Interactions of 12-lipoxygenase with phospholipase A_2 isoforms following platelet activation through the glycoprotein VI collagen receptor

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Received 26 July 2004; revised 1 September 2004; accepted 7 September 2004

Available online 18 September 2004

Edited by Veli-Pekka Lehto

Abstract Recent studies implicate the collagen receptor, glycoprotein VI (GPVI) in activation of platelet 12-lipoxygenase (p12-LOX). Herein, we show that GPVI-stimulated 12-hydro(peroxy)eicosatetraenoic acid (H(P)ETE) synthesis is inhibited by palmityl trifluromethyl ketone or oleyloxyethylphosphocholine , but not bromoenol lactone, implicating secretory and cytosolic, but not calcium-independent phospholipase A_2 (PLA_2) isoforms. Also, following GPVI activation, 12-LOX co-immunoprecipitates with both cytosolic and secretory PLA_2 ($sPLA_2$). Finally, venoms containing $sPLA_2$ acutely activate p12-LOX in a dose-dependent manner. This study shows that platelet 12-H(P)ETE generation utilizes arachidonate substrate from both c- and $sPLA_2$ and that 12-LOX functionally associates with both PLA_2 isoforms.

Keywords: Platelet; 12-Lipoxygenase; Phospholipase A_2; 12-Hydro(peroxy)eicosa-tetraenoic acid; Glycoprotein VI

1. Introduction

Lipoxygenases (LOX) play central roles in vascular disease via synthesis of hydro(peroxy)eicosatetraenoic acids (H(P)ETEs) from unsaturated fatty acids [1]. Currently, little is known regarding acute control of LOX turnover in mammalian cells. Activation has been previously reported for neutrophil 5-LOX by fMLP, and platelet 12-LOX (p12-LOX) in platelets by collagen, and we recently reported that the collagen receptor, glycoprotein VI (GPVI), activates p12-LOX in platelets via *src* tyrosine kinases, PI3 kinase and calcium mobilization [2–6]. Direct activation of cellular LOXs is thought to involve (i) oxidation of the ferrous iron and (ii) translocation of the cytosolic enzyme to plasma or nuclear membranes. In addition, provision of arachidonate substrate by phospholipase A_2 (PLA_2) isoforms is a critical determinant of H(P)ETE synthesis, especially for the p12-LOX isoform which is unable to directly oxidize AA that is esterified to membrane phospholipids. Several PLA_2 isoforms are expressed by mammalian cells and previous studies have found a role for cytosolic calcium dependent PLA_2 ($cPLA_2$) in supply of arachidonate to the 5-LOX isoform [7]. However, the source(s) of arachidonate substrate for the p12-LOX and its interactions with PLA_2 isoforms following GPVI activation have not been explored.

Platelets possess several PLA_2 isoforms, including $cPLA_2$, calcium-independent ($iPLA_2$) and group II secretory phospholipase A_2 ($sPLA_2$) [8–10]. Studies on the mechanism of GPVI-mediated activation of platelet 12-H(P)ETE synthesis were undertaken to determine which of these PLA_2 isoforms are responsible for provision of arachidonate to platelet 12-LOX. The study shows that GPVI-dependent 12-H(P)ETE generation requires s- and $cPLA_2$ isoforms, and that following collagen or CRP activation, 12-LOX co-immunoprecipitates with both isoforms. Finally, $sPLA_2$ isoforms purified from *Apis mellifera* or *Bungarus multicinctus* acutely stimulated 12-H(P)ETE synthesis in a dose-dependent manner, indicating that exogenous $sPLA_2$ could also activate 12-LOX product generation.

2. Materials and methods

2.1. Reagents

Collagen (Type I) was from Mascia Brunelli, Milan, Italy. Collagen related peptide (CRP) was synthesized to a known sequence [11]. 12-Hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxy-11Z,13E-eicosadienoic acid (15-HEDE), bromoenol lactone (BEL), palmityl trifluromethyl ketone ($PACOCF_3$), oleyloxyethyl phosphorylcholine (OOEPC), $sPLA_2$ from *Apis mellifera* (>98% pure), β-bungarotoxin $sPLA_2$ from *Bungarus multicinctus* (>98% pure) and anti-$sPLA_2$ IgG were from Alexis Chemicals Ltd., Nottingham, UK. Anti-$cPLA_2$ IgG was from AbCam Ltd., Cambridge, UK. Anti-p12-LOX IgG was from Oxford Biomedical Ltd., USA. All other reagents were from Sigma Ltd., Poole, UK unless otherwise stated.

