# Lipoxin synthase activity of human platelet 12-lipoxygenase

Mario ROMANO,* Xin-Sheng CHEN,† Yoshitaka TAKAHASHI,‡ Shozo YAMAMOTO,‡ Colin D. FUNK† and Charles N. SERHAN*§

*Hematology–Oncology Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, U.S.A.,
†Division of Pharmacology, Vanderbilt University, Nashville, TN 37232, U.S.A.,
‡Department of Biochemistry, Tokushima University School of Medicine, Tokushima 770, Japan

Human platelets and megacaryocytes generate lipoxins from exogenous leukotriene $A_4$ ($LTA_4$). We examined the role of human 12-lipoxygenase (12-LO) in lipoxin generation with recombinant histidine-tagged human platelet enzyme (6His-12-LO), partially purified 12-LO from human platelets (HPL 12-LO) and, for the purposes of direct comparison, permeabilized platelets. Recombinant and HPL 12-LO catalysed the conversion of intact $LTA_4$ into both lipoxin $A_4$ ($LXA_4$) and lipoxin $B_4$ ($LXB_4$). In contrast, only negligible quantities of $LXA_4$ were generated when recombinant 12-LO was incubated with the non-enzymic hydrolysis products of $LTA_4$. 6His-12-LO also converted a non-allylic epoxide, 5(6)-epoxy-(8*Z*,11*Z*,14*Z*)-eicosatrienoic acid. The apparent $K_m$ and $V_{max.}$ for lipoxin synthase activity of 6His-12-LO were estimated to be $7.9 \pm 0.8\ \mu M$ and $24.5 \pm 2.5$ nmol/min per mg respectively, and the $LXB_4$ synthase activity of this enzyme was selectively regulated by suicide inactivation. Aspirin gave a 2-fold increase in lipoxin formation by platelets but did not enhance the conversion of $LTA_4$ by the recombinant 12-LO. These results provide direct evidence for $LXA_4$ and $LXB_4$ synthase activity of human platelet 12-LO. Moreover, they suggest that 12-LO is a dual-function enzyme that carries both oxygenase and lipoxin synthase activity.

## INTRODUCTION

Human 12-lipoxygenase (12-LO) catalyses oxygenation of arachidonic acid at its C-12 position to give (12*S*)-hydroperoxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HPETE), a product first described in isolated platelets [1]. 12-LO activity was subsequently detected in many other mammalian cells including porcine leucocytes (reviewed in ref. [2]). However, human platelet 12-LO and porcine leucocyte 12-LO represent two distinct isoforms with different biochemical and immunological properties [2]. cDNAs recently obtained for porcine leucocyte 12-LO [3] and human platelet 12-LO [4–6] show that they share 65% predicted amino acid sequence homology [4–6]. Although 12-LO-derived products elicit biological activities *in vitro* (reviewed in ref. [7]), the biological role of platelet 12-LO remains to be fully appreciated.

Lipoxins are tetraene-containing eicosanoids with selective bioactions [8] that can be generated by single cell types [9] or during cell–cell interactions by transcellular metabolism. Multiple biosynthetic routes can lead to lipoxin. One route of lipoxin generation by transcellular biosynthesis involves conversion of leucocyte 5-LO-derived leukotriene $A_4$ ($LTA_4$) by platelets [10–13]. Evidence to date suggests that human platelet 12-LO is involved in lipoxin generation in that lipoxin-generating activity co-localizes with 12-LO activity, and lipoxin generation by intact platelets is inhibited by an LO inhibitor, esculetin [10]. Platelets isolated from patients with myeloproliferative disorders show reduced ability to generate lipoxin as well as reduced 12-LO activity during blastic crisis [12]. In addition, COS cells transfected with human platelet 12-LO cDNA generate lipoxin from $LTA_4$ [13]. Taken together these results suggest that human platelet 12-LO may play a pivotal role in lipoxin formation when exposed to cells that can both generate and emit $LTA_4$ to the extracellular milieu.

Lipoxin generation by platelets may be relevant in multifactorial events such as thrombosis, inflammation and atherosclerosis when transcellular biosynthesis between platelets and neutrophils is likely to occur *in vivo* [14]. This route of production is also of interest as both lipoxin $A_4$ ($LXA_4$) and lipoxin $B_4$ ($LXB_4$) are vasoactive [8] and $LXA_4$ may regulate neutrophil function via occupancy of specific recognition sites [15]. Although lipoxin generation is observed when either human platelets or megacaryocytes are exposed to $LTA_4$ [10–13], direct evidence for the role of the platelet 12-LO has not been obtained. Here, we report that recombinant platelet 12-LO (6His-12-LO) as well as partially purified human platelet 12-LO (HPL 12-LO) can generate both $LXA_4$ and $LXB_4$.

