Lipoprotein-associated phospholipase A_2, platelet-activating factor acetylhydrolase, generates two bioactive products during the oxidation of low-density lipoprotein: use of a novel inhibitor

Colin H. MACPHEE*[1], Kitty E. MOORES*, Helen F. BOYD†, Dash DHANAK‡, Robert J. IFE‡, Colin A. LEACH‡, David S. LEAKE§, Kevin J. MILLINER*, Rebecca A. PATTERSON§, Keith E. SUCKLING*, David G. TEW† and Deirdre M. B. HICKEY‡

*Department of Vascular Biology, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, The Pinnacles, Harlow, Essex CM19 5AW, U.K., †Department of Molecular Recognition, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, The Pinnacles, Harlow, Essex CM19 5AW, U.K., ‡Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, The Pinnacles, Harlow, Essex CM19 5AW, U.K., and §Cell and Molecular Biology Research Division, School of Animal and Microbial Sciences, The University of Reading, Whiteknights, Reading, Berks. RG6 6AJ, U.K.

A novel and potent azetidinone inhibitor of the lipoprotein-associated phospholipase A_2 (Lp-PLA_2), i.e. platelet-activating factor acetylhydrolase, is described for the first time. This inhibitor, SB-222657 ($K_i = 40 \pm 3$ nM, $k_{obs}/[I] = 6.6 \times 10^5$ $M^{-1} \cdot s^{-1}$), is inactive against paraoxonase, is a poor inhibitor of lecithin:cholesterol acyltransferase and has been used to investigate the role of Lp-PLA_2 in the oxidative modification of lipoproteins. Although pretreatment with SB-222657 did not affect the kinetics of low-density lipoprotein (LDL) oxidation by Cu^{2+} or an azo free-radical generator as determined by assay of lipid hydroperoxides (LOOHs), conjugated dienes and thiobarbituric acid-reacting substances, in both cases it inhibited the elevation in lysophosphatidylcholine content. Moreover, the significantly increased monocyte chemoattractant activity found in a non-esterified fatty acid fraction from LDL oxidized by Cu^{2+} was also prevented by pretreatment with SB-222657, with an IC_{50} value of 5.0 ± 0.4 nM. The less potent diastereoisomer of SB-222657, SB-223777 ($K_i = 6.3 \pm 0.5$ μM, $k_{obs}/[I] = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$), was found to be significantly less active in both assays. Thus, in addition to generating lysophosphatidylcholine, a known biologically active lipid, these results demonstrate that Lp-PLA_2 is capable of generating oxidized non-esterified fatty acid moieties that are also bioactive. These findings are consistent with our proposal that Lp-PLA_2 has a predominantly pro-inflammatory role in atherogenesis. Finally, similar studies have demonstrated that a different situation exists during the oxidation of high-density lipoprotein, with enzyme(s) other than Lp-PLA_2 apparently being responsible for generating lysophosphatidylcholine.

Key words: chemoattractant, lysophosphatidylcholine, oxidized fatty acids.

INTRODUCTION

There is a significant body of evidence suggesting a critical role for the oxidative modification of low-density lipoprotein (LDL) in atherogenesis [1,2]. Much of this has been based on the many observations demonstrating a pro-inflammatory activity of oxidized LDL when compared with native unmodified LDL. One of the earliest biological activities attributed to oxidized LDL was its ability to be a chemoattractant for circulating monocytes [3]. This was an important observation because the continued infiltration of monocytes into the intima is recognized as a critical process in the pathophysiology of atherosclerosis [4]. Thus, in addition to being responsible for the formation of foam cells via uptake by the macrophage scavenger receptors, oxidized LDL was shown to regulate monocyte function directly. Other disease-relevant biological activities have subsequently been ascribed specifically to oxidized LDL and include smooth-muscle proliferation [5], endothelial dysfunction [6,7], vascular cell cytotoxicity [8] and macrophage mitogenic activity [9].

The precise mechanism(s) by which oxidized LDL transmits a pro-inflammatory oxidative signal remain poorly understood, but the enzyme platelet-activating factor (PAF) acetylhydrolase, which associates predominantly with LDL in plasma, has been shown to be involved in the oxidative modification of LDL [10,11]. This enzyme is recognized as a member of the rapidly growing phospholipase A_2 (PLA_2) superfamily and distinguishes itself from most other members by being Ca^{2+}-independent [12]. It also shares with serine proteases an active-site serine residue that, with a histidine residue and an aspartic residue, forms a catalytic triad [13]. In addition to PAF hydrolysis, this still only partly characterized PLA_2 can hydrolyse a broad spectrum of substrates including oxidized and polar phosphatidylcholines [11,14]. Unlike the PAF receptor, it has equal preference for phosphatidylcholines containing an ether or ester linkage at the *sn*-1 position [11]. Because this enzyme has properties far beyond the hydrolysis of PAF, we have suggested that a more general name such as LDL- or lipoprotein-associated PLA_2 (Lp-PLA_2) should be used to reflect more accurately its broad substrate properties [11,15].

One well-established consequence of LDL oxidation is that its lysophosphatidylcholine (lyso-PtdCho) content markedly increases; a significant amount of evidence has accumulated in favour of lyso-PtdCho being a proatherogenic mediator. Previous research has demonstrated that the increased lyso-PtdCho content of oxidized LDL can be completely accounted for by Lp-PLA_2 [11]. We have therefore proposed that this enzyme is intimately involved in the increased atherogenicity of oxidatively modified LDL. This lysophospholipid product of Lp-PLA_2 action

Abbreviations used: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LOOH, lipid hydroperoxide; Lp-PLA_2, lipoprotein-associated phospholipase A_2; lyso-PtdCho, lysophosphatidylcholine; PAF, platelet-activating factor; PLA_2, phospholipase A_2; TBARs, thiobarbituric acid-reacting substances.

[1] To whom correspondence should be addressed (e-mail Colin-H-Macphee@sbphrd.com).

has been shown, for example, to be capable of promoting monocyte recruitment either directly by functioning as a chemoattractant itself [16] or indirectly via the selective induction of endothelial leucocyte adhesion molecules [17,18]. Lyso-PtdCho has also been identified as the component of oxidized LDL that promotes both smooth muscle [19] and macrophage [20] proliferation, induces endothelial dysfunction in various arteries [6,21] and is involved in the antigenicity of oxidized LDL [22].

There has been considerable discussion over whether Lp-PLA_2 primarily has a pro-inflammatory (generation of lyso-PtdCho) or anti-inflammatory (degradation of PAF or PAF-like lipids) role in atherogenesis [15,23,24]. To help elucidate the biological role of Lp-PLA_2, we describe here the use of a novel, selective and extremely potent azetidinone inhibitor of this enzyme. These inhibitors were specifically designed to target the active-site serine residue of the enzyme [25]. Azetidinones represent a major advance, as previously only high concentrations of relatively non-selective inhibitors such as PMSF have been available. We have therefore used these novel pharmacological tools to investigate the role played by Lp-PLA_2 in the oxidative modification of both LDL and high-density lipoprotein (HDL) and to determine whether the enzyme can generate biologically active products. We present evidence to support a proinflammatory role for this enzyme, showing that it is capable of generating two biologically active products from oxidized LDL: Lyso-PtdCho and oxidatively modified non-esterified fatty acid(s).

