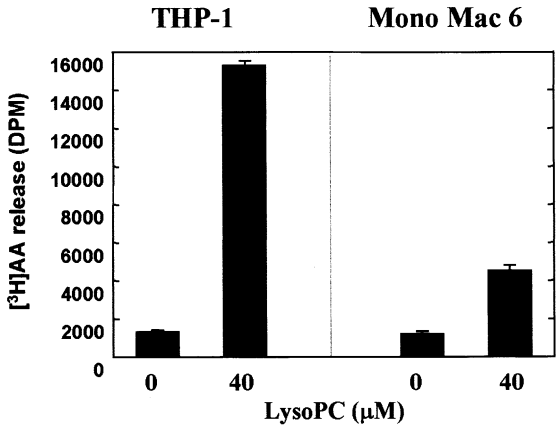

Fig. 1. LysoPC stimulates [^{3}H]AA release in human monocytic cell lines. THP-1 and Mono Mac6 cells prelabelled with [^{3}H]AA or [^{14}C]OA, were treated with lysoPC (40 and 30 μM, respectively) for 15 min.

3.2. $sPLA_2$, $cPLA_2$ and group VI $iPLA_2$ are expressed in THP-1 and Mono Mac6 cells

RT-PCR analysis with primers for different forms of PLA_2 enzymes was performed on total RNA extracted from THP-1 and Mono Mac6 cells. Transcripts were detected for group IId, IVα, and VI PLA_2 in undifferentiated THP-1 cells (Fig. 4, lane 2), and in Mono Mac6 cells (Fig. 4, lane 1). Transcript for group V PLA_2 was detected only in THP-1 (Fig. 4, lane 2). Upon PMA differentiation of THP-1 cells, transcripts for group IId, IV and VI PLA_2 were found to be constitutively expressed (Fig. 4, lanes 3 and 4), while expression of group V $sPLA_2$ was down-regulated upon differentiation (Fig. 4, lanes 3 and 4). Group IIa and group X $sPLA_2$ were not detected in any of the monocytic cell lines.

3.3. Both $sPLA_2$ and $cPLA_2$ enzymes regulate [^{3}H]AA release

Several specific PLA_2 inhibitors were examined for their ability to reduce agonist-induced fatty acid release. The active site-directed $sPLA_2$ inhibitor, SB203347 [22], strongly inhibited lysoPC-stimulated [^{3}H]AA release dose dependently in both THP-1 and Mono Mac6 cell lines with a maximum of 80% and 90% inhibition, respectively (Fig. 5A,B). The $sPLA_2$ enzymes have, in in vitro assays, not been shown to display any acyl chain selectivity [23] and SB203347 equally reduced both lysoPC-stimulated [^{3}H]AA and [^{14}C]OA release, confirming a role for the group IId and V $sPLA_2$ in monocyte AA release.

Using MAFP, a dual $cPLA_2$/group VI $iPLA_2$ inhibitor [24], the lysoPC-stimulated [^{3}H]AA release was also dose depen-

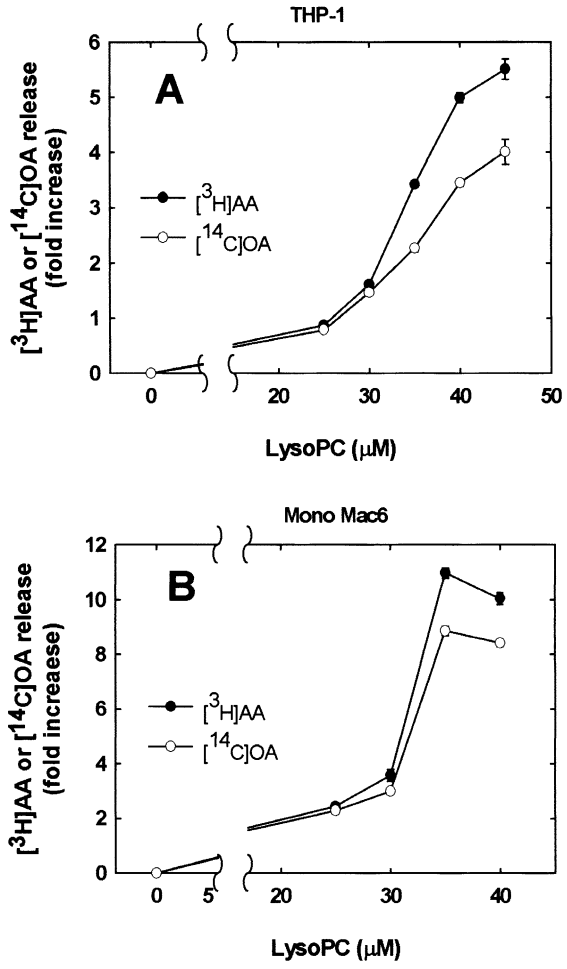

Fig. 2. LysoPC stimulates [^{3}H]AA and [^{14}C]OA release in a dose-dependent manner in THP-1 and Mono Mac6 cells. Prelabelled (A) THP-1 and (B) Mono Mac6 cells were treated with 25–45 μM lysoPC for 10 min.

