

15-Lipoxygenation of phospholipids may precede the *sn*-2 cleavage by phospholipases A₂: reaction specificities of secretory and cytosolic phospholipases A₂ towards native and 15-lipoxygenated arachidonoyl phospholipids

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Abstract Reticulocyte-type 15-lipoxygenase is known to dioxygenate phospholipids without preceding action of phospholipases A₂ (PLA₂). Therefore we studied the reaction of the secretory PLA₂s (sPLA₂) from pancreas and snake venom, and of the human cytosolic PLA₂ (cPLA₂) with 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) and their 15-lipoxygenated species (PAPC-OOH and PAPC-OH) either alone or as equimolar mixtures. These PLA₂s cleaved PAPC-O(O)H with higher (sPLA₂) or similar rates (cPLA₂) as compared with native PAPC. In mixtures, however, PAPC proved to be the preferred, albeit not exclusive substrate for all three PLA₂s. Thus, partial 15-lipoxygenation of phospholipids may also trigger liberation of arachidonic acid.

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Key words: 15-Lipoxygenase; Phospholipase A₂; Cytosolic phospholipase A₂; Secretory phospholipase A₂; Peroxidized phospholipid

1. Introduction

Phospholipases A₂ (PLA₂s) constitute a family of signal transduction enzymes, which catalyse fatty acid hydrolysis from the *sn*-2 position of phospholipid with concomitant production of lysophospholipid, a key event in the production of inflammatory lipid mediators, e.g. prostanoids, leukotrienes and platelet-activating factor. Several PLA₂ isoforms are currently considered as potential targets for this role [1–3]. While the calcium-independent PLA₂ (iPLA₂) does not seem to mobilise fatty acids, both cytosolic PLA₂ (cPLA₂) and secretory PLA₂s (sPLA₂s) contribute to the total fatty acid turnover on the basis of spatial and temporal characteristics of activation and substrate preferences [4]. The liberated fatty acid is then transformed by a number of eicosanoid-synthesising enzymes to lipoxygenase products and prostanoids. Thus, arachidonic acid (AA) can be converted by 15-lipoxygenases to (15*S*)-hydro(pero)xy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid (15-H(p)ETE) for which a number of biological actions have been described [5]. Among the enzymes of the polyenoic fatty acid metabolism, the 15-lipoxygenase of the reticulocyte type including that occurring in human neutrophils as well as soybean lipoxygenase-1 exhibit the peculiarity to react not only with non-esterified polyenoic fatty acids but also with phos-

pholipids in the absence of preceding action of PLA₂ [6–12]. By this way, phospholipids bearing a (15*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoyl residue or another hydroperoxy-polyenoic fatty acid residue at the *sn*-2 position are formed, which may be subsequently reduced by phospholipid hydroperoxide glutathione peroxidase to the corresponding hydroxy derivatives [13,14]. Since the latter products have been detected in rabbit reticulocytes [15,16], which contain high levels of 15-lipoxygenase, 15-lipoxygenated phospholipids should be considered as potential biological substrates for PLA₂s. A number of studies have been reported in which a stimulation of the PLA₂-mediated phospholipid breakdown upon non-enzymatic lipid peroxidation or in the presence of lipoxygenated phospholipid was observed [8,17–24]. From these reports it could be assumed that a basic function of PLA₂s may be that of repairing enzymes against peroxidative damage to membrane phospholipids in the cell. However, many of these studies were inconsistent owing to the use of chemically undefined substrates (e.g. when the phospholipids were subjected to non-enzymatic lipid peroxidation) or by insufficient analytical data to characterise quantitatively and kinetically the PLA₂ action. Moreover, most of the work was done with snake venom PLA₂ whereas mammalian PLA₂s were used in only a few cases [8,22,23]. In the present study, we investigated the reactions of representatives of two main subfamilies of PLA₂, the 14 kDa sPLA₂ (group I) and the 85 kDa cPLA₂ (group IV) [2], on 1-palmitoyl-*sn*-2-arachidonoyl-phosphatidylcholine (PAPC) and its 15-lipoxygenated derivatives and provide comparative quantitative data on the liberation of arachidonic acid and of its 15-lipoxygenase products.