MATERIALS AND METHODS

Materials

Sodium cholate, Apo-A1, human and bovine (fraction V) serum albumin, phosphatidylcholine (egg yolk), 2-mercaptoethanol, Folin–Ciocalteau phenol reagent, 1,1,3,3-tetraethoxypropane, 1,1,3,3-tetramethoxypropane, 2-(*p*-toluidino)naphthalene-6-sulphonic acid and α-naphthyl-butyrate esterase kit were obtained from Sigma. Cholesterol oxidase/iodide cholesterol colour reagent, high-performance TLC plates (silica gel 60 without fluorescent indicator) and TLC plastic sheets were obtained from Merck. PBS, RPMI 1640, Hanks balanced salt solution and fetal calf serum were purchased from Gibco. PAF was from Cascade Biochemicals (Reading, Berks., U.K.). Paraoxon was obtained from Promochem Ltd. (Welwyn Garden City, Herts., U.K.) and dextran T500 was purchased from Pharmacia. NycoPrep$^{TM1.068}$ (1.068 refers to the density) was bought from Nycomed. Diff-Quik stain was obtained from B. M. Browne Ltd. and Bond Elut columns were purchased from Jones Chromatography (Hengoed, Mid Glamorgan, U.K.). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Polysciences (Warrington, PA, U.S.A.). All other reagents were of analytical grade.

Lipoprotein isolation and oxidation

Blood from normolipaemic volunteers was collected into EDTA (4 mM final concentration) and centrifuged at 1200 ***g*** for 10 min at 4 °C in a Beckman TJ6 centrifuge. LDL and HDL were prepared from the resulting plasma by ultracentrifugation by using a slightly modified protocol of Redgrave et al. [26] that allowed 12 ml of plasma to be processed from each centrifuge tube. In brief, 3 vol. of the various KBr solutions were used including the KBr-adjusted plasma ($d = 1.21$) and all solutions contained 20 μM butylated hydroxytoluene. Ultracentrifugation on a Beckman J6-B centrifuge with a SW28 rotor was at 84000 ***g*** for 65 h at 8 °C. The bottom of each tube was then pierced with a needle, the contents were displaced by pumping through a 5 M KBr solution at 0.75 ml/min, and fractions were collected every 1 min via an on-line fraction collector. Protein concentration was estimated by a modified Lowry method [27] and peak lipoprotein fractions were pooled for further analysis.

Pooled lipoprotein fractions were dialysed for 48 h at 4 °C against 20 mM PBS to remove the KBr and EDTA, then stored at 4 °C in the dark and used within 24 h. For the experiments involving AAPH, LDL was isolated as described by Wilkins and Leake [28]. LDL or HDL, diluted to 150 μg/ml protein, was pretreated with either inhibitor or vehicle (DMSO) for 15 min at 37 °C before Cu^{2+} (5 μM $CuSO_4$)-stimulated oxidation. The extent of oxidation was followed by monitoring three separate parameters: the rate of conjugated diene formation at 234 nm, lipid hydroperoxide (LOOH) generation [29] and the formation of thiobarbituric acid-reacting substances (TBARs) [28,30]. The concentration of TBARs was expressed as equivalents of either 1,1,3,3-tetraethoxypropane or 1,1,3,3-tetramethoxypropane, which were used as standard. Oxidation proceeded until completion as monitored, in parallel, by the rate of conjugated diene formation at 234 nm (routinely 3–4 h).

In separate studies, the free-radical generator AAPH was used as an alternative means of oxidizing LDL [31]. In these experiments LDL (100 μg/ml protein) was oxidized with 4 mM AAPH for 4 h at 37 °C in PBS containing Ca^{2+} and Mg^{2+} plus the highly effective metal chelator diethylenetriaminepenta-acetic acid (100 μM), to inhibit chain cleavage of oxidized polyunsaturated fatty acids. For these 1 ml incubations, which also included LDL oxidized by Cu^{2+}, butylated hydroxytoluene (20 μM final) was added to terminate oxidation and two 250 μl aliquots were removed immediately for determinations of TBARs and LOOH. The remaining 500 μl was used for lyso-PtdCho analysis as outlined below.

Lipid extraction and monocyte chemotaxis

Lipids were extracted under acidic conditions, essentially by the method of Bligh and Dyer [32]. The 1 ml lipoprotein incubations were terminated by the addition of 3.75 ml of chloroform/methanol/HCl (1:2:0.0032, by vol.) containing 100 μM EDTA and vortex-mixed. Two phases were obtained by adding 1.25 ml of 0.1 M HCl and 1.25 ml of chloroform, vortex-mixing vigorously and centrifuging in a bench-top low-speed centrifuge. The contents of all the incubations were made identical after the addition of organic solvents by adding Cu^{2+} or AAPH and/or Lp-PLA_2 inhibitor where necessary. With inhibitor dose-dependence studies, the highest concentration of inhibitor was routinely added to control incubations after termination with organic solvents.

By using a positive-displacement pipette, 2 ml (78 % of total) of the organic phase was removed for further analyses. This lipid extract was split in half when parallel measurements were required, for example for lyso-PtdCho content and monocyte chemotaxis. Lyso-PtdCho content was determined exactly as described previously by using a fluorescence indicator [11]. The non-esterified fatty acid fraction was isolated by the method of Kuhn et al. [33]. In brief, the lipid extracts were evaporated to dryness with nitrogen gas, reconstituted with 1 ml of 50 mM phosphate buffer (pH 7.4)/methanol (50:50, v/v) solution, and loaded on Bond Elut (C_{18}, 200 mg) columns that had been preconditioned first with 2 ml of methanol and then with 2 ml of the phosphate/methanol solution. Columns were then washed with 4 ml of the phosphate/methanol solution. Fatty acids were eluted with 5 ml of methanol/water (50:50, v/v) solution. Samples were concentrated with nitrogen gas, then redissolved in 2 ml of chloroform/methanol (3:2, v/v) and stored at −70 °C until use.

To determine monocyte chemoattractant activity, total lipids or fatty acids were dried down under nitrogen, vortex-mixed in 1 and 2 ml of Hanks balanced salt solution containing 0.1 % BSA (fraction V) respectively and assayed immediately. Monocyte chemotaxis was measured by the method outlined by Quinn et al. [3], with the exception that fresh human monocytes were isolated on a Nycodenz gradient rather than Ficoll/Hypaque. It should also be noted that SB-222657 (e.g. 300 nM final) added directly to the chemotactic chamber did not influence monocyte chemotaxis (results not shown).

Enzyme assays

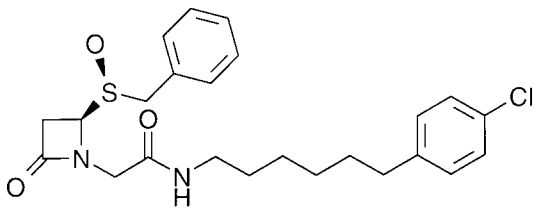
Lp-PLA_2 was assayed by using either PAF or 1-decanoyl-2-(4-nitrophenylglutaryl)phosphatidylcholine as substrate, exactly as described previously [11]. Plasma and serum enzyme assays routinely used 50 μM PAF as substrate. Studies investigating the kinetics of enzyme inhibition with standard methodologies used 1-decanoyl-2-(4-nitrophenylglutaryl)phosphatidylcholine as substrate, and K_i and k_{obs}/[I] values were determined as reported previously [25].

Assay of recombinant human Type IIa 14 kDa PLA_2 was performed exactly as described previously [34]. Paraoxonase activity was determined by using a modification of the method of Mackness et al. [35]. Generation of *p*-nitrophenol from paraoxon was monitored at 405 nm in 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM $CaCl_2$. The final substrate concentration during the enzyme assay was 2 mM. To assay for lecithin:cholesterol acyltransferase (LCAT), 15 μl aliquots of human plasma were assayed by using the proteoliposome substrate method, exactly as described by Gillett and Owen [36]. All results are means $\pm$ S.D. unless stated otherwise.