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## MATERIALS AND METHODS

Aspirin was obtained from Sigma (St. Louis, MO, U.S.A.). Prostaglandin H synthase (PGH synthase) from ram seminal vesicles was obtained from Oxford Biomedical Research Inc. (Oxford, MI, U.S.A.). H.p.l.c.-grade solvents were from Doe and Ingalls (Medford, MA, U.S.A.). Sep-Pak C18 cartridges were from Waters Associates (Milford, MA, U.S.A.). Diazo-

Abbreviations used: 12-HETE, (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 12-HPETE, (12*S*)-hydroperoxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; $LTA_4$, leukotriene $A_4$ [(5*S*)-*trans*-5,6-oxido-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid]; $LTB_4$, leukotriene $B_4$ [(5*S*,12*R*)-dihydroxy-(6*Z*,8*E*,10*E*,14*Z*)-eicosatetraenoic acid]; $LXA_4$, lipoxin $A_4$ [(5*S*,6*R*,15*S*)-trihydroxy-(7*E*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid]; $LXB_4$, lipoxin $B_4$ [(5*S*,14*R*,15*S*)-trihydroxy-(6*E*,8*Z*,10*E*,12*E*)-eicosatetraenoic acid]; 5(6)-EpETrE, (±)-5,6-epoxy-(8*Z*,11*Z*,14*Z*)-eicosatrienoic acid; LO, lipoxygenase; 6His-12-LO, 12-LO containing an additional six N-terminal His residues; PGH synthase, prostaglandin H synthase (cyclo-oxygenase); BSTFA, *NO*-bis(trimethylsilyl)trifluoracetamide.

§ To whom correspondence should be addressed.

methane was prepared from *N*-methyl-*N*-nitrosoguanidine purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). *NO*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Arachidonic acid was from NuCheck Prep (Elysian, MN, U.S.A.). Synthetic(±)-5(6)-epoxy-(8*Z*,11*Z*,14*Z*)-eicosatrienoic acid [5(6)-EpETrE] was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). $LTA_4$ methyl ester, $LXA_4$, 11-*trans*-$LXA_4$, $LXB_4$, 8-*trans*-$LXB_4$ and (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE) standards were obtained from Biomol Research Laboratories (Philadelphia, PA, U.S.A.). Saponification of $LTA_4$ methyl ester was carried out as described by Serhan and Sheppard [10].

### Preparation of histidine-tagged human platelet 12-LO

A DNA sequence encoding six histidines was attached to the 5′ end of human platelet 12-LO cDNA [4] by PCR. This cDNA was cloned in *Nhe*I-digested pETL to generate the baculovirus transfer vector pETL/6His-12-LO carrying the full-length 6His-12-LO DNA sequence under transcriptional control of the polyhedrin promotor. Recombinant baculovirus 6His-12-LO was produced by *in vivo* homologous recombination by co-transfection of Sf9 monolayers with wild-type viral DNA and transfer vector pETL/6His-12-LO followed by plaque assay. For purification of native 6His-12-LO, Sf9 cells (approx. $2.5 \times 10^7$ cells/plate) were infected with recombinant baculovirus 6His-12-LO for 48–60 h. Cells were washed with Dulbecco's PBS supplemented with phenylmethanesulphonyl fluoride (1 mM), $MgCl_2$ (10 mM) and DNAase (50 ng/ml) and incubated on ice for 5 min. Additional NaCl was added to a final concentration of 600 mM and incubated for 5 min. The cell suspension was homogenized (30–40 strokes) with a Pyrex Ten Broeck homogenizer, and Tween 20 was added (final concentration 0.2%), followed by centrifugation for 15 min at 15000 *g*. The supernatant was incubated with a portion of $Ni^{2+}$-$N(CH_3COOH)_3$ agarose (approx. 0.5 ml/$2 \times 10^8$ cells) for 1 h with continuous agitation. The agarose pellets were packed after centrifugation (1 min at 2500 *g*) into columns and washed twice with 40 vol. of imidazole (25 mM) in buffer containing NaCl (150 mM) and sodium phosphate (50 mM, pH 8.0) (buffer B). The column was washed twice with 40 vol. of imidazole (25 mM) in buffer B and eluted with 5 vol. of imidazole (100 mM) in buffer B to obtain 6His-12-LO. Protein fractions were stored at −70 °C in the presence of glycerol (17%). Purity of the preparation assessed by SDS/PAGE was greater than 95%.

### Isolation of human platelet and porcine leucocyte 12-LO

Human platelets isolated from 10 units of buffy coats were suspended in Tris/HCl (20 mM, pH 7.4) and sonicated at 20 kHz twice for 15 s on ice using a Branson sonifier, model 185D [16]. The lysate was centrifuged at 10000 *g* for 10 min, and then at 105000 *g* for 60 min. The supernatant (approx. 40 ml) was mixed with solid $(NH_4)_2SO_4$ at 35% satn. and stirred in an ice bath for 30 min. The precipitate was dissolved in 1 ml of Tris/HCl (20 mM, pH 7.4) and used as the partially purified human platelet 12-LO. Porcine leucocyte 12-LO was partially purified as in [19]. Briefly, porcine leucocytes obtained from 1 litre of aortic blood were suspended in Tris/HCl (20 mM, pH 7.4) and sonicated at 20 kHz twice for 30 s. The cell lysate was centrifuged as described above. Solid $(NH_4)_2SO_4$ was added to the supernatant (approx. 40 ml), and the fraction obtained at 25–50% satn. was suspended in 1 ml of Tris/HCl (pH 7.4), which was used as partially purified porcine leucocyte 12-LO.

### Preparation of platelets

Platelets were isolated from fresh peripheral blood and permeabilized as described by Romano and Serhan [17]. Briefly, platelet-rich plasma was mixed with EDTA (7 mM) and centrifuged (1100 *g* for 15 min). Cells were washed twice with Hepes/Tyrode buffer (pH 7.4) in the presence of EDTA (7 mM) and suspended in the same buffer containing human fatty acid-free albumin (0.1%). Platelets were enumerated using a Coulter counter (model ZF, Coulter Electronics Inc., Hialeah, FL, U.S.A.) and permeabilized using freeze–thawing (full cycle 30 min).