2. Materials and methods

2.1. Preparation of 1-palmitoyl-2-[(15*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoyl]-phosphatidylcholine (PAPC-OOH) and 1-palmitoyl-2-[(15*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoyl]-phosphatidylcholine (PAPC-OH)

PAPC-OOH was prepared by the use of soybean lipoxygenase according to the procedure of Brash and co-workers [25] with some modifications. Briefly, 780 µg of 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) were deprived of solvent and dissolved in 6 ml 10 mM sodium deoxycholate and 13 ml 0.2 M sodium borate buffer, pH 9.0, under sonication for 5 min. The reaction was started by addition of 250 µg of soybean lipoxygenase (Sigma, Germany) per 5.0 ml sample at room temperature. After 10 min the reaction was stopped by addition of 10 ml methanol and 5 ml chloroform. Thereafter 5 ml water were added to produce phase separation. The combined organic phases were evaporated and dissolved in the HPLC solvent methanol/acetonitrile/water 90:6:4 (v/v/v) containing 20 mM choline chloride

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and subjected to reverse-phase HPLC on a Nucleosil 1005 C₈ column (Macherey and Nagel, Germany) on a LC-6A instrument (Shimadzu, Japan) equipped with a diode-array detector SPD 1040A (Hewlett-Packard, USA). The fractions were monitored at 210 nm for non-converted substrate, 235 nm for lipoxygenated product and 270 nm for conjugated trienes. Crude P_{APC}-OOH was eluted at a retention time of about 7 min (flow rate 0.8 ml/min) and was purified by straight-phase HPLC (Zorbax Sil column 4.6 mm × 150 mm; DuPont) twice with the solvent *n*-hexane/2-propanol/water 4:6:1 (v/v/v). Pure P_{APC}-OOH was eluted at 12.5 min (flow rate 0.8 ml/min) and stored at -80°C under argon until used.

P_{APC}-OH was prepared similarly as P_{APC}-OOH except that the enzymatic reaction product was reduced with 151 mM sodium borohydride prior to extraction. The product was eluted at about 4 min (flow rate 0.8 ml/min) and at 14.2 min (flow rate 0.6 ml/min) in reverse-phase and straight-phase HPLC separations, respectively. The yields were approximately 45% for P_{APC}-OOH and 64% for P_{APC}-OH, respectively.

2.2. Phospholipase A₂ assay

sPLA₂ from *Crotalus adamanteus* venom (400 IU/mg protein) and bovine pancreas sPLA₂ (45 IU/mg protein) were purchased from Sigma, Germany. The enzymes were dissolved in 10 mM sterile Tris-HCl buffer, pH 8.0 to a concentration of 100 IU/ml. Purified human recombinant cPLA₂ was a kind gift of Dr. Tibes (Boehringer Mannheim) and used in a concentration of 184 µg protein/ml.

For the experiments with pure substrates, 25 µM of either P_{APC}, P_{APC}-OOH or P_{APC}-OH was sonicated twice for 1 min in 1.0 ml assay medium containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 5 mM CaCl₂ under argon atmosphere. The sPLA₂ from *C. adamanteus* venom exhibited a very little activity at pH 8.0 and was therefore measured at pH 9.0. The concentration of the phospholipids was determined by fatty acid analysis upon alkaline hydrolysis. After preincubation for 5 min the reaction was started with either 1 µl cPLA₂, or 0.55 IU sPLA₂ from bovine pancreas at 37°C or 0.25 IU sPLA₂ from *C. adamanteus* venom at 25°C. At various intervals 100 µl aliquots were taken, mixed with 200 µl methanol-acetic acid 98:2 containing 75 µM triphenylphosphine, and analysed for the liberation of fatty acids by reverse-phase HPLC on a Nucleosil 100-7C₁₈ column (Macherey and Nagel, Germany) with a precolumn 5-C₁₈-AB using the mobile phase methanol/water/acetic acid 85:15:0.1 (v/v). The peaks eluted at about 5 min (free 15-HETE) and 11.8 min (AA), respectively with a flow rate of 1.0 ml/min were quantitated by peak area using calibration curves with authentic standards. For some experiments, substrate mixtures consisting of 12.5 µM P_{APC} and 12.5 µM of either P_{APC}-OOH or P_{APC}-OH were used and treated as above.