2.2. Preparation and activation of washed human platelets

Platelets were isolated as described [12] from consenting donors who were NSAID-free for two weeks prior to donating. Washed platelets were resuspended in Ca^{2+}-free Tyrodes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4). 3×10^8 platelets in 100 μl Tyrodes, 1 mM $CaCl_2$, were stimulated with collagen (10 μg ml^{-1}), CRP (5–10 μg ml^{-1}), bee $sPLA_2$ (0–50 ng ml^{-1}) or snake $sPLA_2$ (0–10 μg ml^{-1}) at 37 °C with agitation for 10 min. Experiments using PLA_2 inhibitors included a 10 min pre-incubation step at 37 °C prior to addition of agonist. Incubations were stopped by addition of 200 μl ice-cold methanol and samples spun at 10 000 × *g* for 10 min at 4 °C. Supernatants containing 12-H(P)ETE were recovered and supplemented with 200 ng 15(S)-HEDE per sample as an internal standard.

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.09.007

2.3. Reverse phase HPLC analysis of platelet 12-LOX products

Samples (equivalent to 2×10^7 cells) were separated on a 150 mm × 4.6 mm, 5 m C_{18} ODS2 (Waters Ltd., Ireland) column using 50–90% B over 20 min (A = water:acetonitrile:acetic acid, 75:25:0.1, B = methanol:acetonitrile:acetic acid, 60:40:0.1) at 1 ml min^{-1}. Absorbance was monitored at 235 nm. Products were identified and quantified using 200 ng 12(S)-HETE standard run in parallel under the same conditions.

2.4. Immunoprecipitation and Western blotting

Washed platelets (3×10^8 in 100 μl Tyrodes, pH 7.4, containing 1 mM $CaCl_2$) treated with agonists and/or inhibitors at 37 °C for 10 min with agitation were lyzed by resuspension in modified RIPA buffer (1% (v/v) nonidet P40, 0.1% (w/v) SDS, 0.1% (w/v) sodium deoxycholate, and PBS, pH 7.4) containing protease inhibitors at 4 °C, at a concentration of 1.5×10^9 cells/ml, and passing through a 21-gauge needle twice, before addition of anti-c- or $sPLA_2$ antibodies, SDS–PAGE and immunoblotting (probing for p12-LOX) according to existing protocols [13–15].

2.5. Statistical analysis

Data are representative of at least three separate donors, with samples run in triplicate for each donor (means ± S.E.M.). Significance was examined using unpaired t test at the 95% confidence interval, where $P \leqslant 0.05$ was considered significant (denoted by '*' in figures). Activity in the presence of inhibitors is expressed as % relative to the collagen or CRP or response, as appropriate.

3. Results

3.1. GPVI-mediated p12-LOX activity is sensitive to PLA_2 inhibition

To determine which PLA_2 isoforms provide arachidonate substrate to 12-LOX following its activation by GPVI, platelets were preincubated with inhibitors selective for particular PLA_2 isoforms and generation of 12-H(P)ETE in response to collagen or CRP determined. Inclusion of BEL, which is selective for $iPLA_2$, was without effect on either collagen or CRP activation of 12-LOX ruling out a role for this isoform (up to 500 nM, Fig. 1 and data not shown). In contrast, 12H(P)ETE synthesis was sensitive to inhibitors of c- or $sPLA_2$, although to different extents depending on the agonist. In particular, collagen activation was approximately 80% inhibited by $PACOCF_3$, but only approximately 35% by OOEPC, whereas CRP activation was inhibited 60% by OOEPC, but only 30% by $PACOCF_3$ (Fig. 1). This indicates that $cPLA_2$ may be the predominant source of arachidonate for p12-LOX in collagen-activated platelets, whereas both $cPLA_2$ and $sPLA_2$ appear important when selectively activating via GPVI.