### Incubations and product analysis

Intact and permeabilized platelets were incubated with $LTA_4$ as described by Romano and Serhan [17]. 6His-12-LO as well as HPL 12-LO were incubated with $LTA_4$, 5(6)-EpETrE or arachidonic acid (0.8–13 nmol) for 10 s over 30 min in 50 mM Tris/HCl buffer (0.125–0.5 ml), pH 7.4. These incubations were performed in the presence and absence of 0.1% human fatty acid-free albumin. The non-enzymic hydrolysis products of $LTA_4$ were obtained by exposure to Tris buffer for 20 min at 37 °C. Product formation was examined by either real-time u.v. spectroscopy or after reversed-phase h.p.l.c. Real-time determinations were performed by monitoring changes in absorbance at either 234 nm or 316 nm in a model 8452 A spectrophotometer (Hewlett–Packard G.m.b.H.), equipped with a diode array detector. For reversed-phase h.p.l.c. analysis, incubations were terminated by the addition of $NaBH_4$ (0.5 mg/ml, 15 min), to reduce any remaining hydroperoxides to their corresponding alcohols, followed by cold methanol (2 vol.). Samples were extracted and eluted from Sep-Pak C18 cartridges [17]. U.v. spectra from the methyl formate fractions were recorded in methanol. Lipoxins were routinely resolved by reversed-phase h.p.l.c. using a model 484 u.v. detector and a pump model 501 (Waters Associates). The column, C18 (4.6 cm × 25 cm; J. T. Baker Inc., Phillipsburg, NJ, U.S.A.), was eluted with methanol/water/acetic acid (60:40:0.01, by vol.) at a flow rate of 1 ml/min with the detector set at 300 nm to monitor the tetraene chromophores. Products from 5(6)-EpETrE incubations were chromatographed using the same equipment, and the column was eluted with methanol/water/acetic acid (65:35:0.01, by vol.) at 1 ml/min with the u.v. detector set at 234 nm. For 12-HETE determinations, the column was resolved with methanol/water/acetic acid (75:25:0.01, by vol.) at a flow rate of 1 ml/min with the u.v. detector set at 234 nm. Products were routinely identified and quantified as described in [17]. For analysis of products formed by 6His-12-LO, another h.p.l.c. system was also used which consisted of a dual pump and solvent control apparatus (LKB Instruments, Bromma, Sweden) linked to a diode array detector HP 1040 M series II (Hewlett–Packard G.m.b.H.). Post-h.p.l.c. analyses were performed utilizing an h.p.l.c.$^{3D}$ Chemstation (DOS series) data system. Isomerization of $LXA_4$ and $LXB_4$ to their respective all-*trans* isomers was quantified during work-up procedures by adding synthetic $LXA_4$ and $LXB_4$ to cell-free buffer controls in parallel, and percentage isomerization was determined after h.p.l.c. [17].

For g.c.–m.s. analysis, compounds collected after h.p.l.c. were treated with diazomethane for 20 min at room temperature followed by BSTFA for 12 h at room temperature in the dark. The gas chromatograph was a Hewlett–Packard 5890 series II with a Hewlett–Packard 5971A mass-selective detector quadrupole equipped with m.s. Chemstation software (Hewlett–Packard 1030A). This instrument was also equipped with a Hewlett–Packard Ultra 2 (25 m × 0.2 mm × 0.33 μm), and the gas chromatograph temperature program was initiated at 150 °C

and reached 250 °C after 10 min and 325 °C after 20 min. Data were collected at 1.6 scans/s in the scan mode.

## RESULTS AND DISCUSSION

To examine lipoxin-generating activity of human platelet 12-LO, recombinant enzyme (6His-12-LO) was incubated with $LTA_4$ and product formation was either monitored in real time by u.v. spectroscopy or analysed by reversed-phase h.p.l.c. after extraction. A time-dependent increase in absorbance at 316 nm was observed when $LTA_4$ was added to 6His-12-LO (Figure 1a), indicating that $LTA_4$ was transformed to tetraene-containing products. The reversed-phase h.p.l.c. profile of products formed during these incubations showed materials eluted with the retention times of $LXA_4$, $LXB_4$ and their respective all-*trans*-isomers (Figure 1b). U.v. spectra of these materials recorded on-line displayed triplets of absorption consistent with tetraene-containing eicosanoids (Figure 1c). For purposes of direct comparison, these products were also formed when either HPL 12-LO or permeabilized platelets were exposed to $LTA_4$ (Figure 2). In parallel incubations, neither heat-inactivated 6His-12-LO nor HPL 12-LO (100 °C, 60 min) transformed $LTA_4$ (results not shown). When 6His-12-LO (10 $\mu$g) was co-incubated with permeabilized platelets ($1.5 \times 10^8$) and exposed to $LTA_4$ (9 nmol), a total amount of $490.4 \pm 20.1$ pmol of lipoxin was formed which corresponded to the sum of lipoxin generated by either platelets ($252.1 \pm 52.2$ pmol) or 12-LO alone ($286.9 \pm 30.5$ pmol; $n = 3$). This observation diminishes the possible contribution of other enzymes carrying lipoxin synthase activity from human platelets