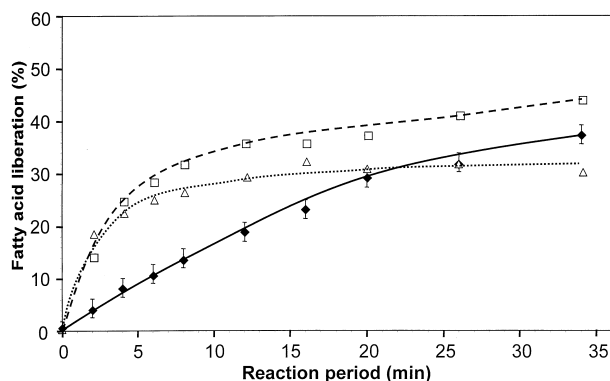


Fig. 1. *sn*-2 cleavage of pure non-lipoxygenated (P_{APC}) and 15-lipoxygenated (P_{APC}-OOH and P_{APC}-OH) substrate by the sPLA₂ from bovine pancreas. Conditions as described in Section 2. The values of AA liberation (◆) represent the mean ± S.D. of four separate experiments. The values of 15-HpETE (□) and 15-HETE (△) represent the mean of two experiments under identical conditions as for AA.

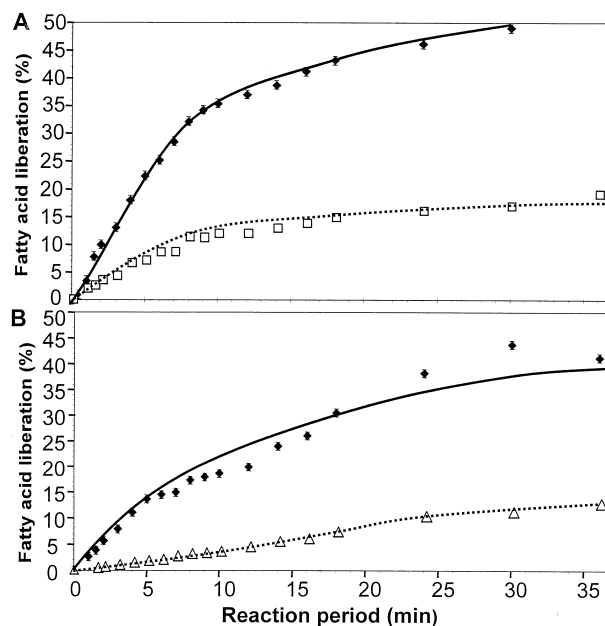


Fig. 2. *sn*-2 cleavage of equimolar mixtures of non-lipoxygenated (P_{APC}) and 15-lipoxygenated (P_{APC}-OOH and P_{APC}-OH) substrates (12.5 µM each) by the sPLA₂ from bovine pancreas. A: Equimolar mixture of P_{APC} and P_{APC}-OOH. B: Equimolar mixture of P_{APC} and P_{APC}-OH. The values for the liberation of AA (◆), 15-HpETE (□) and 15-HETE (△) are expressed as molar percentages of the initial total amount of phospholipids in the sample. AA values represent the mean ± S.D. of four separate experiments. The values of 15-HpETE and 15-HETE represent the mean of two experiments. Other conditions as in Fig. 1.