3.2. Platelet 12-LOX co-immunoprecipitates with intracellular PLA_2 isoforms in agonist-treated washed platelets

To examine whether p12-LOX co-localizes with PLA_2 isoforms following activation, human platelets were treated with collagen or CRP before being subjected to immunoprecipitation with anti-$cPLA_2$ or anti-$sPLA_2$ and then probing for p12-LOX. Some immunoreactivity was observed in untreated platelets indicating basal association, however, this was substantially increased following collagen or CRP activation of platelets (Fig. 2A and B).

3.3. Venom $sPLA_2$ acutely activates p12-LOX

To examine whether exogenous $sPLA_2$ can activate p12-LOX, $sPLA_2$s from *Apis mellifera* (bee) or *Bungarus multicinctus* (snake) were added to platelets and 12-H(P)ETE generation determined. Both $sPLA_2$ isoforms acutely activated

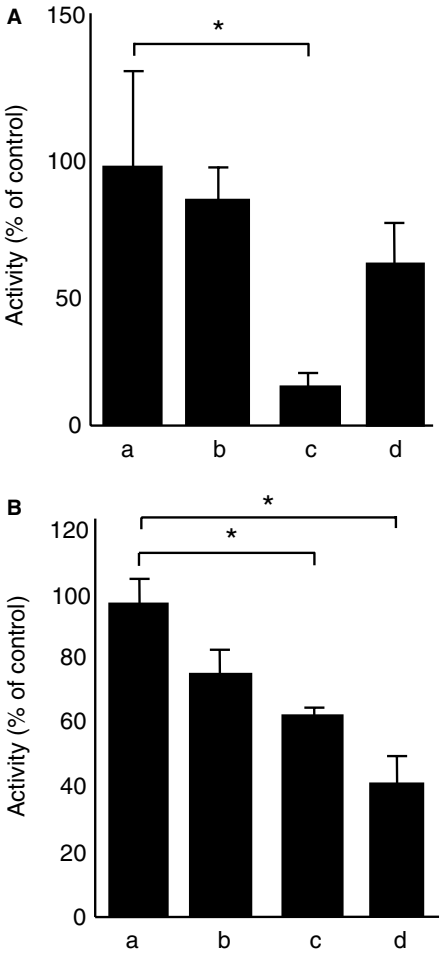

Fig. 1. Collagen and CRP-mediated p12-LOX activity is sensitive to c- and $sPLA_2$ inhibition. Samples were analyzed for 12-H(P)ETE content as described. Platelets were pre-incubated with the $iPLA_2$ inhibitor BEL (50 nM); the $cPLA_2$ inhibitor $PACOCF_3$ (10 μM) or the $sPLA_2$ inhibitor OOEPC (2 μM) before adding collagen (10 μg ml^{-1}) or CRP (10 μg ml^{-1}). (A) Inhibition of collagen-mediated p12-LOX by PLA_2 inhibitors. Platelets + (a) collagen; (b) + collagen + BEL; (c) + collagen + $PACOCF_3$; (d) + collagen + OOEPC. (B) Inhibition of CRP-mediated p12-LOX activity by PLA_2 inhibitors. Platelets + (a) CRP; (b) + CRP + BEL; (c) + CRP + $PACOCF_3$; (d) + CRP + OOEPC.

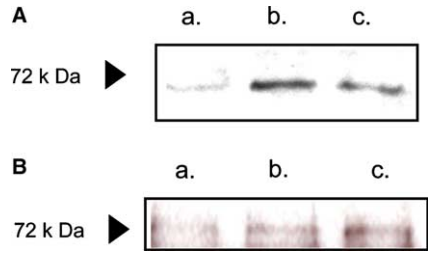

Fig. 2. Platelet 12-LOX co-immunoprecipitates with $cPLA_2$ and $sPLA_2$ in agonist-treated washed platelets. (A) p12-LOX co-precipitates with $cPLA_2$ in agonist-treated washed platelets. Western blot probing for p12-LOX following immunoprecipitation with anti-$cPLA_2$ (a) untreated plateles; (b) + CRP (5 μg ml^{-1}); (c) + collagen (10 μg ml^{-1}). (B) p12-LOX co-precipitates with $sPLA_2$ in agonist-treated washed platelets. Western blots probing for p12-LOX following immunoprecipitation with anti-$sPLA_2$ (a) untreated platelets; (b) + CRP (5 μg ml^{-1}); (c) + collagen (10 μg ml^{-1}).