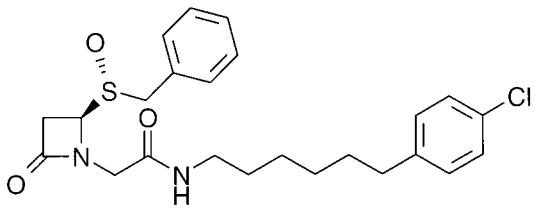
RESULTS

Effect of SB-222657 pretreatment on oxidized LDL phospholipid composition

Figure 1 provides the structure of the novel and potent azetidinone inhibitor of Lp-PLA_2, SB-222657, and its diastereoisomer SB-223777. From determinations (Figure 2) of the reversible K_i before inactivation, the inhibition is stereoselective because the diastereoisomer was over two orders of magnitude less potent than SB-222657. The calculated K_i values for SB-222657 and SB-223777 were 40 ± 3 nM and 6.3 ± 0.5 μM respectively ($n = 3$). This stereoselectivity is echoed in the second-order rate constants for inactivation: $k_{obs}/[I] = 6.6 \times 10^5$ $M^{-1} \cdot s^{-1}$ for SB-222657 and $k_{obs}/[I] = 1.6 \times 10^4$ $M^{-1} \cdot s^{-1}$ for SB-223777. In addition, and as predicted, SB-222657 was completely inactive against the human synovial fluid Type IIa 14 kDa PLA_2 when used at concentrations up to 10 μM (results not shown). This enzyme, unlike Lp-PLA_2, is a Ca^{2+}-dependent PLA_2 that does not share an active-site serine residue [12] and was therefore not expected to be inhibited by mechanism-based azetidinone inhibitors. Both compounds were then used to investigate the role of Lp-PLA_2 in the oxidative modification of lipoproteins prepared by density-gradient ultracentrifugation.



SB-222657



SB-223777

Figure 1 Structure of SB-222657, a novel and potent azetidinone inhibitor of Lp-PLA_2, and its diastereoisomer, SB-223777

The data in Table 1 and Figures 3 and 4 demonstrate that preincubation of LDL with SB-222657 greatly inhibits the increase in lyso-PtdCho formed during oxidation by both AAPH and Cu^{2+}. This effect of SB-222657 was achieved without influencing the kinetics of oxidation as measured in parallel by various assays (Figure 4 and Table 1). AAPH-mediated oxidation was conducted in the presence of diethylenetriaminepenta-acetic acid to inhibit chain cleavage of oxidized polyunsaturated fatty acids. This was confirmed by the large increase in LOOHs but only a modest increase in TBARs during AAPH-mediated oxidation (Table 1); some of these TBARs might have been due to LOOHs' giving rise to colour development during the TBARs assay [37]. LDL oxidation by Cu^{2+}, in contrast, demonstrated a large increase in both TBARs and LOOHs. The finding that SB-222657 was able to greatly inhibit the increased lyso-PtdCho content of LDL oxidized by AAPH in these studies suggests that Lp-PLA_2 is able to recognize phosphatidylcholines containing intact oxidized polyunsaturated fatty acids in addition to fragmented forms.

The inhibition of lyso-PtdCho generation within oxidized LDL by the azetidinone was both concentration-dependent and stereoselective. SB-222657 inhibited the increased lyso-PtdCho content of LDL oxidized by Cu^{2+} with an IC_{50} of 11.7 ± 1.0 nM ($n = 3$) (Figure 5B). In contrast, SB-223777, the significantly less potent diastereoisomer, was inactive at 30 nM. The actual increase in lyso-PtdCho content from 4.8 to 25 μg/mg of LDL protein in the absence of inhibitor shown in Figure 5 is equivalent to a 6 μM total increase (from 1.4 to 7.5 μM) in the lysophospholipid when corrected for the actual concentration of LDL used, i.e. 150 μg/ml protein.

Lp-PLA_2 inhibition influences oxidized LDL-mediated monocyte chemotaxis

When the total lipid extract from LDL modified by Cu^{2+} was assayed in human monocyte chemotaxis assays, preincubation with SB-222657 was found to potently inhibit the increased chemoattractant activity of oxidized LDL at similar concentrations to those that inhibited the generation of lyso-PtdCho (Figures 5A and 6A) ($IC_{50} = 5.5 \pm 0.81$ nM, $n = 5$). SB-223777 was again inactive at 30 nM in this assay. It was noted that these lipid extracts from oxidized LDL had a calculated maximal lyso-PtdCho concentration in the chemotaxis assay of approx. 3 μM. This concentration of lyso-PtdCho is below its reported EC_{50} in this assay and would be predicted to elicit only a marginal response ([38], and results not shown). Indeed, the data in Figure 5 suggested a lack of a correlation between lyso-PtdCho content and chemoattractant activity. Therefore it seemed likely

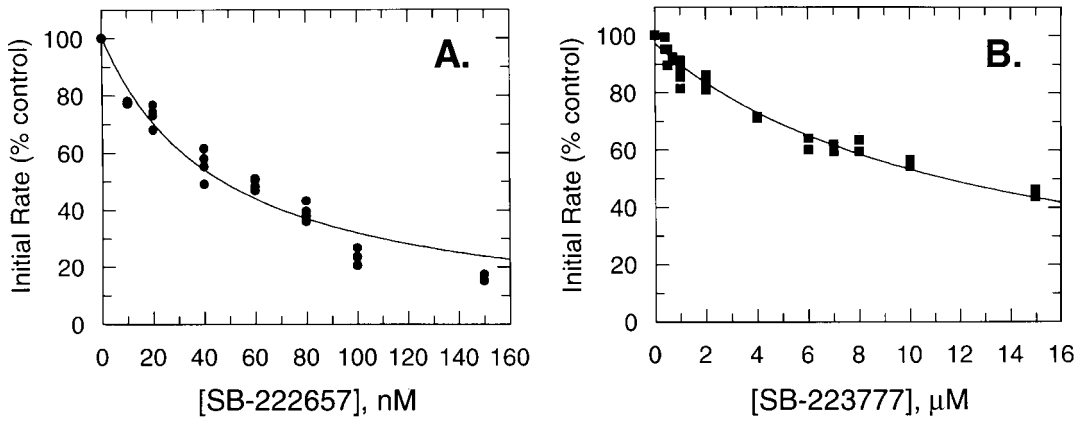


Figure 2 K_i determinations for SB-222657 and SB-223777 by using recombinant human Lp-PLA_2

Standard methodologies were employed as outlined in the Materials and methods section; enzyme activity in the absence of inhibitor was $(9.1 \pm 0.4) \times 10^{-3}$ absorbance units/min. The experiment was repeated on two further occasions and the calculated reversible K_i values before inactivation for SB-222657 and its diastereoisomer SB-223777 were 40 ± 3 nM and 6.3 ± 0.5 μM respectively.

Table 1 AAPH-stimulated LDL oxidation in the presence of diethylene-triaminepenta-acetic acid

LDL (100 μg/ml protein final concentration) was modified with AAPH or $CuSO_4$ (5 μM) for 4 h in the presence or absence of 100 nM SB-222657, and phospholipids were extracted and analysed as outlined in the Materials and methods section. The TBARs and LOOH values given are the changes from zero time. Results are means $\pm$ S.D. for three different LDL preparations. $^*P < 0.05$; $^{**}P < 0.005$ compared with control LDL.

Condition	TBARs (nmol/mg)	LOOH (nmol/mg)	Lyso-PtdCho (μg/mg)
Control LDL	1.2 ± 3.4	7.7 ± 6.3	4.0 ± 0.4
AAPH	$8.1 \pm 0.6^*$	$417 \pm 131^*$	$22.3 \pm 8.3^*$
AAPH + SB-222657	$8.4 \pm 1.4^*$	$375 \pm 76^{**}$	7.5 ± 2.3
Cu^{2+}	$54.5 \pm 11^{**}$	$644 \pm 159^{**}$	$45.7 \pm 11.1^{**}$
Cu^{2+} + SB-222657	$57.6 \pm 9^{**}$	$636 \pm 159^{**}$	6.9 ± 2.9

that an alternative component of oxidized LDL was responsible for the increased monocyte chemoattractant activity.

