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A PHOSPHOLIPASE A_2 ISOENZYME PROVOKES LIPOXIN B FORMATION FROM ENDOGENOUS SOURCES OF ARACHIDONIC ACID IN PORCINE LEUKOCYTES

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SUMMARY: Porcine leukocytes incubated with an isoenzyme of phospholipase A_2 (PLA_2) (isolated from snake venom) produced several trihydroxytetraene- containing compounds which were derived from endogenous sources of arachidonic acid. The formation of these compounds was dose-dependent with an EC_{50} of approximately 1.25×10^{-8}M. At this concentration of the isoenzyme and time of explosure the cells remained viable as determined by the exclusion of trypan blue. The compounds were purified by HPLC and their identities were determined by physical criteria which included U.V. spectrometry, GC/MS and by comparison with both synthetic and authentic materials. The biologically derived compounds proved to be lipoxin B (5S,14R,15S-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid) and its two structural isomers (8-trans-LXB and 14S-8-trans-LXB). Of interest, only small amounts of lipoxin A and its isomers were found in these incubations. Results of the present study indicate that porcine leukocytes can generate lipoxin B and its isomers from endogenous sources of arachidonic acid. Moreover, they suggest that certain PLA_2 isoenzymes may initiate the formation of lipoxins and related compounds.

The lipoxins, a new series of trihydroxytetraenes (1,2) and trihydroxypentaenes (3) derivatives of arachidonic acid (AA) and eicosapentaenoic acid (EPA), were initially isolated following incubation of either 15-HPETE or 15-HPEPE with leukocytes, respectively. These lipoxygenase products display several biological activities. For example, at submicromolar concentrations, lipoxin A stimulates superoxide anion

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Abbreviations Used: 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 15-HPEPE, 15-hydroperoxyeicosapentaenoic acid; AA, Arachidonic acid; EPA, Eicosapentaenoic acid; RP-HPLC, Reverse phase-High Performance Liquid Chromatography; GC/MS, Gas Chromatography/Mass Spectrometry; LXA, lipoxin A: (5S,6R,15S)-5,6,15-trihydroxy-7,9,13--trans-11-cis-eicosatetraenoic acid); LXB, lipoxin B: (5S,14R,15S)-5,14,15-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid; 8-trans-LXB, (5S,14R,15S)-5,14,15-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid; 14S-8-trans LXB, (5S,14S,15S)-5,14,15-trihydroxy-6,8,10.12-trans-eicosatetraenoic acid; PLA_2, Phospholipase A_2; PBS, phosphate buffered saline.

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generation and some degranulation in human neutrophils without provoking substantial aggregatory responses (2,6). At a similar dose range both lipoxin A and lipoxin B inhibit natural killer cell activity (7). In addition, lipoxin A induces contraction of lung parenchymal strips and stimulates microvascular changes (8). More recently, lipoxin A (LXA) has been found to activate isolated preparations of human placental-derived protein kinase C (in vitro) (9) and proved to be 30 times more potent than diacylglycerol, a proposed intracellular signal for the activation of protein kinase C (10). Like lipoxin A, lipoxin A_5 derived from 15-HPEPE also induces superoxide anion generation with similar potency to that of LXA without causing aggregation in canine neutrophils (11).

Although both series of eicosanoids have distinct biological activities different than those of either LT or PGs in several systems (12,13,14), their importance as mediators or regulators has been questioned since these compounds had not yet been isolated from endogenous sources (4,15). Recently, we have demonstrated that lipoxins of the 4 series (AA derived products) and 5 series (EPA derived products) can be isolated from porcine leukocytes exposed to either AA or EPA, respectively (11). The results of these studies (11) taken together with the stereospecific nature and spectrum of biological activities observed with synthetic and authentic lipoxins suggest that lipoxins may play a role in inflammation. Recently, Yamamoto et al. (16,17) had demonstrated that multiple oxygenation of AA by 5-, 12- and 15-lipoxygenases resulted in the formation of lipoxins. However, the mechanism involved in the generation of lipoxins from endogenous sources remained obscure. Using an isoenzyme of PLA_2 purified from snake venom (Vipera Russelli), we present evidence indicating that this PLA_2 provokes the formation of LXB from endogenous sources of AA in porcine leukocytes.