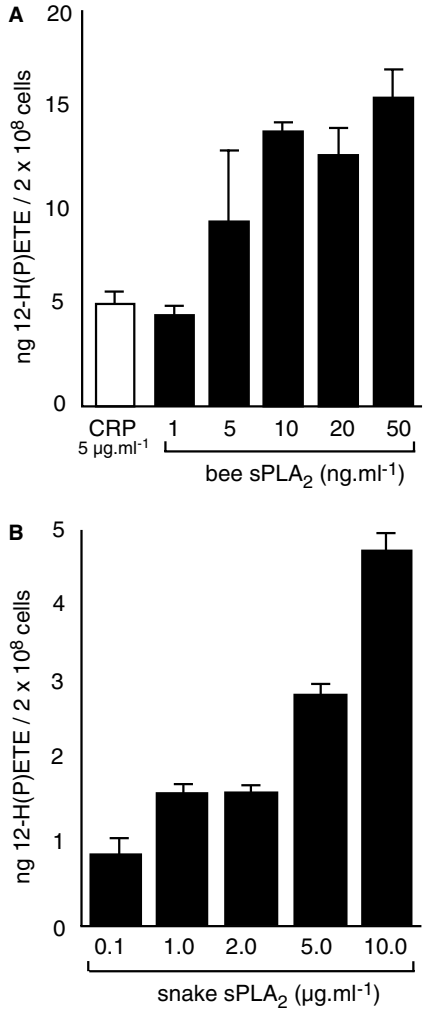

Fig. 3. Platelet 12-LOX is activated in a dose-dependent manner by bee and snake venom $sPLA_2$. (A) Dose-dependent activation of p12-LOX by bee venom $sPLA_2$. 3×10^8 platelets in Tyrodes buffer (pH 7.4), 1 mM $CaCl_2$ were treated with $sPLA_2$ (1–50 $ng\,ml^{-1}$), or CRP (5 $\mu g\,ml^{-1}$) as described in Section 2. (B) Dose-dependent activation of p12-LOX by snake venom $sPLA_2$. 3×10^8 platelets in Tyrodes buffer (pH 7.4), 1 mM $CaCl_2$ were activated with $sPLA_2$ (0.1–10 $g\,ml^{-1}$).

H(P)ETE generation in a dose-dependent manner (18.87 ± 2.00 and 6.85 ± 0.96 ng/2×10^8 platelets, for bee and snake isoforms, respectively, mean $\pm$ S.D., $n = 3$, Fig. 3A and B). In contrast, bovine pancreatic $sPLA_2$ was unable to stimulate generation of p12-LOX products (not shown). These data indicate that exogenous $sPLA_2$ isoforms can also activate p12-LOX, in addition to platelet $sPLA_2$.

4. Discussion

The detailed signaling pathways that activate LOX turnover and H(P)ETE synthesis acutely in mammalian cells are only now being characterized. We recently found a role for the collagen receptor, GPVI, in mediating acute activation of the platelet 12-LOX isoform and demonstrated a role for several downstream intracellular signaling pathways, including *src* tyrosine kinases, calcium mobilization and PI3 kinase [2], while a separate study found that collagen-induced arachidonate release requires H_2O_2 generation [16]. The present study further characterizes the pathway of GPVI activation of p12-LOX, with particular regard to which PLA_2 isoforms provide p12-LOX with arachidonate. Use of pharmacological inhibitors demonstrated that $iPLA_2$ is not involved in this pathway (Fig. 1). In contrast, both c- and $sPLA_2$ were required for full 12-LOX turnover (Fig. 1). Further evidence for their involvement was provided by immunoprecipitation experiments that demonstrated that either PLA_2 isoform was associated with p12-LOX following activation with collagen or CRP (Fig. 2).