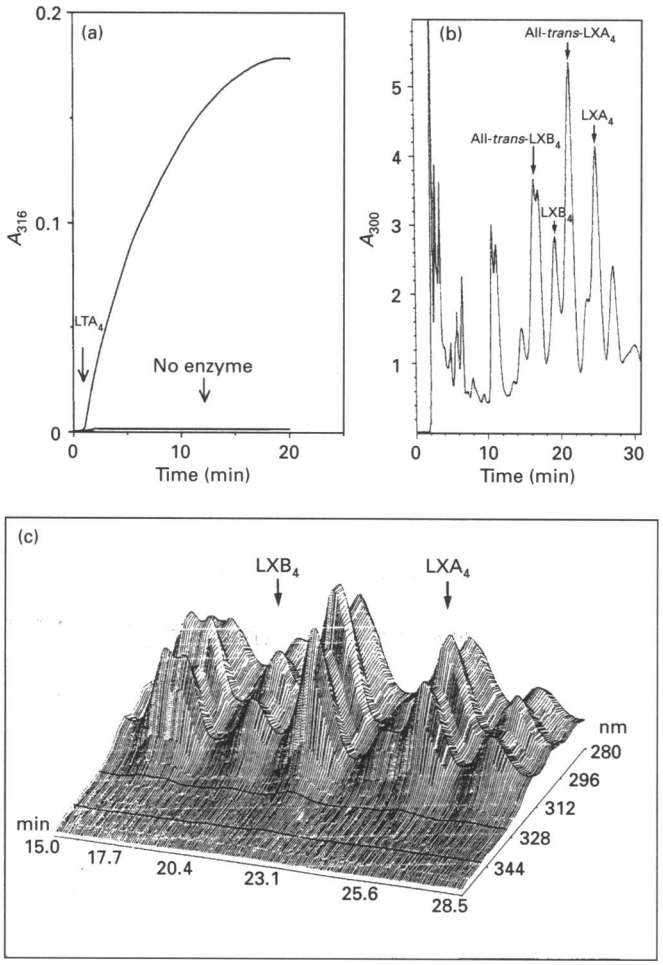


**Figure 1 Transformation of $LTA_4$ to lipoxins by recombinant 12-LO**

(**a**) Time course. $LTA_4$ (5.6 nmol) was added to 6His-12-LO (2.3 $\mu$g) in Tris/HCl buffer (pH 7.4; 0.2 ml). Changes in absorbance at 316 nm were monitored for 20 min (10 s cycles). The tracing is representative of $n = 10$. (**b**) Reversed-phase h.p.l.c. profile. 6His-12-LO (10 $\mu$g in 0.125 ml of Tris buffer) was incubated with $LTA_4$ (12 nmol) for 20 min at 37 °C. Incubations were terminated and products analysed by reversed-phase h.p.l.c. as described in the Materials and methods section. The tracing is representative of 42 determinations. (**c**) Three-dimensional u.v. spectra of products. Enlargement of the chromatographic region between 15 and 28.5 min with a three-dimensional Chemstation plot of acquired u.v. spectra. Materials eluted at 12 min did not carry tetraene chromophores.

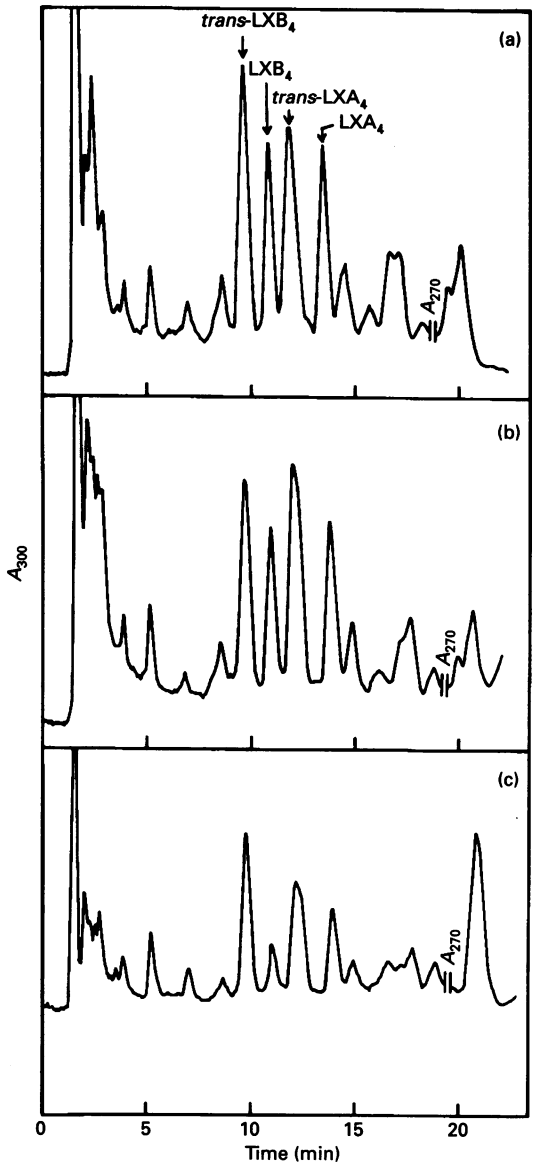


**Figure 2 Comparison of $LTA_4$ conversion into lipoxin**

Permeabilized platelets ($2.5 \times 10^8$ in 0.5 ml of Hepes/Tyrode buffer) (**a**), HPL 12-LO (230 $\mu$g in Tris buffer) (**b**), or 6His-12-LO (10 $\mu$g in Tris buffer) (**c**) were incubated with $LTA_4$ (9 nmol) for 20 min at 37 °C. Incubations were terminated and products identified and quantified as described in the Materials and methods section. Retention times of authentic lipoxins are denoted by arrows ($n = 5$ with 15 determinations).