Monocyte chemotactic activity resides in a non-esterified fatty acid fraction

Because lipids were extracted under acidic conditions, the extracts also contained significant amounts of free oxidized fatty acid products of Lp-PLA_2. To evaluate their contribution to monocyte chemotaxis, the oxidized non-esterified fatty acids were separated from phospholipid and assayed for human monocyte chemotactic activity (Figure 6). The data in Figure 6(B) demonstrate that most of the monocyte chemoattractant activity resided within the non-esterified fatty acid fraction of oxidized LDL and, importantly, was Lp-PLA_2-dependent because the response could be blocked by prior incubation of LDL with SB-222657 with an IC_{50} of 5.0 ± 0.4 nM ($n = 3$). This IC_{50} was identical to that observed for inhibition of Lp-PLA_2 activity in the same incubations ($IC_{50} = 4.9 \pm 0.7$ nM, $n = 3$) (Figure 6B). From calculations of the lyso-PtdCho content within oxidized LDL, it follows that a 1.5–3 μM equivalent of oxidized non-esterified fatty acid was capable of generating a highly significant increase in monocyte chemotactic activity.

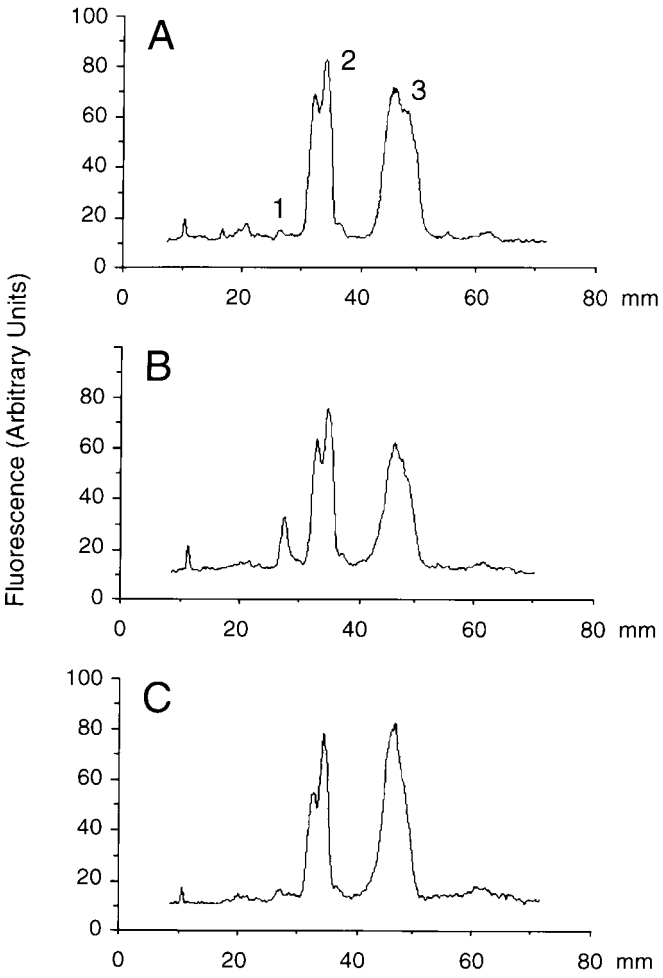


Figure 3 SB-222657 is able to inhibit completely the increased lyso-PtdCho content of LDL oxidized by Cu^{2+}

Freshly isolated LDL that had been pretreated with either vehicle (**B**) or 100 nM SB-222657 (**C**) for 15 min at 37 °C was then subjected to oxidation by 5 μM Cu^{2+} for 4 h. A control LDL sample (**A**) containing no additions was run in parallel. Incubations were terminated, then lipids were extracted and separated on high-performance TLC plates as outlined in the Materials and methods section. Peak 1, Lyso-PtdCho; peak 2, sphingomyelin; peak 3, phosphatidylcholine.

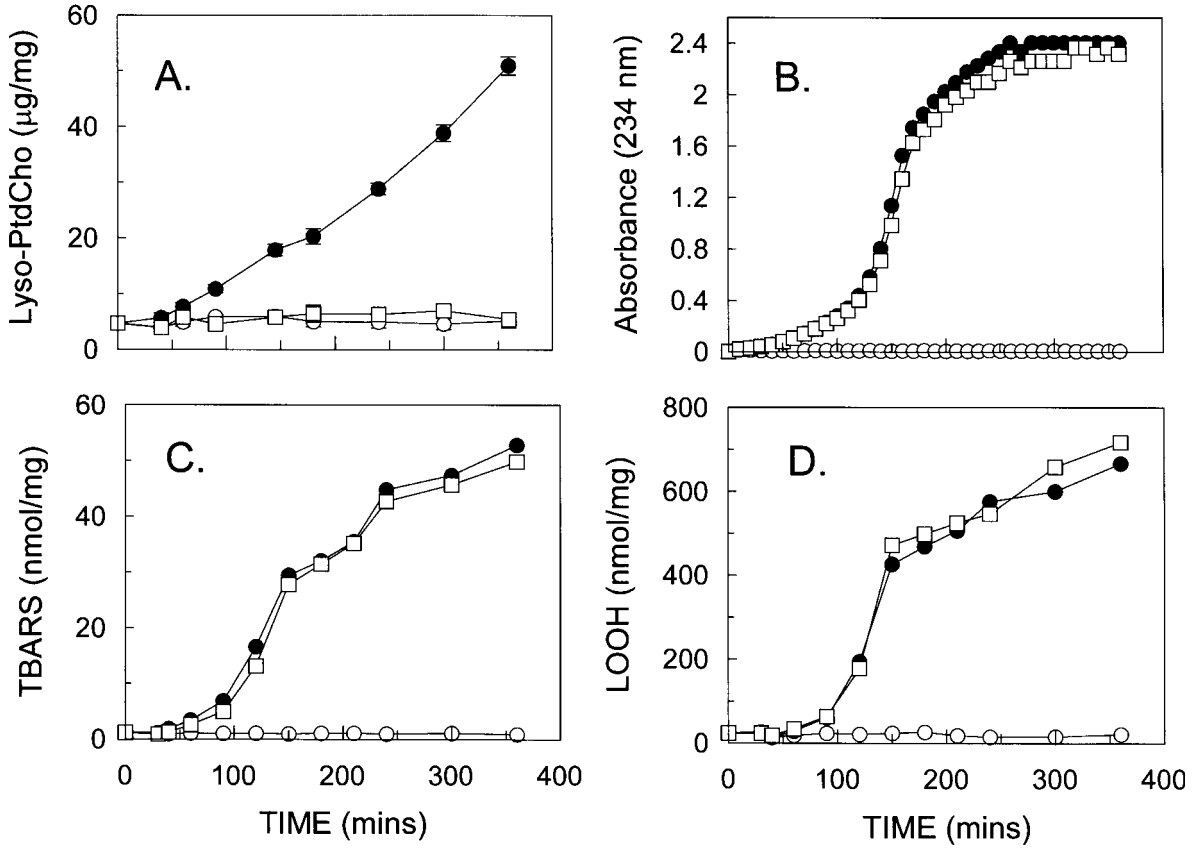


Figure 4 SB-222657 inhibits lyso-PtdCho generation without altering the kinetics of LDL oxidation by Cu^{2+}

Freshly isolated LDL (150 μg/ml protein) either remained untreated (○) or was subjected to oxidation by Cu^{2+} in the absence (●) or presence (□) of 100 nM SB-222657. The following parameters were monitored in parallel, as described in the Materials and methods section: lyso-PtdCho (**A**), conjugated dienes (**B**), TBARs (**C**) and LOOHs (**D**). For measurements of conjugated dienes only, incubations were blanked against the native lipoprotein sample just before the addition of Cu^{2+}. The average Lp-PLA_2 activity for native LDL was 13.9 ± 2 nmol/min per mg of protein ($n = 4$). Values are means $\pm$ S.D. for a representative experiment performed in triplicate. The experiment was repeated three times with similar results.