MATERIALS AND METHODS

Phospholipase A_2 isoenzyme

Phospholipase A_2 isoenzymes were prepared from Vipera Russelli venom (Sigma Chemical, St. Louis, MO), according to the method of Salach et al. (18), by isoelectric focusing and further purified by HPLC using a Protein-PAK DEAE 5 PW column and 20 mM Tris-acetate Buffer, pH 7.8 with 5% glycerol as mobile phase (Waters Assoc., Milford, MA). The isoenzyme with an isoelectric point of 8.8 to 9.0 was used in this study (Molecular weight of this isoenzyme was estimated to be 15,000 Dalton as determined By SDS-gel electrophoresis).

Cell preparation, incubation condition and separation

Leukocytes were prepared as previously described (3) and was suspended in PBS. After preincubation of the leukocytes (10-20 ml) for 5 min at 37°C in a shaking water bath, phospholipase A_2 suspended in deionized distilled water was added and the incubation continued for 10 min with continuous shaking. The incubations were terminated by addition of ice cold ethanol followed by immediate centrifugation at 300 g for 20 min. The ethanolic solution was evaporated under vacuum and the residues was redissolved in 3 ml of distilled water. After being acidified with 1N HCl to a pH range of 3.5 to 4.0, the residue was extracted with 9 volumes of ethylacetate. The ethylacetate extracts were dried under nitrogen. The residue was dissolved in 50 μl methanol and then separated by RP-HPLC on a Water's Associates Dual Pump System equipped with an RP-HPLC ultrasphere ODS column (C_{18}-ODS, 5μ, 4.6 mm x 25 cm, Beckman, Palo Alto, CA), a U6-K injector and a 481 max variable wavelength detector. The products were eluted on a linear gradient of methanol-water/acetic acid (50:50:0.05, v/v) (solvent A) to methanol (solvent B) for 15 min at a flow rate of 1 ml/min. Column effluents were monitored with a Water's Associates λ 481 max variable wavelength detector set at 301 nm. PGB_2 (2 μg) was added to the incubation after termination as internal standard. Following treatment of the tetraene-containing fraction with diazomethane (CH_3N_2), samples were repurified by a second RP-HPLC using a solvent system as described (5). Viability of porcine leukocytes was also tested by Trypan blue exclusion method after treatment with various concentrations of PLA_2 isoenzyme (10^{-9} to 3 x 10^{-7}M).

Gas Chromatography-Mass Spectrometry

The methylesters of the tetraene containing materials (Fig 1A and 1B) were converted to trimethylsilyl ethers by addition of 25 μl of pyridine followed by 50 μl of trimethylchlorosilane and 50 μl of hexamethyldisilazine (Supelco). The mixtures were kept at room temperature for 20 min and dried under N_2. The samples were dissolved in 5 μl hexane and injected into the gas chromatograph-mass spectrometer. GC/mass spectrometry was performed with a Dani 3800 gas chromatograph HR PRV-2CH equipped with a fused silica capillary column (20m x 0.32, Orion) SE-30 and 7070E VG analytical mass spectrometer. The electron energy was set at 22.5 eV, with an oven temperature of 230°C.

RESULTS AND DISCUSSION

Preliminary studies with crude PLA_2 from snake venom incubated with leukocytes indicated that several novel products were generated from leukocytes. Therefore, the PLA_2 isoenzymes responsible for this activity were isolated (18). Porcine leukocytes generated several tetraene containing materials following exposure to the isolated PLA_2 (Fig. 1A). The formation of these materials by PLA_2 was dose dependent as indicated in Fig 2A. At the highest dose of PLA_2 (3 x 10^{-7} M), the viability of leukocytes after 10 min of incubation was between 65 and 72% (Fig 2B) (n=3). Following 10 min exposure to 10^{-8}M of PLA_2 the viability of the leukocytes was greater than 90%, and the formation of these materials from these cells was more than half of the maximum response (EC_{50}:1.25 x 10^{-8}M) (Fig 2B). This result suggests that the generation of these tetraene containing compounds is not likely a result of cell injury or cellular toxicity caused by PLA_2.