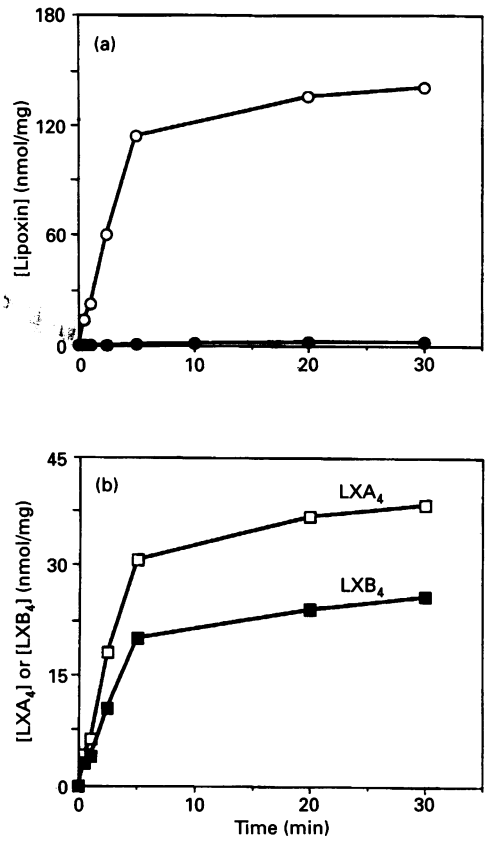


**Figure 3 Time course of $LTA_4$ conversion into $LXA_4$ and $LXB_4$ by recombinant 12-LO**

6His-12-LO (30 $\mu$g) was incubated at 37 °C in 0.75 ml of Tris/HCl buffer (50 mM, pH 7.45) with either intact $LTA_4$ (54 nmol) (○) or non-enzymic hydrolysis products of $LTA_4$ (54 nmol) (●). At the indicated intervals, samples (0.125 ml) were removed and reactions terminated. After extraction, products eluted in the methyl formate fractions were identified and quantified by reversed-phase h.p.l.c. as in Figure 1 and the Materials and methods section. (**a**) Total lipoxins (sum of $LXA_4$, $LXB_4$ and their all-*trans* isomers); (**b**) individual $LXA_4$ (□) and $LXB_4$ (■) formed during incubation of 6His-12-LO with $LTA_4$. Values reported take into account *trans* isomerization of native $LXA_4$ and $LXB_4$ as in [17]. Data are representative of two separate experiments.

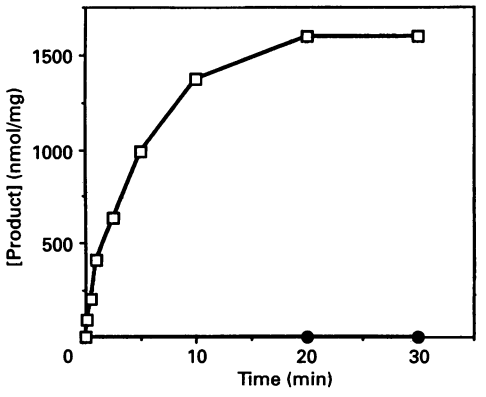


**Figure 4 Time course of 5(6)-EpETrE transformation by recombinant 12-LO**

5(6)-EpETrE (54 nmol) was incubated for the indicated times with either 6His-12-LO (1 $\mu$g) (□) or heat-denatured enzymes (100 °C, 60 min) (●) using similar conditions to those in Figure 3. Products were identified and quantified after reversed-phase h.p.l.c. Data represent the sum of the two major products (see the text).

that may have participated in the conversion of $LTA_4$ into lipoxin. Moreover, when samples of HPL 12-LO and partially purified porcine leucocyte 12-LO that exhibited comparable catalytic activity with arachidonic acid were incubated with $LTA_4$, the leucocyte enzyme generated approx. 10 times more lipoxin than the platelet enzyme (results not shown). These findings are consistent with the rank order of activities previously obtained with immunoaffinity-purified enzyme [16].

Results in Figures 1 and 2 establish that the human platelet 12-LO carries both $LXA_4$ and $LXB_4$ synthase activity. A previous study with 12-LOs of human and bovine platelets incubated with $LTA_4$ did not demonstrate definite lipoxin peaks on h.p.l.c., and concluded that the platelet enzymes had much lower, if any, activities of lipoxin production from $LTA_4$ as compared with the leucocyte enzymes [16]. In the present study, we used human platelet 12-LO that was more concentrated by $(NH_4)_2SO_4$ precipitation, rather than the immunoaffinity-purified enzyme utilized previously, and thus $LTA_4$ was incubated with a higher enzyme activity. In addition, a purified recombinant enzyme (6His-12-LO) was used that clearly demonstrates lipoxin synthesis. It is also apparent that the recombinant 12-LO carries both $LXA_4$ and $LXB_4$ synthase activity with $LTA_4$, as co-incubation of the enzyme with platelets resulted in an additive rather than a synergistic effect on lipoxin formation. Taken together, these results are consistent with those obtained with COS M6 cells transfected with human 12-LO cDNA [13] and provide further support for the proposed lipoxin-biosynthetic scheme where both $LXA_4$ and $LXB_4$ are generated by 12-LO-catalysed conversion of $LTA_4$ into a common delocalized cation intermediate [10,13]. Transformation of $LTA_4$ to lipoxin represents an additional function of 12-LO distinct from its originally described oxygenase activity [1] with arachidonic acid as substrate because it includes a multistep process to generate $LXA_4$ and $LXB_4$; namely, the initial hydrogen abstraction from the C-13 position of $LTA_4$ followed by oxygenation at C-15 exemplifies the oxygenase activity of this enzyme [1], which, from the present results, also includes abstract hydrogen at C-13 of $LTA_4$. The unique events in this process are that the 12-LO can also direct or guide water attacks on the substrate (i.e. envisaged to be a delocalized cation intermediate). Thus 12-LO-directed addition of the water-derived alcohol at C-6 of $LTA_4$ gives rise to $LXA_4$; hence the $LXA_4$ synthase activity of 12-LO, and addition of the alcohol group at C-14 of $LTA_4$ to give $LXB_4$ constitutes the enzyme's $LXB_4$ synthetase activity. Taken together, the present results indicate that platelet 12-LO is a dual-function enzyme joining several other enzymes of the eicosanoid cascade that possess dual activities. For example, 5-LO carries $LTA_4$ synthase activity [8], 12-LO from rat basophilic leukaemia cells shows $LTA_4$ synthase activity [18], 12-LO from porcine leucocytes converts 15-HPETE into 14,15-$LTA_4$ [19] and $LTA_4$ hydrolase also serves as an aminopeptidase [20].