Previous studies have suggested that $cPLA_2$ is an important source of arachidonate for the 5-LOX and 12/15-LOX isoforms [7,17], but investigations into associations of $cPLA_2$ with platelet 12-LOX have not been carried out. Our observations suggest a critical role for $cPLA_2$ in providing substrate to the p12-LOX also. This is in agreement with a recent study which showed that deletion of $cPLA_2$ causes 80% reduction in serum 12-HETE levels, although in mice this metabolite will not exclusively originate from platelet 12-LOX, as 12/15-LOX will also be a significant source [18].

A role for $sPLA_2$ in providing arachidonate for 12-H(P)ETE generation was also found, indicating that this isoform may be functionally coupled to p12-LOX following GPVI activation in platelets. To our knowledge, this is the first report of $sPLA_2$ involvement in GPVI signaling. Previous reports have shown that 12-H(P)ETE generated by leukocyte-type 12/15-LOX (following provision of substrate by $cPLA_2$) can activate $sPLA_2$ to release more AA, which is in turn metabolized to H(P)ETE by 12/15-LOX [17,19]. This was proposed to mediate a paracrine amplification of inflammation and showed a pathway, whereby $cPLA_2$ can activate $sPLA_2$ during cell signaling [20,21]. It is possible that a similar mechanism also exists in platelets since (i) exogenously added platelet-derived $sPLA_2$ is unable to mobilize arachidonate from resting platelets and (ii) addition of lipid hydroperoxide to rabbit platelets can overcome the inability of exogenous group II $sPLA_2$ to hydrolyze platelet membrane phospholipids [9,22]. The relative involvement of c- or $sPLA_2$ in 12-H(P)ETE synthesis varied with agonist used, with $cPLA_2$ being the predominant arachidonate source in collagen-activated platelets, and both $cPLA_2$ and $sPLA_2$ being similarly involved when selectively activating GPVI (Fig. 1). This difference may reflect signaling via additional receptors when using collagen as agonist, including α(IIb)β(3) integrin which can also activate thromboxane generation [23].

Our observation of co-precipitation of p12-LOX with $sPLA_2$ suggests that these enzymes are localized together during 12-H(P)ETE synthesis. Previous studies have shown that several $sPLA_2$ isoforms act internally to release arachidonate. For example, group IV $sPLA_2$ acts on the perinuclear membrane, where it co-localizes with 5-LOX [24]. Additionally, $sPLA_2$-IIA is sorted into caveolin-rich vesicular and perinuclear compartments during its secretion and internalization through association with glypican, a GPI-linked form of heparan sulfate proteoglycan [25]. Finally, $sPLA_2$-IIF localizes to the plasma membrane in HEK293 cells, and is coupled to downstream synthesis of both cyclooxygenase and 5-LOX independently of glypican [26].

Finally, we found that bee or snake venom $sPLA_2$, but not bovine pancreatic $sPLA_2$ could acutely activate 12-H(P)ETE generation in platelets. This is similar to previous observations

that bovine pancreatic or platelet $sPLA_2$s do not activate platelets, unlike venom isoforms, and has been suggested to be related to both origin (venom versus mammalian) and the relative inability of mammalian isoforms to hydrolyze phosphatidylcholine, the major phospholipid of the outer leaflet of the plasma membrane, in the absence of $cPLA_2$ turnover [9,17,20,27].

The biological significance of p12-LOX remains unknown, but a pathophysiological involvement in autoimmune thrombocytopaenia was recently suggested [28]. Herein, sPLA2 was utilized as a tool for dissecting LOX activation mechanisms, however, following exposure to venom $sPLA_2$ through bites or stings, it is possible that p12-LOX product generation may play a role in the thrombocytopaenia which can result [29].

In summary, this study shows that c- and $sPLA_2$ isoforms associate with and provide substrate to p12-LOX following activation of platelets by the collagen receptor GPVI and demonstrates a significant involvement of $sPLA_2$ in collagen signaling in platelets.

Acknowledgements: Funding from British Heart Foundation (M.J.C., V.B.O.) and Wellcome Trust (V.B.O.) is gratefully acknowledged.

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