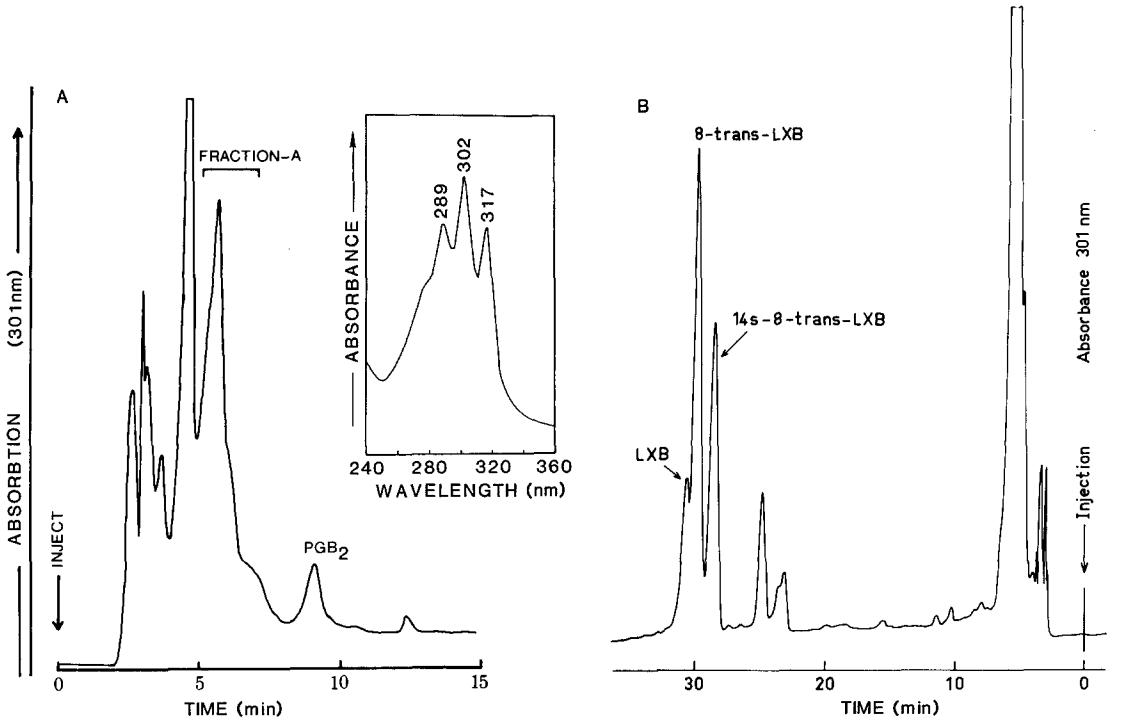

Fig. 1. RP-HPLC chromatograms and U.V. spectrum of products extracted from incubation of porcine leukocytes with PLA_2 isoenzyme ($1x10^{-7}$M) A) RP-HPLC chromatogram. The column (Beckman, Ultrasphere ODS, 5 μM, 4.6 mm x 2.5 cm) was eluted on a linear gradient of MeOH/H_2O/HAC, 50/50/0.05 (V/V) to methanol at 1.0 ml/min; (B) RP-HPLC chromatogram of methylated fraction A. The column was eluted with MeOH/H_2O/65/35 (v/v), at a flow rate of 1.0 ml/min. (insert) U.V. spectrum of materials under Fraction A. The spectrum was recorded in ethanol.

To further substantiate the identity of this tetraene-containing material, samples eluted from RP-HPLC (fraction A) (Fig 1A) were treated with diazomethane and