$LTA_4$ is an allylic epoxide which undergoes non-enzymic hydrolysis in aqueous medium to yield 6-*trans*-$LTB_4$ and 6-*trans*-12-epi-$LTB_4$ as major products as well as two 5,6-DiHETEs [(5*S*,6*R*/*S*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acids] in lesser amounts [21]. Platelets have been recently shown to convert (5*S*,6*R*/*S*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acids into $LXA_4$ and (6*S*)-$LXA_4$, which illustrates yet another novel route in their biosynthesis [22]. Here, we determined whether conversion of (5*S*,6*R*/*S*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acids could account for lipoxin formation during transformation of $LTA_4$. To this end, 6His-12-LO was incubated with equimolar amounts of intact $LTA_4$ or $LTA_4$ that had previously undergone non-enzymic hydrolysis in Tris buffer (20 min at 37 °C). $LXA_4$ and $LXB_4$ generation was observed with

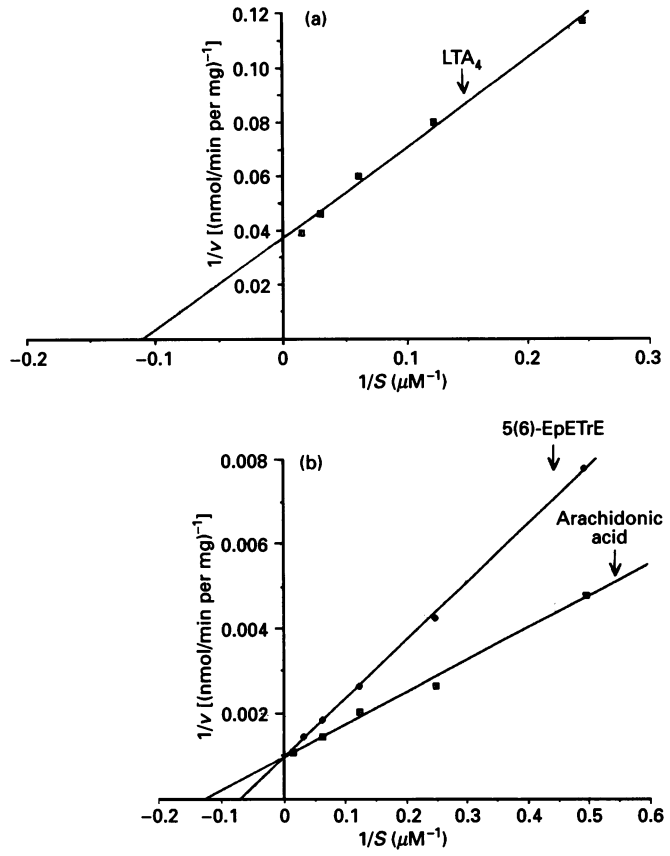


**Figure 5 Lineweaver–Burk plots of $LTA_4$, 5(6)-EpETrE and arachidonic acid conversion**

(**a**) 6His-12-LO (5 $\mu$g) was incubated in 0.2 ml of Tris/HCl buffer (pH 7.4) with $LTA_4$ (0.8–13 nmol). (**b**) 6His-12-LO (2 $\mu$g) was incubated in 0.2 ml of Tris/HCl buffer (pH 7.4) with either 5(6)-EpETrE (0.8–13 nmol) or arachidonic acid (0.8–13 nmol). Initial velocities were determined spectrophotometrically from the rate of increase in absorbance at 316 nm for $LTA_4$ (as in Figure 1a) and 234 nm for 5(6)-EpETrE and arachidonic acid without albumin present.