3. Results

3.1. Experiments with secretory phospholipases A₂

In Figs. 1 and 2 are shown representative examples of a series of experiments with sPLA₂ from bovine pancreas. This enzyme cleaved the pure 15-lipoxygenated phospholipids with about 3–4-fold higher initial rates as compared with the same concentration (25 µM) of P_{APC} within the first 5 min of reaction period (Fig. 1). Following this period there occurred a sharp decline of the cleavage of lipoxygenated phospholipids, so that after 30 min two thirds or three quarters of them were found to be uncleaved, respectively. This deviation from linearity, which was much less pronounced with P_{APC} (Fig. 1), was apparently in part due to a product inhibition by free 15-HETE or 15-HpETE, respectively, as a partial inhibition by 9 µM 15-HETE (the final concentration obtained under the experimental conditions in Fig. 1) of the *sn*-2 cleavage of P_{APC} was observed (data not shown). Comparatively much higher reaction rates of sPLA₂s (from porcine pancreas and bee venom) with defined oxygenated linoleyl phospholipids versus the parent one were also observed by van den Berg et al. who applied an indirect lipid monolayer technique for measuring PLA₂ activity, but did not analyse the fatty acids liberated [22].

A quite opposite behaviour was observed when equimolar mixtures of lipoxygenated and non-lipoxygenated phospholipids were used as substrate. Under these conditions P_{APC} turned out to be the strongly preferred substrate as compared with its 15-lipoxygenated derivatives with respect to both initial rates and final values for various reaction periods (Fig. 2). The initial rates of the liberation of arachidonic acid were

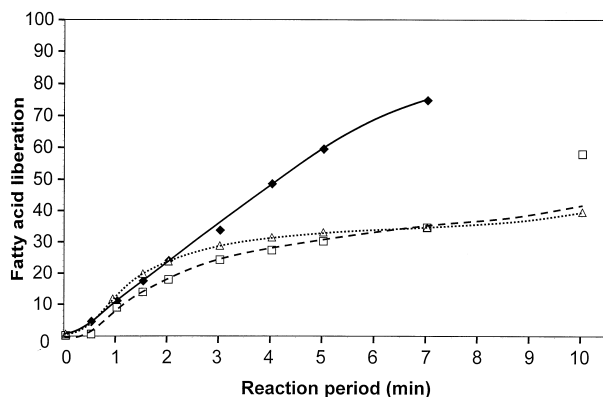


Fig. 3. *sn*-2 cleavage of pure non-lipoxygenated (PAPC) and 15-lipoxygenated (PAPC-OOH or PAPC-OH) substrates by the 85 kDa cPLA₂. Conditions and symbols as in Fig. 1. The values for the liberation of AA represent the mean \pm S.D. of four separate experiments, for that of 15-HpETE and 15-HETE the mean of two experiments.

approximately three and eight times higher than that of 15-HpETE and 15-HETE, respectively (Fig. 2). Moreover, the initial rates of the liberation of AA with the equimolar mixtures of PAPC and PAPC-OOH or PAPC-OH were about two to three times higher than with pure PAPC (cf. Figs. 1 and 2). Similar, albeit less pronounced effects were obtained with 1:10 mixtures of lipoxygenated and parent phospholipids (not shown). These observations are in sharp contrast to the work of Jung et al. who interpreted their results with porcine pancreas sPLA₂ as a preferred action on the lipoxygenated phospholipid on an analogous equimolar mixture within the first 30 s [8]. However, these authors measured the activity at 10°C at which the physicochemical structure of the phospholipids is different from that under physiological conditions in mammalian cells. Our data suggest a triggering by 15-lipoxygenation of the PLA₂-mediated liberation of AA from phospholipids.