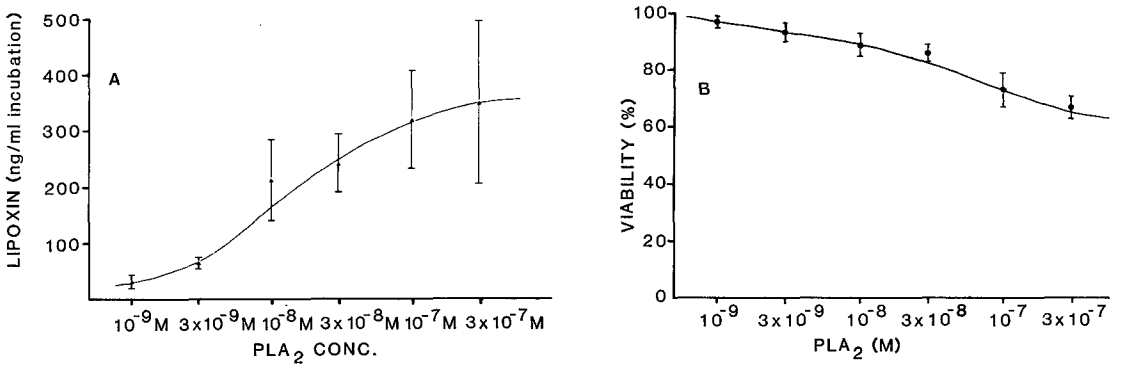

Fig. 2. A) Endogenous release of tetraene-containing materials from porcine leukocytes after stimulatin with various doses of PLA_2 isoenzyme. Amount was calculated from U.V. absorbance using E=50,000 at 302 nm.
Error bar indicates SEM (n=3)
B) Viability of porcine leukocytes after incubation with various doses of PLA_2 isoenzymes for 10 min at 37°C. Viability was determined by exclusion of Trypan blue. Error bar indicates SEM (n=3).

re-chromatographed on a second RP-HPLC as recently described for the separation of LXB (5,26). As shown in Figure 1b, the material was resolved into three components. These biologically generated materials co-migrated with authentic standards of lipoxin B (LXB), 8-trans-lipoxin B and 14s-8-trans-lipoxin B (5). Interestingly, only small amounts of material co-migrated with authentic standards of lipoxin A and its isomers. Each of the materials eluted under the three peaks were collected separately and their UV spectra were recorded. Each material displayed a U.V. spectrum typical of a conjugated-tetraene (Fig 1 insert). To further elucidate the structure of these three materials, samples were derivatized with TMS and analysed by GC/MS.

GC/MS structural analysis was performed on the material eluted at 28.5 mins from RP-HPLC. On GC/MS this compound gave a C value of 24.0, identical to that previously reported for lipoxin B (2,5). Its mass spectrum showed ions of high intensities at m/e 173 (base peak, [Me_3 SiO^+ = CH-$(CH_2)_4$ - CH_3] and 203 [Me_3 SiO^+ = CH-$(CH_2)_3$ - $COOCH_3$]. Ions of lower intensities were observed at m/e 582[M], 492[M-90, loss of Me_3SiOH], 482[M-100], 409[M-173], 379[M-203], 319[409-90] and 289[379-90]. These fragmentation ions and the C value are consistent with those reported for lipoxin B (Fig 3A) (5). GC/MS analysis of material collected under the peak, with retention time of 30.6 mins (C value=28.3) showed ions of high intensities at m/e 173 (base peak) and 203. Ions of lower intensities were observed at m/e 582, 492, 482, 409, 379, 319 and 289. These findings were essentially identical to the authentic and reported spectrum of 8-trans-lipoxin B (Fig 3B) (5). GC/MS analysis of the TMS derivatives of the material eluted at 31.2 min gave a C value of 28.0. Its mass-spectrum showed prominent ions at m/e, 173 [base peak, Me_3 SiO^+ = CH-$(CH_2)_4$ - CH_3], 203[Me_3 SiO^+ = CH - $(CH_2)_3$ - $COOCH_3$)]. Ion of lower intensities were observed at m/e 582 (M), 492[M-90], 482[M-100; rearrangement followed by loss of O=HC-$(CH_2)_4$ - CH_3), 409[M-173], 379[M-203] and 289[379-90]. The mass spectrum as well as the C value was essentially identical to that reported for 14S-8-trans-lipoxin B (Fig 3C) (5). Thus, the tetraene-containing materials formed by porcine leukoctyes was identified unequivocally as lipoxin B and its nature stereoisomers.

lipoxins were first isolated and their basic structures determined following incubation of 15-HPETE and human leukocytes (1). The stereochemistry of human leukocyte