**Table 1 Kinetic constants of transformations catalysed by recombinant 12-LO**

Kinetic constants were determined from the data in Figure 5. Results are means ± S.E.M. of two experiments with four determinations. Two different batches of 12-LO gave comparable results.

| Substrate | $K_m$ ($\mu$M) | $V_{max.}$ (nmol/min per mg) |
|---|---|---|
| $LTA_4$ | 7.9 ± 0.8 | 24.5 ± 2.5 |
| 5(6)-EpETrE | 14.3 ± 0.3 | 984.6 ± 58.3 |
| Arachidonic acid | 6.2 ± 1.8 | 1057.0 ± 20.5 |

$LTA_4$ as substrate in a time-dependent fashion. In contrast, incubations with the hydrolysed products generated low amounts of $LXA_4$ and isomers that were detected only at the later time intervals. In these incubations, neither $LXB_4$ nor its isomers were generated (Figure 3). The profile of lipoxins obtained when 6His-12-LO was incubated with (5*S*,6*R*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid (not shown) was consistent with those recently reported with human platelets [22]. Also, recombinant 12-LO converted $LTA_4$ methyl ester into both $LXA_4$ and $LXB_4$ methyl esters (results not shown). These results clearly indicate that $LTA_4$ is used by 6His-12-LO as an intact epoxide.

To determine whether reaction of 12-LO with epoxide-containing eicosanoids was restricted to the allylic form of the epoxide, 6His-12-LO was incubated with a cytochrome *P*-450-derived non-allylic epoxide. Among non-allylic epoxides of biological interest is 5(6)-EpETrE which is formed by cytochrome $P_{450}$-catalysed oxygenation of arachidonic acid [23]. It contains an epoxide at C-5 and -6, like $LTA_4$, but is non-allylic and was recently shown to be metabolized by human platelets [24]. When incubated with 6His-12-LO, 5(6)-EpETrE was converted in a time- and concentration-dependent fashion (Figures 4 and 5 respectively). Two products that gave parallel kinetics of formation and were resolved by reversed-phase-h.p.l.c. (methanol/water/acetic acid; 65:35:0.01, by vol.) had retention times of 19.2 min and 34.5 min respectively. These materials showed u.v. spectra with $\lambda_{max.}^{methanol}$ of 234 nm and were not formed by the heat-denatured recombinant enzyme (100 °C, 60 min) (Figure 4). Mass spectra obtained from their methyl ester *O*-trimethylsilyl derivatives were consistent with those recently reported by Balazy [24] for 5(6)epoxy-12-hydroxyeicosatrienoic acid and 5,6,12-trihydroxyeicosatrienoic acid. Thus, 6His-12-LO catalyses insertion of oxygen at C-12 of 5(6)-EpETrE, indicating that this non-allylic epoxide is also a substrate of 12-oxygenase activity rather than lipoxin synthase activity of the enzyme.

The kinetic constants of 6His-12-LO reactions with $LTA_4$ and 5(6)-EpETrE and, for the purposes of comparison, with arachidonic acid, were determined. Figure 5 shows the Lineweaver–Burk plot of $LTA_4$, 5(6)-EpETrE and arachidonic acid transformation by 6His-12-LO, and the apparent $K_m$ and $V_{max.}$ values for these reactions are summarized in Table 1. It is apparent that arachidonic acid and 5(6)-EpETrE are essentially equally efficient as substrates for 6His-12-LO, whereas conversion of $LTA_4$ was approx. 35-fold slower. It is important to point out that, because of its non-allylic structure, 5(6)-EpETrE is a more stable epoxide than $LTA_4$. As a result of $LTA_4$ instability, catalytic constants of reactions involving $LTA_4$ as in previous studies [21] are difficult to obtain because of substrate loss caused by rapid non-enzymic hydrolysis. In the present experiments, 45 ± 8% (mean ± S.E.M., $n = 4$) of the $LTA_4$ was hydrolysed within the first minute of addition to the incubations (pH 7.45) giving rise to products that cannot participate in lipoxin generation (i.e. 12-epi-6-*trans*-$LTB_4$ and 6-*trans*-$LTB_4$) (Figure 3). This loss of substrate may affect the kinetic parameters for $LTA_4$ conversion, resulting in an increase in the apparent $K_m$. Thus the affinity of 12-LO for $LTA_4$ is likely to be higher *in situ* because non-enzymic aqueous hydrolysis of $LTA_4$ in these incubations was not taken into account in the present calculations (Table 1). On the other hand, the apparent $K_m$ for $LTA_4$ conversion by 6His-12-LO was within the same order of magnitude of the apparent $K_m$ for arachidonic acid (Table 1), indicating that the affinity of 12-LO for $LTA_4$ *in vitro* is comparable with that for arachidonic acid. These findings are consistent with those obtained with permeabilized platelets [17] and suggest that lipoxin synthase may represent a major activity of 12-LO in human platelets. Thus when $LTA_4$ is released by 5-LO-carrying cells, either amplification of leukotriene formation by $LTA_4$ hydrolase [20,21] or lipoxin generation may occur depending on the enzymes present in the neighbouring cell type(s), particularly in transcellular biosynthetic exchanges. Consistent with this scenario are results obtained from *in vivo* studies that indicate that both $LXA_4$ and (5*S*,12*S*)-dihydroxy-(6*Z*,8*E*,10*E*,14*Z*)-

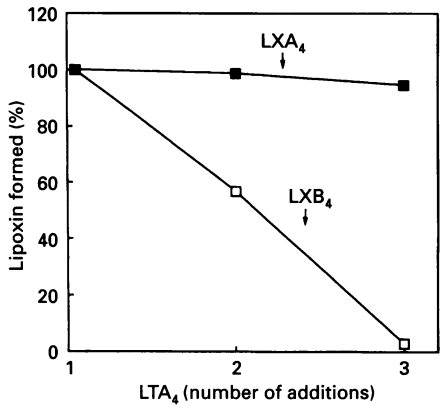


**Figure 6 Selective suicide inactivation of $LXB_4$ formation by 6His-12-LO**

Three samples of 6His-12-LO (1 $\mu$g) were incubated with $LTA_4$ (18 nmol) at 37 °C. After 20 min, one incubation was terminated, while the other two incubations received a second addition of $LTA_4$ (18 nmol) and were incubated for an additional 20 min at 37 °C. At the end of this period, one incubation was terminated, while the other received an additional portion of $LTA_4$ (18 nmol) and continued for 20 min. Data represent ng of lipoxins formed during the second and third incubations expressed as the percentage of lipoxin formed during the first incubation. Results are representative of three separate experiments.