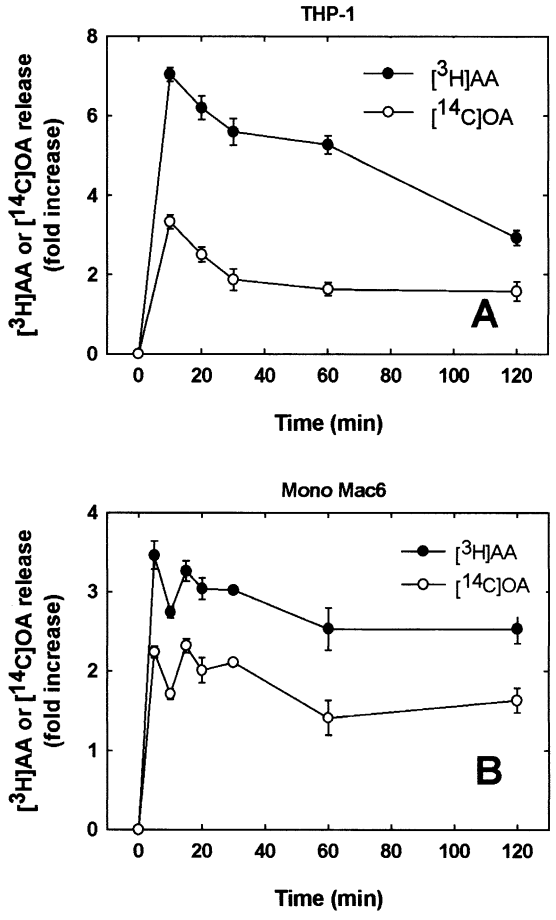

Fig. 3. LysoPC challenge of THP-1 and Mono Mac6 cells gives a peak in [^{3}H]AA and [^{14}C]OA release after, respectively, 10 and 5 min. Prelabelled (A) THP-1 or (B) Mono Mac6 cells were treated with (A) 40 or (B) 30 μM lysoPC for 0–120 min.

dently inhibited (Fig. 5C,D) in both THP-1 and Mono Mac6 cells with a maximum of 90% and 98% inhibition, respectively. BEL is a potent group VI iPLA$_2$ inhibitor [25] and manifests a 1000-fold selectivity for inhibition of group VI iPLA$_2$ versus cPLA$_2$ in vitro. BEL partially inhibited lysoPC-induced [^{3}H]AA release (Fig. 5E,F) in THP-1 and Mono Mac6 cells. To exclude that BEL exerts its reduction in lysoPC-stimulated AA release by inhibiting phosphatidic acid phosphohydrolase (PAP) [26], propranolol, a well established PAP inhibitor [27] was employed. LysoPC-stimulated AA release was, indeed, found to be reduced in the presence of propranolol (Fig. 5G), while dual inhibition with BEL and propranolol showed no additive effect (Fig. 5H). Therefore the initial inhibition observed with the dual cPLA$_2$/group VI iPLA$_2$ inhibitor MAFP is due to inactivation of the cPLA$_2$ enzyme which was further evidenced by assay of cPLA$_2$ enzyme activity (Fig. 6).

3.4. Involvement of G-proteins in lysoPC-stimulated AA release

The G$_i$-protein inhibitor PTX was used on prelabelled THP-1 cells. PTX inhibited the lysoPC-induced [^{3}H]AA release by a maximum of 50% (Fig. 7), suggesting that a G$_i$-protein partly mediates the lysoPC response in THP-1 cells.

4. Discussion

It is known that lysoPC mediate AA release in endothelial cells [7], and that a major pathway for AA release from agonist-stimulated cells is via hydrolysis of phospholipids by

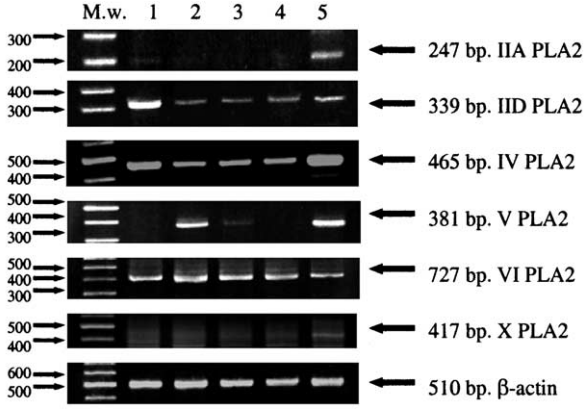

Fig. 4. RT-PCR detection of groups IId, IV, V, VI PLA$_2$ in THP-1 cells and groups IId, IV and VI in Mono Mac6. Lane 1 is Mono Mac6 cells, lane 2 is non-differentiated THP-1 cells, lanes 3 and 4 are THP-1 cells differentiated for 72 and 120 h, respectively, and lane 5 is positive control.