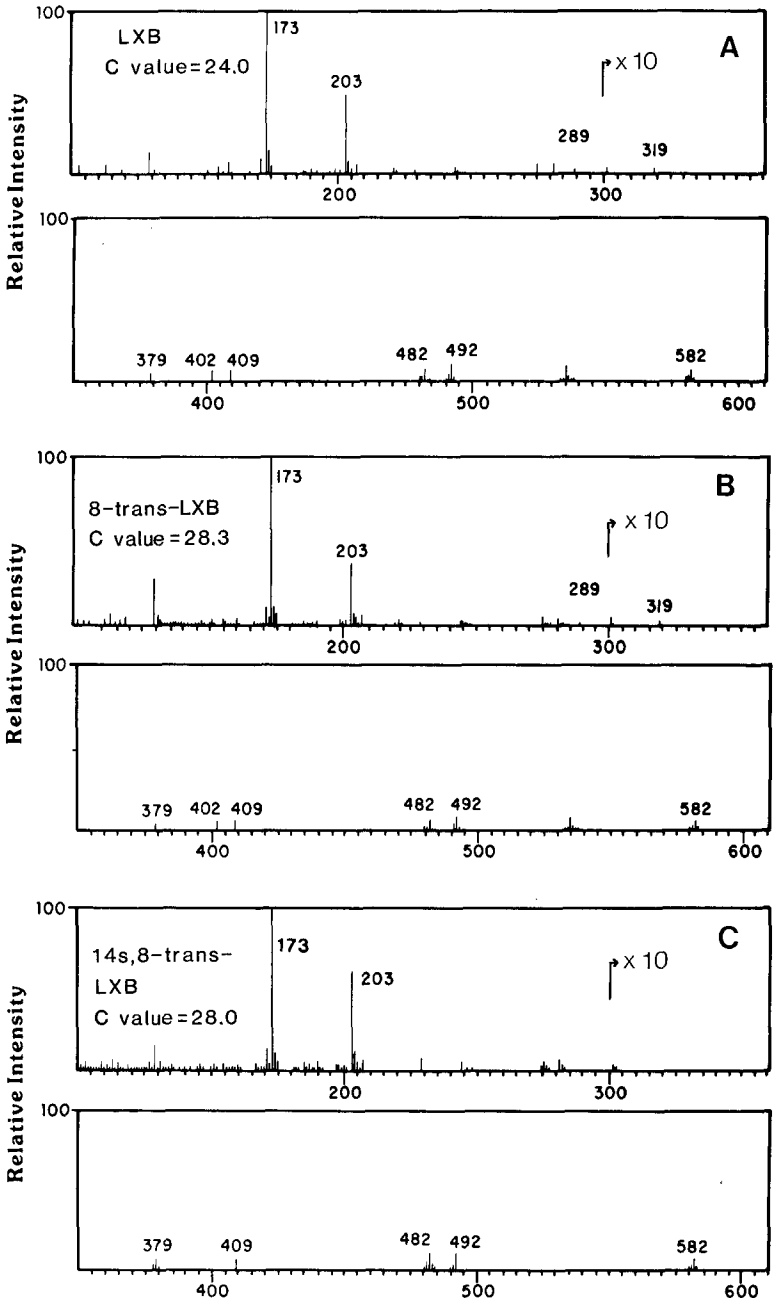

Fig. 3. Mass spectra of the Me_3Si derivatives of methylesters of materials eluted at retention time: A) 28.5 min, B) 30.6 min; and C) 31.2 min under Fig. 1B.

derived lipoxin A [(5S,6R,15S)-5,6,15-trihydroxy-7,9,13- trans-11 trans-eicosatetraenoic acid) and lipoxin B (5S,14R,15S)-5,14,15- trihydroxy6,10,12-trans-8-cis-eicosatetraenoic acid)] as well as several of their isomers have been determined (5,19). In addition to both AA and 15-HPETE serving as a substrate for the formation of LXA and LXB, 15-HETE has