Similar results were also obtained with snake venom sPLA₂ (Table 1). Again, higher enzymatic rates were observed with the pure 15-lipoxygenated phospholipids than with non-lipoxygenated PAPC, but in equimolar mixtures of PAPC and PAPC-OOH or PAPC-OH the PAPC proved to be the preferred substrate. Unlike the sPLA₂ from bovine pancreas, however, in our hands the snake venom enzyme did not reveal a facilitated liberation of AA from PAPC in the presence of equimolar amounts of 15-lipoxygenated phospholipids. This difference may be due to different experimental conditions when having measured this enzyme (higher pH, lower temperature, see Section 2). An enhancement of the AA liberation by *C. adamanteus* PLA₂, however, has been reported by other

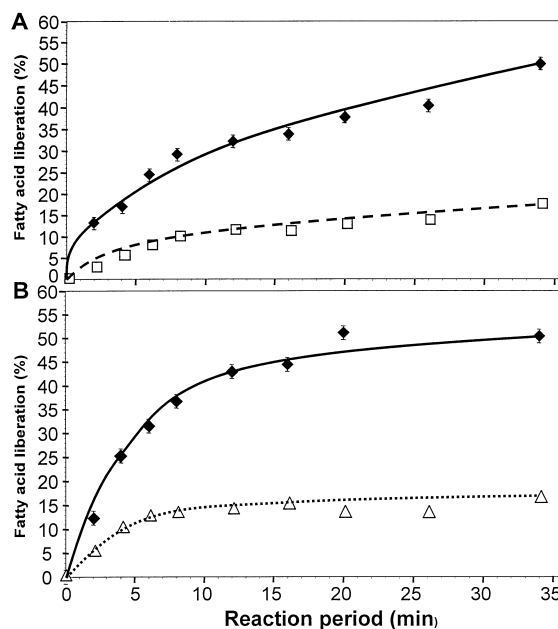


Fig. 4. *sn*-2 cleavage of equimolar mixtures of non-lipoxygenated (PAPC) and 15-lipoxygenated (PAPC-OOH or PAPC-OH) substrates by the 85 kDa cPLA₂. A: Equimolar mixture of PAPC and PAPC-OOH. B: Equimolar mixture of PAPC and PAPC-OH. Conditions and symbols as in Fig. 2. The values for the liberation of AA represent the mean \pm S.D. of three separate experiments, for that of 15-HpETE and 15-HETE sets of single experiments.

authors in the presence of 8% 1-palmitoyl-2-hydroperoxylinoleyl phosphatidylcholine at pH 7.8 and 37°C [23]. Therefore it is reasonable to assume that triggering of the liberation of AA by the presence of lipoxygenated phospholipids is a general property of sPLA₂s.

3.2. Experiments with the 85 kDa cytosolic phospholipase A₂

Upon reaction of cPLA₂ with lipoxygenated and non-lipoxygenated phospholipids, comparable initial rates of fatty acid liberation were obtained from pure non-lipoxygenated and parent phospholipids (Fig. 3). The time courses of these reactions were principally similar to those obtained with pancreatic sPLA₂ (Fig. 1) and may reflect progressive inhibition of cPLA₂ by accumulating 15-H(p)ETE. To our knowledge, this is the first direct evidence that cPLA₂ is capable of liberating 15-H(p)ETE from the corresponding phospholipids. This property is not in line with the general assumption in the literature that the action of cPLA₂ would be specific for AA release, which was solely deduced from the fact that cPLA₂ liberated selectively AA from a mixture of cellular phospholipids [26].

Table 1

Action of sPLA₂ from *C. adamanteus* venom on non-lipoxygenated and 15-lipoxygenated *sn*-2-arachidonoyl phosphatidylcholines in pure state and in equimolar mixtures

| Substrate(s) | AA liberation (μ M/min) | 15-H(p)ETE liberation (μ M/min) |
|---|------------------------------|--------------------------------------|
| 25 μ M PAPC | 2.82 (2) | – |
| 25 μ M PAPC-OOH | – | 4.08 (2) |
| 25 μ M PAPC-OH | – | 5.20 (2) |
| 12.5 μ M PAPC+12.5 μ M PAPC-OOH | 2.00 \pm 0.41 (3) | 0.55 \pm 0.02 (3) |
| 12.5 μ M PAPC+12.5 μ M PAPC-OH | 2.01 \pm 0.28 (3) | 0.76 \pm 0.02 (3) |

The time courses of the liberation of AA and of 15-H(p)ETE were obtained in a similar manner as in the experiments in Figs. 1 and 2. The values representing the linear parts of the kinetics after 1 min are given. Numbers of experiments are denoted in parentheses.