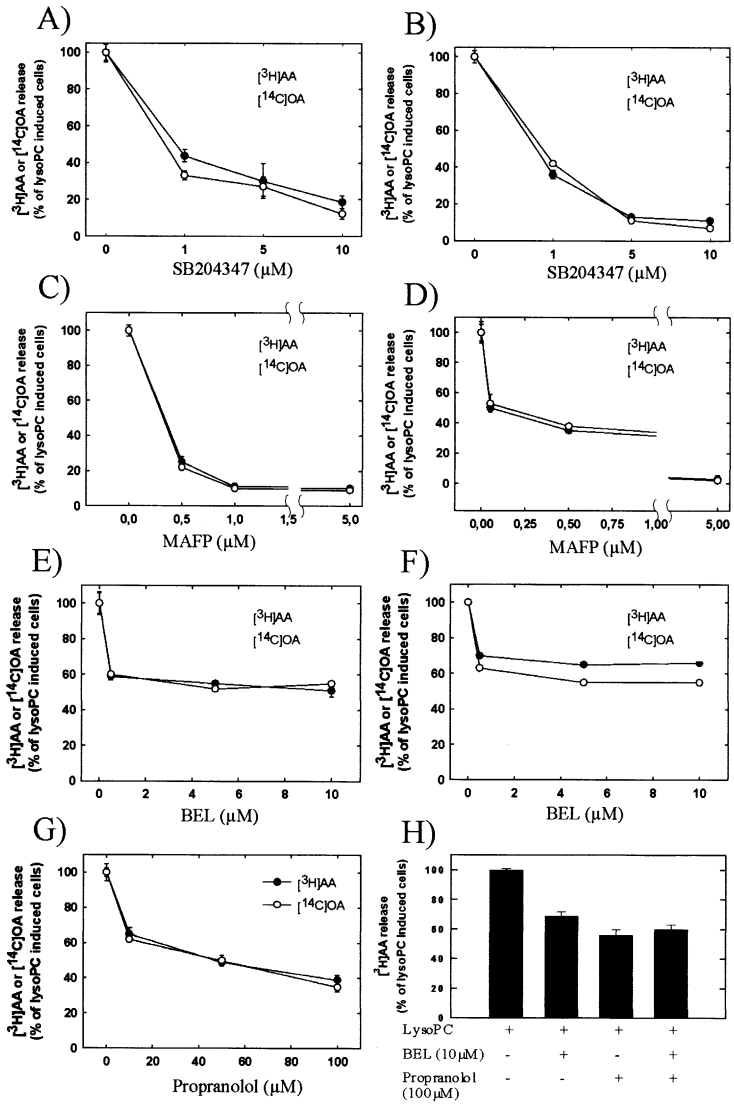

Fig. 5. Different PLA_2 inhibitors reduce lysoPC-induced [^{3}H]AA and [^{14}C]OA release. THP-1 cells and Mono Mac6 cells were incubated with different inhibitors as marked in the figure before treatment with lysoPC.

PLA_2 [28]. We show here for the first time that the group IV $cPLA_2$ enzyme is of importance in lysoPC-induced monocyte AA release, due to the successful action of MAFP and the out-ruling of partial contribution of the group VI $iPLA_2$ by the PAP inhibitors BEL and propranolol. This was also confirmed by the $cPLA_2$ assay. Furthermore, evidence is also provided for the participation of $sPLA_2$ enzymes in lysoPC-stimulated AA release based on concomitant AA and OA release. As shown by Balsinde et al. [29], AA/OA release is due to the action of secretory enzymes on the outer plasma membrane. Whether this release is due to the action of the group IId or V $sPLA_2$ enzyme is uncertain because the SB203347 inhibitor has been shown to inhibit both enzymes [30].