been found to be transformed to LXA and LXB in activated human leukocytes (5,11,20). The results of these studies suggest that lipoxin can be formed in part by transcellular metabolism of 15-HETE (5,19). It is obvious that several biosynthetic routes could yield tetraene compounds. Thus it remained to be determined whether lipoxins can be formed from endogenous AA released from cellular pools. Recently, we demonstrated that porcine leukocytes incubated with either AA or EPA generated lipoxins (0.05% for AA and 0.1% for EPA) which is about 15-30% of the amount of leukotriene B_4 produced. These results were based on RP-HPLC, U.V. and GC/MS data (Wong et al., unpublished data). We therefore hypothesized that lipoxins may also be formed from endogenous AA if appropriate stimuli are applied as in the case with bradykinin stimulation of human platelets to release 15-lipoxygenase products from endogenous sources (21). In this study we have found that Ca^{++} ionophore A23187 (1-10 μM) was relatively weak stimulus for lipoxin production by porcine leukocytes (less than 50$\pm$10 ng of lipoxin B and its isomers were isolated from each 100 x 10^6 cells incubation, n=8). Bradykinin was not active with the doses tested (up to 10 μM). In contrast, the PLA_2 isoenzyme (pI=8.9) provokes the formation of lipoxin B and its isomers (approximately 30 ng/ml; 100 x 10^6 cells) from porcine leukocytes at doses as low as 10^{-9}M.

The use of purified PLA_2 isoenzyme in the present study is based on the following: (1) granule associated PLA_2 of inflammatory cells may be released to the extracellular environment upon cell activation (20,22-27); (2) that similar PLA_2 isoenzyme has previously been demonstrated to induce leukotriene release in isolated perfused guinea pig lung (28); (3) we have found that other PLA_2 isoenzymes (other than pI of 8.9) isolated from crude Russelli Vipera's venom do not induce the release of lipoxins from porcine leukocytes; finally, 4) we have also found that this isoenzyme provokes the formation of these compounds by certain cell types, i.e., porcine and human leukocytes, rat PMN and not by guinea pig PMNs, guinea macrophage and rat macrophages (Wong et al, unpublished data). The responses of porcine leukocytes to exogenously added PLA_2 as shown in this study may mimick the <u>in situ</u> pathophysiologic response of leukocytes exposed to extracellular PLA_2 which may be released by macrophages or leukocytes during cell activation and phagocytosis or in antigen induced chronic inflammation (24,25). Furthermore, PLA_2 have been reported to be released by rat platelets during platelet

aggregation (26) and has been found at site(s) of inflammation and in lymph draining nodes with tuberculin reactions in rabbits (27). Thus, PLA_2 and its isoenzymes released from macrophages, platelets or isolated from snake venom may be useful tools in studying the formation of eicosanoids from endogenous sources during cell activation.

In the present study, the identities of these materials were established by U.V., HPLC, and by GC/MS and comparison with synthetic materials. The formation of lipoxin B and its isomers provoked by PLA_2 was dose dependent (Fig. 2A). Maximal recovery of LXB was achieved at about $1x10^{-7}M$ PLA_2. At this dose, approximately 70-80% of leukocytes were viable after 10 min of incubation at 37°C as determined by the Trypan blue exclusion technique (Fig. 2b). Similar results were also obtained from lactate dehydrogenase release assay (data not shown). The selective formation of large amounts of LXB but not LXA, may reflect the involvement of a specific biosynthetic pathway in porcine leukocytes. Previous studis in human leukocytes using 15-HETE suggested that lipoxins can be formed via 5,6-epoxide tetraene intermediates (5,19). The high activity of 12-lipoxygenase in porcine leukocytes (29) may accelerate formation of lipoxins, since 12-lipoxygenase can also metabolize 15-HPETE to form 14,15 DHETE (29) and may represent another biosynthetic pathway for LXB formation. It is of interest to note that purified 12-lipoxygenase from porcine leukocytes has recently been reported to generate LXB from 5,15-DHPETE (17). From the results of the presenty study, however, it is not possible to determine the precise route and intermediates involved in the of formation of LXB and its isomers by porcine leukocytes. Lipoxins have been demonstrated to affect the functions of inflammatory cells, natural killer cells, protein kinase C, and microvascular circulation (2,6,9). Thus, demonstration that lipoxins can be formed from endogenously derived AA (Fig. 1A and 2A) in addition to transcellular metabolism of 15-HETE (5,19) provides further evidence to suggest that lipoxins may serve as lipid mediators or intracellular regulators in various inflammatory diseases and immune responses. Moreover, the result of the present study provide further evidence to support the proposal that the release of PLA_2 activity by inflammatory cells may result in the formation of agents which are active in chronic inflammation.

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