Effect of pretreatment with SB-222657 on oxidized HDL phospholipid composition

Oxidation of HDL by Cu^{2+} produced different results from those observed for LDL (Figure 7). First, LOOHs increased and then decreased; secondly, the generation of lyso-PtdCho, although less than in oxidized LDL when expressed on a protein basis, was relatively unaffected by preincubation with SB-222657. It was also noted that, whereas all of the PAF-hydrolysing activity of LDL could be inhibited by SB-222657, approx. 5 % of the HDL-associated activity was consistently found to be insensitive to the inhibitor. This result therefore suggests that, in addition to Lp-PLA_2, another enzyme activity exists within HDL that is capable of hydrolysing PAF. Although Lp-PLA_2 is found associated with HDL, albeit with activity levels apparently lower than with LDL (Figures 4 and 7) [39], it seems to have little part in the generation of lyso-PtdCho during the HDL oxidation process. In addition, no increased monocyte chemotactic activity was associated with the lipid fraction isolated from Cu^{2+}-modified HDL (results not shown).

In an attempt to characterize this observation further, we investigated whether the inhibitor would still be effective with a mixture of HDL and LDL. The data in Table 2 show that the oxidation of a mixture of the two lipoprotein classes generated a significant elevation of lyso-PtdCho that seemed intermediate to the quantities produced when each lipoprotein was modified individually. Although SB-222657 significantly inhibited the increased lyso-PtdCho content of the oxidized LDL/HDL mixture, the quantity remaining was consistently higher when compared with parallel incubations containing only single lipoproteins (Table 2).

Effect of SB-222657 on LCAT and paraoxonase

Because LCAT, which is associated with HDL, was an obvious candidate that could be involved in the observed changes in phospholipid metabolism in HDL, we investigated the ability of SB-222657 to inhibit LCAT activity. When assayed in freshly isolated human plasma, SB-222657 inhibited Lp-PLA_2 and LCAT with IC_{50} values of 0.49 ± 0.08 and 120 ± 14 μM respectively ($n = 4$ experiments) (Figure 8A). Because SB-222657 is only a relatively poor inhibitor of LCAT, these data cannot rule out the possibility that this enzyme could contribute to the altered phospholipid composition of oxidized HDL. SB-222657 was also evaluated against paraoxonase even though this important Ca^{2+}-dependent enzyme could not function in the HDL oxidation assay owing to the lack of Ca^{2+} ions. The data in Figure 8(B) clearly demonstrate that SB-222657, although able to inhibit Lp-PLA_2 ($IC_{50} = 0.52 \pm 0.02$ μM), was completely

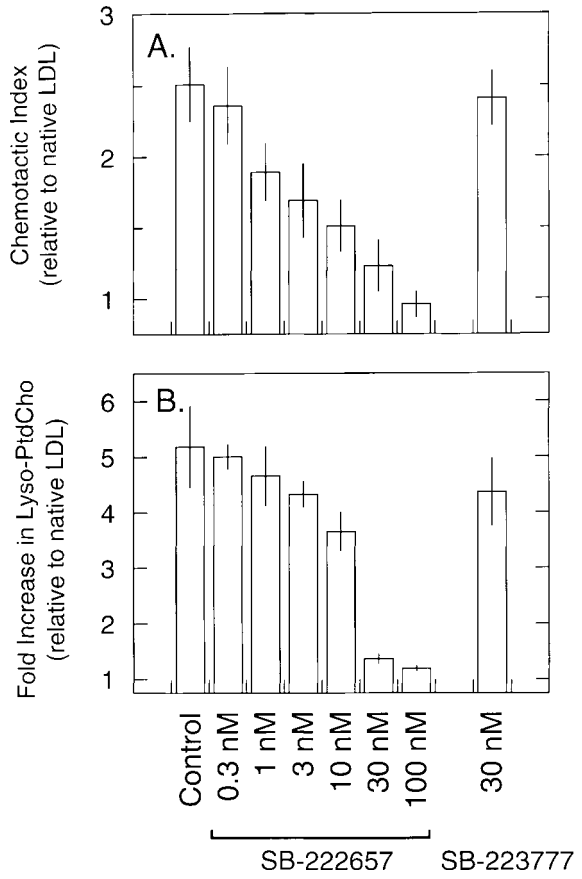


Figure 5 SB-222657 inhibits the enhanced monocyte chemotactic activity and increased lyso-PtdCho content of oxidized LDL in a similar dose-dependent manner

Freshly isolated LDL was pretreated with the indicated concentrations of inhibitor and oxidized for 4 h, then lipids were extracted as outlined in the legend to Figure 4. Lipid extracts were split into two portions with one used for monocyte chemotaxis activity (**A**) and the other to determine lyso-PtdCho content (**B**). The monocyte count and lyso-PtdCho content for native LDL were 15 ± 3 cells per field and 4.8 ± 0.3 μg/mg of protein respectively. Results are means $\pm$ S.D. for triplicate determinations from a representative experiment that was repeated with similar results.

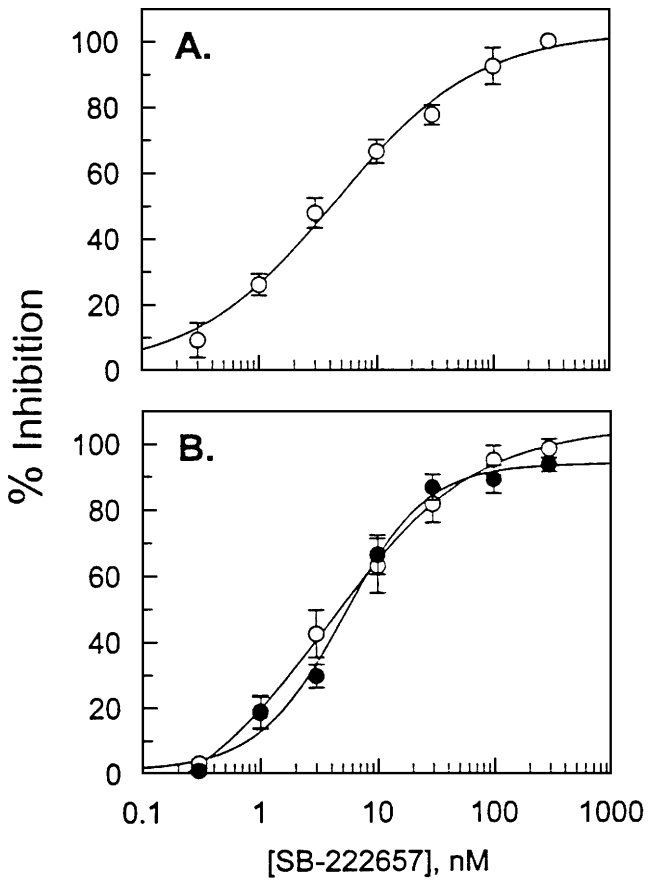


Figure 6 SB-222657 prevents the generation of oxidized non-esterified fatty acid monocyte chemoattractants during LDL oxidation

LDL was pretreated with either vehicle or the indicated concentrations of SB-222657 and oxidized by Cu^{2+}. The phospholipids were extracted and either the crude lipid extract (**A**) or the non-esterified fatty acid fraction (**B**) assayed for monocyte chemoattractant activity (○). Lp-PLA_2 activity was also measured at the end of the incubation period and is shown in (**B**) (●). Results are expressed as percentage inhibition of the oxidized LDL-mediated increase in monocyte chemotaxis in the absence of inhibitor. The average monocyte counts for native and oxidized LDL in the studies shown were 14 ± 3 and 37 ± 5 for the crude extract ($n = 5$) and 12 ± 2 and 39 ± 3 ($n = 3$) for the fatty acid fraction respectively.

inactive against paraoxonase when assayed in freshly prepared human serum.