There are two observations indicating that a sequential relationship may exist between the secretory and cytosolic enzymes, (1) the AA release is totally attenuated by either an $sPLA_2$ inhibitor (SB203347) or a $cPLA_2$ inhibitor (MAFP) and (2) the $cPLA_2$ inhibitor has equal potency on both AA and OA release, despite the high arachidonyl selectivity of the enzyme. Such relationships between $sPLA_2$ and $cPLA_2$ en-

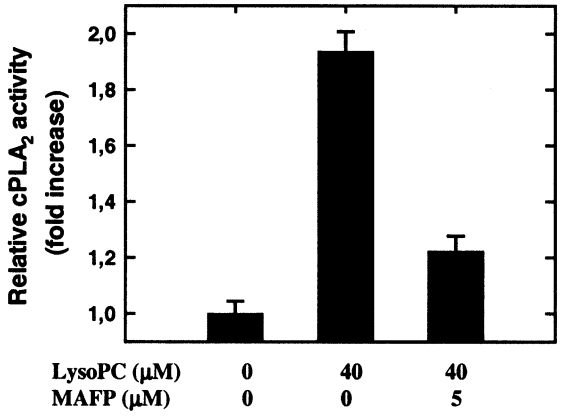

Fig. 6. LysoPC enhances $cPLA_2$ activity in human-derived THP-1 cells. $cPLA_2$ enzyme assays were done on cell lysates treated with lysoPC or MAFP and lysoPC.

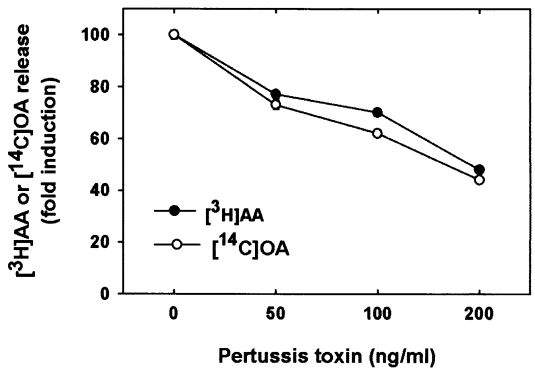

Fig. 7. LysoPC-induced [^{3}H]AA and [^{14}C]OA release in THP-1 cells is partly inhibited by the G-protein inhibitor PTX.

zymes were reported by us and others earlier [20,31]. Whether the secretory enzyme precedes the cytosolic or vice versa is uncertain.

Cellular expression of specific PLA_2 enzymes depends on cell type and state of differentiation, and there is limited information on PLA_2 distribution patterns in primary monocytes and monocytic cell lines. Specific expressions of $cPLA_2$ enzymes include: group IV in human primary monocytes [15], U937 [32] and THP-1 cells [33], while group VI $iPLA_2$ is reported from murine P388D macrophages [34]. Among the $sPLA_2$ enzymes constitutive expression of the group V from the murine macrophage cell line P388D [35] and primary derived monocytes [15] are reported, while expression of group IIa $sPLA_2$ is observed in primary, in vitro differentiated monocytes stimulated with minimally oxLDL [15]. We systematically examined the expression patterns of groups IIa, IId, IV, V, VI and X in THP-1 and Mono Mac6 cells and show for the first time expression of the group IId $sPLA_2$ in monocytes and the coexistence of groups IId, IV and VI PLA_2 independently of monocyte differentiation. Moreover, the group V $sPLA_2$ is constitutively expressed in undifferentiated THP-1 cells, and we show for the first time that its expression level diminishes upon PMA differentiation and that its expression is undetectable in Mono Mac6 cells representing more differentiated monocytes compared to THP-1.

The cellular molecular mechanism of lysoPC is not completely understood, but it has been indicated that lysoPC may elicit biological responses through the G-protein-coupled PAF receptor [8], or on specific lysoPC receptors [9,10]. Taking into account the structure of lysoPC it is important to know if the cellular actions of lysoPC are due to a specific response or to its amphiphatic property. Our results indicate involvement of a G_i-protein-coupled receptor due to the inhibitory effect of PTX; however, further investigations are needed to address the question of which receptor is involved in the response.

In summary, we have shown that lysoPC, a product of enzymatic modification of LDL, participates in proinflammatory activation of human-derived monocytes by activating $sPLA_2$ and $cPLA_2$. Additionally, we observed that expression of specific PLA_2 enzymes changed with differentiation of monocytes and that the proinflammatory activation of monocytes by lysoPC was partly mediated through a G_i-protein. Our results have provided a better molecular understanding of the specific role of lysoPC in proinflammatory monocyte activation, and may in the future provide new potential targets for drug development.

Acknowledgements: We thank James Winkler, Smith Kline Beecham (Pharmaceuticals, PA, USA) for kindly providing the $sPLA_2$ inhibitor SB203347. This work was supported by grants from the Norwegian Public Health program no. 1997/68, and a fellowship from the Norwegian University of Science and Technology no. 6228.

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