**Table 2 Impact of aspirin on lipoxin formation from $LTA_4$**

Platelets ($5.0 \times 10^8$) in Hepes/Tyrode buffer (0.5 ml) were treated with either aspirin (0.5 mM) or vehicle control for 15 min at 37 °C and then exposed to thrombin (1 unit/ml) and $LTA_4$ (9 nmol) for 20 min at 37 °C. 6His-12-LO (2.5 $\mu$g) was incubated in Tris buffer (0.125 ml) with either aspirin or vehicle control. $LTA_4$ (9 nmol) was subsequently added for 20 min at 37 °C. PGH synthase (1–10 $\mu$g) in buffer (0.125 ml) was exposed to $LTA_4$ (9 nmol) for 20 min at 37 °C. Incubations were terminated and products quantified as described in the Materials and methods section. Lipoxin represents the sum of $LXA_4$, $LXB_4$ and their all-*trans* isomers. Results are means ± S.E.M. of three separate experiments. $^*P < 0.05$ compared with the control (Student's *t* test). ND, not determined.

| | Lipoxin (pmol) | |
|---|---|---|
| $LTA_4$ | Control | Aspirin |
| Platelets | 191.9 ± 18.1 | 363.3 ± 56.4* |
| 6His-12-LO | 201.3 ± 4.8 | 187.6 ± 15.4 |
| PGH synthase | 0 ± 0 | ND |

eicosatetraenoic acid, products of the interactions between 5- and 12-LOs, are generated during angioplasty in the coronary artery [25].

$LXB_4$ formation by platelets is regulated by suicide inactivation [17]. To examine whether this mechanism involves a direct action on 12-LO, the recombinant enzyme was subjected to sequential additions of $LTA_4$. $LXB_4$ formation but not $LXA_4$ was selectively reduced after consecutive exposures to $LTA_4$ (Figure 6), indicating that regulation of $LXB_4$ formation by suicide inactivation results from direct interaction of 12-LO with $LTA_4$. When work-up-induced isomerization of $LXA_4$ and $LXB_4$ to their respective all-*trans* isomers was taken into account (see Figures 1 and 2 and ref. [17]), $LXA_4$ and $LXB_4$ are quantitatively major products of initial 12-LO action with $LTA_4$. The resultant cation intermediate [13] would be attacked by water at either C-6 to give $LXA_4$ or C-14 to give $LXB_4$. It is possible that the interaction of 12-LO with C-14 of the intermediate could block the $LXB_4$-generating centre to yield its inactivation. This finding is intriguing because neither $LXA_4$ (Figure 6) nor 12-HPETE formation [26] by the platelet 12-LO displays suicide inactivation. Regulation of $LXB_4$ biosynthesis but not $LXA_4$ formation by suicide inhibition may be of physiological relevance, as in some cases $LXB_4$ carries biological actions distinct from those elicited by $LXA_4$; for example, recent findings indicate that $LXB_4$ decreases renal plasma flow and glomerular filtration in rats, whereas $LXA_4$ has opposite effects [27].

Arachidonic acid metabolism by 12-LO is enhanced when cyclo-oxygenase activity is inhibited in platelets [28]. We also examined whether lipoxin synthase activity of platelet 12-LO is modulated by cyclo-oxygenase inhibition. To this end, platelets, in either the presence or absence of aspirin, were exposed to $LTA_4$. A statistically significant increase in lipoxin formation was observed with aspirin-treated platelets (Table 2). In contrast, lipoxin synthase activity of 6His-12-LO was not enhanced by aspirin, indicating that its effect on lipoxin formation by platelets was not determined by direct interaction with 12-LO. These results are consistent with the observation of increased $LXA_4$ formation during angioplasty in patients treated with aspirin as compared with untreated subjects [25]. In addition, they indicate that cyclo-oxygenase does not contribute to $LTA_4$ conversion into lipoxin by platelets. Cyclo-oxygenase inserts molecular oxygen at C-15 of arachidonic acid to generate prostaglandin $G_2$; therefore, it could have inserted $O_2$ into $LTA_4$. However, additional evidence for the inability of cyclo-oxygenase to transform $LTA_4$ to lipoxin was obtained after incubating $LTA_4$ with purified cyclo-oxygenase from sheep seminal vesicles (1–10 $\mu$g) (Table 2). Although of potential clinical relevance, the impact of aspirin on enhancing lipoxin biosynthesis by platelets requires further study. Along these lines, the 12-LO has recently been found to be present in epithelial cells from inflamed colon [29], suggesting that these cells may also convert $LTA_4$ into lipoxins.

In summary, the present results establish that the human platelet 12-LO has dual functions, i.e. oxygenase and lipoxin synthase activity. They also provide further steps toward understanding the role of human platelet 12-LO and the products that it can generate. Because circulating platelets are high in number and are a well-documented rich source of 12-LO, they may constitute a major source of lipoxin-generating activity.

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