DISCUSSION

Azetidinones (e.g. SB-222657) have been identified as novel and extremely potent inhibitors of Lp-PLA_2 that are inactive against paraoxonase and only weakly active on LCAT. These inhibitors therefore represent unique tools for investigating the role of Lp-PLA_2 in the altered phospholipid metabolism that takes place during the oxidative modification of lipoproteins. The findings presented here demonstrate unequivocally that Lp-PLA_2 is solely responsible for the elevation of lyso-PtdCho within oxidized LDL.

SB-222657 was able to prevent the hydrolysis of oxidized phosphatidylcholines without influencing the kinetics of LDL oxidation as measured by three distinct assays. This observation is in keeping with the findings of other investigators [10] but contradicts a previous report that described an ability of the enzyme to inhibit the oxidative modification of LDL [40]. This latter study, however, used high concentrations of di-isopropyl fluorophosphate, a relatively non-selective serine hydrolase inhibitor, together with a partly purified erythrocyte enzyme that might well be different from Lp-PLA_2. Others have shown that Lp-PLA_2 activity correlates with a faster and more extensive LDL oxidation [41]. In this respect it is noteworthy that the very atherogenic dense LDL particles that are actually enriched with Lp-PLA_2 [42] have an enhanced susceptibility to oxidation [43]. However, the lack of an effect of SB-222657 treatment on LDL oxidation tends to suggest that the reason for the observed increased sensitivity to oxidation of dense LDL is something other than Lp-PLA_2. This is because by pretreating LDL with SB-222657 we ensured that the LDL preparation completely lacked Lp-PLA_2 activity before and throughout the Cu^{2+}-driven oxidation period.

Lyso-PtdCho accumulation was observed in LDL preparations oxidized by both Cu^{2+} and the free-radical generator AAPH. Lp-PLA_2 involvement was confirmed for both methods of oxidation because lyso-PtdCho accumulation was inhibited by pre-treatment with SB-222657. The results obtained with AAPH were especially interesting because the protocol was designed to

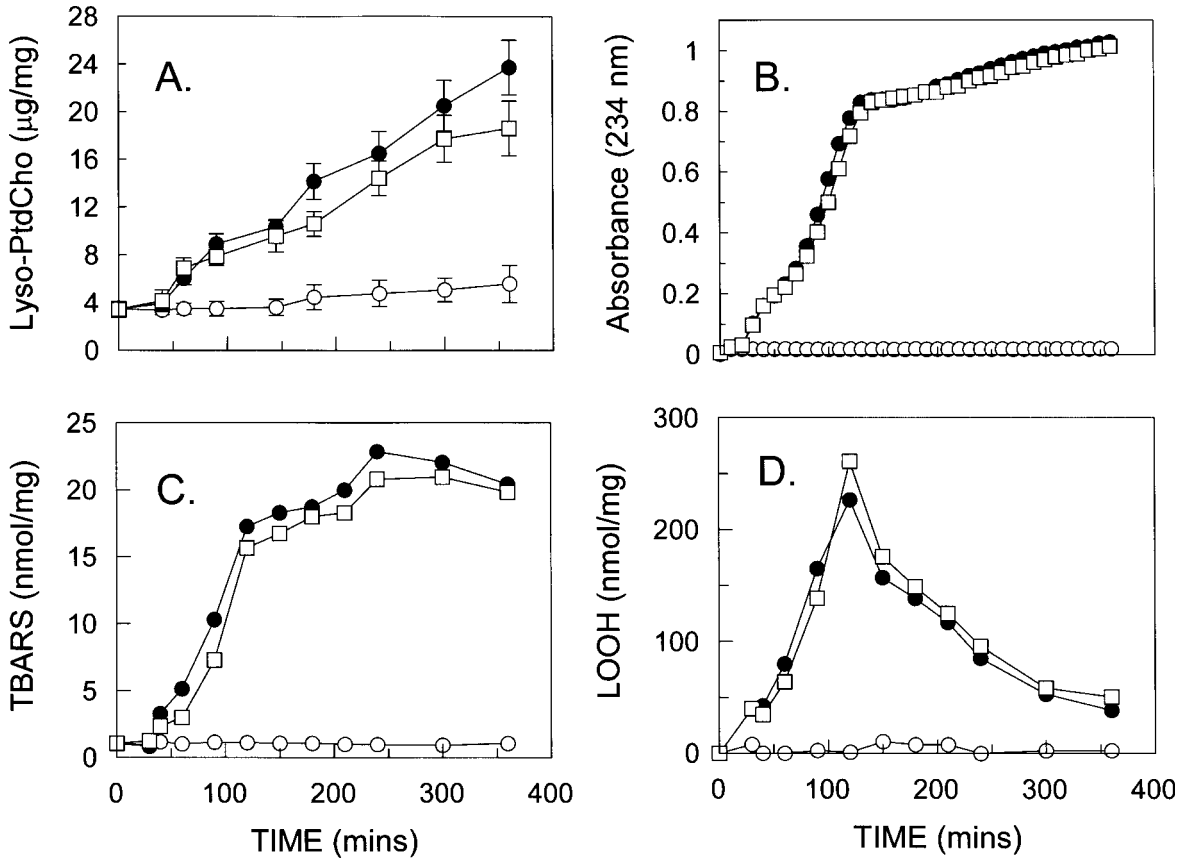


Figure 7 SB-222657 has little effect on the phospholipid changes observed during HDL oxidation

Freshly isolated HDL (150 μg/ml protein) was treated in an identical manner to that outlined for LDL in the legend to Figure 4: (**A**) lyso-PtdCho; (**B**) conjugated dienes; (**C**) TBARs; (**D**) LOOH. The average Lp-PLA_2 activity for native HDL was 1.4 ± 0.6 nmol/min per mg of protein ($n = 4$). Results are means $\pm$ S.D. for triplicate determinations from a representative experiment that was repeated three times with similar results.

Table 2 SB-222657 inhibits lyso-PtdCho generation during oxidation of an LDL/HDL mixture

LDL (150 μg/ml protein), HDL (150 μg/ml protein) or LDL (75 μg/ml protein) plus HDL (75 μg/ml protein) were modified with 5 μM Cu^{2+} for 4 h in the presence or absence of 100 nM SB-222657; the phospholipids were then extracted and analysed as outlined in the Materials and methods section. Results are means $\pm$ S.D. for three different lipoprotein preparations. $^*P < 0.005$ for control lipoprotein compared with oxidized lipoprotein; $\dagger P < 0.05$ for oxidized lipoprotein compared with oxidized lipoprotein with inhibitor.

	Lyso-PtdCho content (μg/mg of protein)		
Condition	LDL	HDL	LDL + HDL
Control	2.4 ± 0.9	1.5 ± 0.2	2.1 ± 0.5
Cu^{2+}-oxidized	$20.5 \pm 3.3^*$	$10.3 \pm 2.4^*$	$17.9 \pm 2.6^*$
Cu^{2+}-oxidized + SB-222657	$6.4 \pm 2.1\dagger$	8.1 ± 1.1	$11.2 \pm 1.5\dagger$

prevent metal-ion-dependent fragmentation of esterified oxidized polyunsaturated fatty acids. The finding that Lp-PLA_2 was able to hydrolyse phosphatidylcholines containing these full-length but oxidized polyunsaturated fatty acids is consistent with the findings of Sukurai et al. [44] and once again highlights the extremely broad substrate capability of this enzyme.