In equimolar mixtures of lipoxygenated and non-lipoxygenated substrates, however, again PAPC turned out to be the preferential substrate, but a small, but significant liberation of 15-H(p)ETE was found under these conditions as well (Fig. 4). Therefore, in cells cPLA₂ may be involved not only in the liberation of AA but also in that of 15-HETE under conditions at which its action is preceded by that of 15-lipoxygenase. Unlike other authors who used complex mixtures of phospholipids supplemented with 10% 1-palmitoyl-2-hydroperoxylinoleyl phosphatidylcholine as substrate for the cPLA₂ [23], we failed to observe a triggering of AA liberation from PAPC by the presence of PAPC-(O)OH.

4. Discussion

Using three different PLA₂s we clearly demonstrated that pure defined 15-lipoxygenated phospholipids are better substrates than the corresponding parent phospholipid which is in line with other reports using other enzymes and methods [22]. The progressive inhibition by liberated 15-H(p)ETE during prolonged reaction periods in the closed system observed by us should not occur in vivo since they are either bound to serum albumin or metabolised. In mixtures of lipoxygenated and non-lipoxygenated phospholipids, however, this preference was reversed with all three enzymes. This observation is not compatible with the putative function of repair enzymes toward peroxidative damage to membrane phospholipids as often proposed, inasmuch as a preferential liberation of AA would give rise to accumulation of esterified oxygenated fatty acids in the phospholipid mixture. It is conceivable that other PLA₂s not studied hitherto are responsible for the removal of oxygenated polyenoic fatty acids from oxidatively damaged membrane phospholipids in the cell. Another explanation could be a modification of the reaction specificity of PLA₂s by other membrane constituents. In this connection we refer to a recent work by Kampayashi et al. who observed a preferential action of *Crotalus adamanteus* venom PLA₂ on hydroperoxylinoleyl phosphatidylcholine if its concentration in the phospholipid mixture was below 10 mol% with concomitant presence of 25 mol% of cholesterol [24]. In the light of this discrepancy our results with defined model substrates construct a fundamental basis for the future work under conditions which correspond to the cellular environment.

Another important observation in our study is that 15-lipoxygenation of phospholipids appears to trigger the liberation of AA rather than that of 15-HETE. This triggering of the liberation of AA by concomitantly present PAPC-O(O)H cannot be accounted for by a detergent-like action of the latter, inasmuch as under our experimental conditions such an effect did not occur in the presence of the corresponding lysophospholipid (data not shown) which is more amphipathic than PAPC-O(O)H. Moreover, with red blood cells we failed to detect any cell-lytic action of PAPC-O(O)H at the concentrations used. It is generally accepted that the different reactions with lipoxygenated and non-lipoxygenated substrates or their mixtures may reflect different structures of the phospholipid liposomes or micelles rather than genuine differences in substrate specificity.

Assuming that this effect of 15-lipoxygenated phospholipids also occurs with the physiologically relevant sPLA₂ types in inflammatory cells, which is currently under investigation in our laboratory, it would reveal a novel possible biological

function of 15-lipoxygenases in the AA metabolic cascade wherein the action of 15-lipoxygenase precedes that of sPLA₂ and stimulates by this way the release of AA via the classical reaction cascade. Besides direct lipoxygenation of phospholipids, 15-lipoxygenated phospholipids can also be formed by esterification of lysophospholipids with the corresponding free hydroxy-polyenoic fatty acid, e.g. 15-HETE. The latter reaction has been reported to influence signal transduction processes [27–32]. In conclusion, 15-lipoxygenase processing of arachidonoyl phospholipids as well as incorporation of 15-HETE into phospholipids may modify the *sn*-2 cleavage by PLA₂s, and thus, signal transduction processes in a complex manner.

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