The observation that Lp-PLA_2 generates two distinct products that are both chemoattractants for monocytes is further evidence that this enzyme has a predominantly pro-inflammatory role in atherogenesis. In addition to lyso-PtdCho, a well-known bioactive lipid, we have demonstrated that Lp-PLA_2 generates one or more biologically active oxidatively modified non-esterified fatty acids. Their characterization and structural elucidation will be an important focus of future research because no Lp-PLA_2-dependent oxidized fatty acid product has yet been identified from oxidized LDL. A potential fruitful avenue for future research would be to investigate whether isoprostanes [45] could be released by Lp-PLA_2 action.

With this new knowledge we can begin to speculate with a greater degree of confidence on what might occur within the clinically relevant, macrophage-enriched, atherosclerotic lesion. We have demonstrated that Lp-PLA_2 protein and mRNA are both up-regulated in atherosclerotic lesions and co-localize with macrophages [46], a cell known to actively secrete this enzyme ([47,48]; C. H. Macphee and K. E. Moores, unpublished work). Thus the intima of atherosclerotic plaques, presumably an environment of high oxidative stress, contains elevated levels of both Lp-PLA_2 and oxidized phospholipid substrate that might undergo rapid hydrolysis. The resulting accumulation of both lyso-PtdCho and oxidatively modified non-esterified fatty acids would probably further augment the inflammatory process.

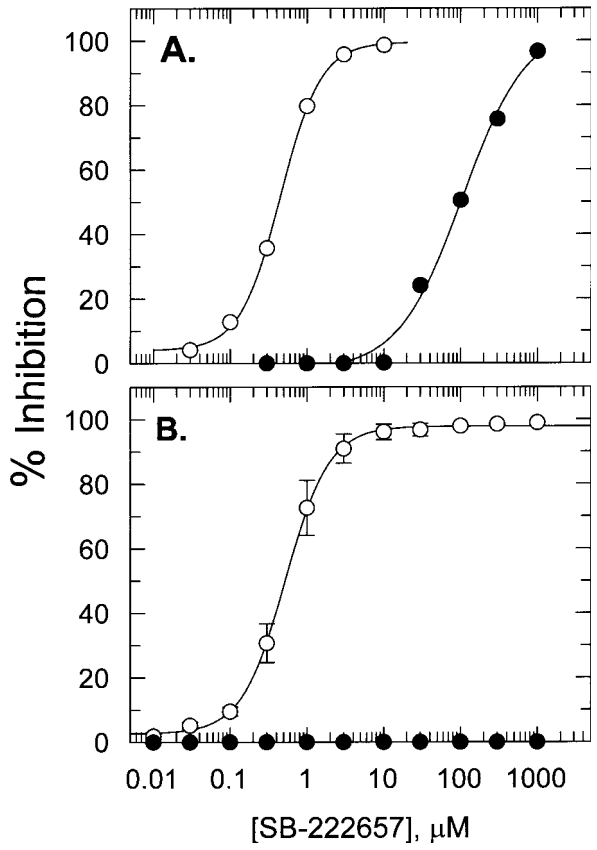


Figure 8 SB-222657 is inactive against paraoxonase and a relatively poor inhibitor of LCAT

(**A**) Freshly isolated human plasma was preincubated for 5 h with or without the indicated concentrations of SB-222657 and assayed simultaneously for LCAT (●) and Lp-PLA_2 (○). Plasma Lp-PLA_2 and LCAT activities without inhibitor present were 21 and 2.5 nmol/min per ml of plasma respectively. Results are means from a representative experiment. (**B**) Freshly prepared human serum was preincubated for 5 h with the indicated concentrations of inhibitor before simultaneously assaying for paraoxonase (●) and Lp-PLA_2 (○). Serum Lp-PLA_2 and paraoxonase activities without inhibitor present were 16 ± 3 and 194 ± 69 nmol/min per ml respectively. Results are means $\pm$ S.D. for three different serum preparations.

Lp-PLA_2 activity is also found on HDL, but to a much smaller extent than on LDL. Our finding that pretreatment with SB-222657 had little influence on the lyso-PtdCho content of oxidized HDL is intriguing. Presumably other mechanisms account for the increased lyso-PtdCho content of oxidized HDL. Candidate enzymes include paraoxonase and LCAT, both of which are found only on HDL and have been suggested to have a role in the ability of HDL to inhibit the consequences of LDL oxidation [49]. SB-222657 was found to be a poor inhibitor of either enzyme. Paraoxonase is thought to be involved in the cleavage of lipid peroxides, among other substrates [50,51], and is therefore thought to have a critical part in HDL's ability to antagonize the biological activities of oxidized LDL [52]. LCAT, in contrast, can generate lyso-PtdCho by a transacylation of a *sn*-2 fatty acid from phosphatidylcholine to the 3-hydroxy group of cholesterol [53]. However, only LCAT could have contributed in our HDL oxidation assay owing to the lack of Ca^{2+}, a critical cofactor for paraoxonase.

An important question is why LCAT should be apparently more active on oxidized HDL than on native HDL. One explanation is that LCAT might have a preference for oxidatively modified esterified fatty acids, as has been suggested [54], and as a result might have a physiological role in the metabolism of oxidized phosphatidylcholine in plasma. Consistent with this notion is the demonstration that HDL is the principal carrier of hydroperoxides, in the form of cholesteryl esters, in plasma [55]. Furthermore these hydroperoxides in HDL are rapidly removed by the liver, all of which supports a possible role for HDL in the detoxification of circulating LOOHs *in vivo* [56]. One possible scenario is that by exchanging hydroperoxides from phospholipid to cholesterol, LCAT is effectively removing potential substrates for Lp-PLA_2 which in our view would be beneficial. The resultant cholesteryl ester hydroperoxides could then be removed by either uptake by the liver, as suggested above, or possibly via degradation by paraoxonase.

In conclusion, Lp-PLA_2 has a significant role in the degradation of oxidatively modified phosphatidylcholines during the oxidation of LDL but apparently not during the oxidation of HDL. It does not, however, influence the overall kinetics of oxidation of either lipoprotein as measured by changes in conjugated dienes, TBARs or LOOHs. The enzyme Lp-PLA_2 is capable of generating two bioactive lipids within modified LDL, lyso-PtdCho and oxidized non-esterified fatty acids. Lp-PLA_2 is in fact able to release oxidized polyunsaturated fatty acid moieties that are biologically active in an analogous fashion to the well characterized, Ca^{2+}-dependent, PLA_2 enzymes that release arachidonic acid for biological signalling [57]. The structural elucidation of these modified fatty acids will be an important area for future research and the new potent inhibitors of Lp-PLA_2 will undoubtedly aid in their identification. Inhibitors such as SB-222657 should also provide an excellent opportunity to further define the role of Lp-PLA_2 in the overall atherosclerotic disease process.

We thank Dr. Lisa Marshall and Dr. Jim Winkler for assaying recombinant human Type IIa 14 kDa PLA_2. R.A.P. was supported by an MRC Collaborative Studentship in association with SmithKline Beecham.

REFERENCES

1 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witzum, J. L. (1989) New Engl. J. Med. **320**, 915–924
2 Berliner, J. A. and Heinecke, J. W. (1996) Free Radicals Biol. Med. **20**, 707–727
3 Quinn, M. T., Parthasarathy, S., Fong, L. G. and Steinberg, D. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2995–2998
4 Davies, M. J. and Woolf, N. (1993) Br. Heart J. **69** S3–S11
5 Lafont, A., Chai, Y. C., Cornhill, J. F., Whitlow, P. L., Howe, P. H. and Chisolm, G. M. (1995) J. Clin. Invest. **95**, 1018–1025
6 Kugiyama, K., Kerns, S. A., Morrisett, J. D., Roberts, R. and Henry, P. D. (1990) Nature (London) **344**, 160–162
7 Flavahan, N. A. (1992) Circulation **85**, 1927–1938
8 Thorne, S. A., Abbot, S. E., Winyard, P. G., Blake, D. R. and Mills, P. G. (1996) Heart **75**, 11–16
9 Yui, S., Sasaki, T., Miyazaki, A., Horiuchi, S. and Yamazaki, M. (1993) Arterioscler. Thromb. **13**, 331–337
10 Steinbrecher, U. P. and Pritchard, P. H. (1989) J. Lipid Res. **30**, 305–315
11 Tew, D. G., Southan, C., Rice, S. Q. J., Lawrence, M. P., Li, H., Saul, H. F., Moores, K., Gloger, I. S. and Macphee, C. H. (1996) Arterioscler. Thromb. Vasc. Biol. **16**, 591–599
12 Dennis, E. A. (1997) Trends Biochem. Sci. **22**, 1–2
13 Tjoelker, L. W., Eberhardt, C., Unger, J., Trong, H. L., Zimmerman, G. A., McIntyre, T. M., Stafforini, D. M., Prescott, S. M. and Gray, P. W. (1995) J. Biol. Chem. **270**, 25481–25487
14 Stremler, K. E., Stafforini, D. M., Prescott, S. M. and McIntyre, T. M. (1991) J. Biol. Chem. **266**, 11095–11103
15 Macphee, C. H., Milliner, K., Moores, K. and Tew, D. G. (1996) Pharmacol. Rev. Commun. **8**, 309–315
16 Quinn, M. T., Parthasarathy, S. and Steinberg, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2805–2809
17 Kume, N., Cybulsky, M. I. and Gimbrone, M. A. (1992) J. Clin. Invest. **90**, 1138–1144

18 Khan, B. V., Parthasarathy, S. S., Alexander, R. W. and Medford, R. M. (1995) J. Clin. Invest. **95**, 1262–1270
19 Chai, Y. C., Howe, P. H., DiCorleto, P. E. and Chisolm, G. M. (1996) J. Biol. Chem. **271**, 17791–17797
20 Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matsumura, T., Kobori, S., Shichiri, M. and Horiuchi, S. (1996) Arterioscler. Thromb. Vasc. Biol. **16**, 600–605
21 Cowan, C. L. and Steffen, R. P. (1995) Arterioscler. Thromb. Vasc. Biol. **15**, 2290–2297
22 Wu, R., Huang, Y. H., Elinder, L. S. and Frostegard, J. (1998) Arterioscler. Thromb. Vasc. Biol. **18**, 626–630
23 Tjoelker, L. W., Wilder, C., Eberhardt, C., Stafforini, D. M., Dietsch, G., Schimp, B., Hooper, S., Trong, H. L., Cousens, L. S., Zimmerman, G. A. et al. (1995) Nature (London) **374**, 549–553
24 Berliner, J., Leitinger, N., Watson, A., Huber, J., Fogelman, A. and Navab, M. (1997) Thromb. Haemostasis **78**, 195–199
25 Tew, D. G., Boyd, H. F., Ashman, S., Theobald, C. and Leach, C. A. (1998) Biochemistry **37**, 10087–10093
26 Redgrave, T. G., Roberts, D. C. K. and West, C. E. (1975) Anal. Biochem. **65**, 42–49
27 Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) Anal. Biochem. **87**, 206–210
28 Wilkins, G. M. and Leake, D. S. (1994) Biochim. Biophys. Acta **1211**, 69–78
29 El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A. Y. and Jurgens, G. (1989) J. Lipid Res. **30**, 627–630
30 Steinbrecher, U., Parasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3883–3887
31 Sato, K., Niki, E. and Shimasaki, H. (1990) Arch. Biochem. Biophys. **279**, 402–404
32 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
33 Kuhn, H., Belkner, J., Wiesner, R. and Brash, A. R. (1990) J. Biol. Chem. **265**, 18351–18361
34 Marshall, L. A., Bauer, J., Sung, M. L. and Chang, J. Y. (1991) J. Rheumatol. **18**, 59–65
35 Mackness, M., Harty, D., Bhatnager, D., Winocour, P. H., Arrol, S., Ishola, M. I. and Durrington, P. (1991) Atherosclerosis **86**, 193–199
36 Gillett, M. P. T. and Owen, J. S. (1992) in Lipoprotein Analysis, A Practical Approach (Converse, C. A. and Skinner, E. R., eds.), pp. 187–202, Oxford University Press, Oxford
37 Ohkawa, H., Ohishi, N. and Yagi, K. (1978) J. Lipid Res. **19**, 1053–1057
38 Quinn, M. T., Kondratenko, N. and Parthasarathy, S. (1991) Biochim. Biophys. Acta **1082**, 293–302
39 Stafforini, D. M., McIntyre, T. M., Carter, M. E. and Prescott, S. M. (1987) J. Biol. Chem. **262**, 4215–4222
40 Stafforini, D. M., Zimmerman, G. A., McIntyre, T. M. and Prescott, S. M. (1994) J. Lipid Mediators Cell Signall. **10**, 53–56
41 Croft, K. D., Williams, P., Dimmitt, S., Abu-Amsha, R. and Beilen, L. J. (1995) Biochim. Biophys. Acta **1254**, 250–256
42 Karabina, S. P., Liapikos, T. A., Grekas, G., Goudevenos, J. and Tselepis, A. D. (1994) Biochim. Biophys. Acta **1213**, 34–38
43 de Graaf, J., Hak-Lemmers, H. L. M., Hectors, M. P. C., Demacker, P. N. M., Hendriks, J. C. M. and Stalenhoef, A. F. H. (1991) Arterioscler. Thrombosis **11**, 298–306
44 Sakurai, T., Yamamoto, Y., Baba, N. and Nakano, M. (1996) Biochim. Biophys. Acta **1301**, 85–89
45 Patrono, C. and Fitzgerald, G. A. (1997) Arterioscler. Thromb. Vasc. Biol. **17**, 2309–2315
46 Luoma, J., Macphee, C., Chamberlain, P., Milliner, K., Tew, D., Rice, S. and Yla-Herttuala, S. (1996) Circulation **94** (Suppl.), I-585
47 Stafforini, D. M., Elstad, M. R., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. (1990) J. Biol. Chem. **265**, 9682–9687
48 Dupuis, F., Denizot, Y., Fixe, P., Dulery, C. and Praloran, V. (1996) Biochim. Biophys. Acta **1311**, 27–32
49 Mackness, M. I. and Durrington, P. N. (1995) Atherosclerosis **115**, 243–253
50 Mackness, M. I., Mackness, B., Durrington, P. N., Connelly, P. W. and Hegele, R. A. (1996) Curr. Opin. in Lipidol. **7**, 69–76
51 Watson, A. D., Berliner, J. A., Hama, S. Y., La Du, B. N., Faull, K. M., Fogelman, A. M. and Navab, M. (1995) J. Clin. Invest. **96**, 2882–2891
52 Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van Lenton, B. J., Frank, J. S., Demer, L. L. et al. (1996) Arterioscler. Thromb. Vasc. Biol. **16**, 831–842
53 Marcel, Y. L. (1982) Adv. Lipid Res. **19**, 85–136
54 Subbaiah, P. V. and Liu, M. (1996) Biochim. Biophys. Acta **1301**, 115–126
55 Bowry, V. W., Stanley, K. K. and Stocker, R. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10316–10320
56 Christison, J., Karjalainen, A., Brauman, J., Bygrave, F. and Stocker, R. (1996) Biochem. J. **314**, 739–742
57 Dennis, E. A. (1994) J. Biol. Chem. **269**, 13057–13060

Received 10 July 1998/8 December 1998; accepted 21 December 1998