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INHIBITION OF HUMAN LEUKOCYTE 5-LIPOXYGENASE BY A 4-HYDROXYBENZOFURAN, L-656,224

EVIDENCE FOR ENZYME REDUCTION AND INHIBITOR DEGRADATION

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Abstract—Detailed studies of the interaction of L-656,224 (2-[(4′-methoxyphenyl)methyl]-3-methyl-4-hydroxy-5-propyl-7-chlorobenzofuran) with 5-lipoxygenase were conducted using the enzymes from human and pig leukocytes. L-656,224 was a potent inhibitor of these 5-lipoxygenases although its efficiency varied with enzyme concentration. L-656,224 also stimulated the pseudoperoxidase activity of 5-lipoxygenase as measured by the consumption of 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), indicating that this compound can reduce the enzyme. Furthermore the inhibitor was degraded rapidly by both cell-free leukocyte extracts and purified 5-lipoxygenase after incubation with 13-HPOD, ATP and calcium ions. The degradation of L-656,224 was also observed during inhibition of the lipoxygenase reaction and occurred mainly after the initial lag phase of the reaction when hydroperoxides begin to accumulate. A single major radioactive product was formed after incubation of [^{3}H]L-656,224 with purified 5-lipoxygenase in the presence of 13-HPOD. This product was unstable and could not be isolated. During the course of the pseudoperoxidase reaction, [^{3}H]L-656,224 covalently labelled the enzyme, suggesting that a chemically reactive species had been formed. These data are consistent with the hypothesis that L-656,224 reduces the oxidized form of the 5-lipoxygenase to an inactive form, with degradation of the inhibitor and regeneration of the active enzyme with hydroperoxides.

The 5-lipoxygenase catalyses the first two steps in leukotriene biosynthesis, including the oxidation of arachidonic acid to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE¶), and the further conversion of 5-HPETE to 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA_4) [1–4]. Because leukotrienes have been implicated in the pathophysiology of allergic and inflammatory diseases, the development of clinically useful 5-lipoxygenase inhibitors could be of value in the therapy of such diseases [5, 6]. However, the development of specific, non-toxic, and potent inhibitors *in vivo* has proven to be a difficult task.

As noted by Thody *et al.* [7], many known inhibitors of 5-lipoxygenase and other lipoxygenases possess antioxidant properties, and often the potency of the compounds as antioxidants correlates with their potency as lipoxygenase inhibitors. Although many possible mechanisms might explain the inhibitory effect of antioxidants on the lipoxygenase reaction, recent studies on the soybean lipoxygenase have shown that at least some of these inhibitors act by reducing the active ferric form of the enzyme to the inactive ferrous state [8, 9]. In this model, the reduced enzyme is converted back to the oxidized form by product hydroperoxy fatty acid through a pseudoperoxidase activity of the enzyme (Fig. 1). Thus, in the presence of both inhibitor and hydroperoxy fatty acid, a cycle ensues in which the enzyme is converted from the reduced to the oxidized form and back with consumption of the hydroperoxide and possibly also the inhibitor.

It is not known for certain whether the mammalian 5-lipoxygenase posesses iron at its active site. However, the kinetic behaviour of the enzyme, including the effects of reducing agents and hydroperoxides [10, 11], suggests that the 5-lipoxygenase is very similar to the iron-containing lipoxygenases. In addition, recent evidence has shown that the 5-lipoxygenase of porcine leukocytes possesses a pseudoperoxidase activity that is stimulated in the presence of a number of enzyme inhibitors [12]. Thus it appears that, at least for some inhibitors, the mechanism shown in Fig. 1 may apply to the mammalian 5-lipoxygenase. In the

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¶ Abbreviations: 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; L-656,224, 2-[(4′-methoxyphenyl)-methyl]-3-methyl-4-hydroxy-5-propyl-7-chlorobenzofuran; L-655,325, 2-(4′-methoxyphenyl)methyl-3-methyl-4-propyl-5-hydroxybenzofuran; 5,8,14-ETE,5,8,14-eicosatrienoic acid; 13-HPOD, 13-hydro-peroxy-9,11-octadecadienoic acid; LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HPETRE, 5-hydroperoxy-6,8,14-eicosatrienoic acid; 5-HETRE, 5-hydroxy-6,8,14-eicosatrienoic acid; 60–90% ppt, protein precipitated from human leukocyte supernatants at 60–90% saturation of ammonium sulfate; and 15-HPEDE, 15-hydroperoxy-11,13-eicosadienoic acid.

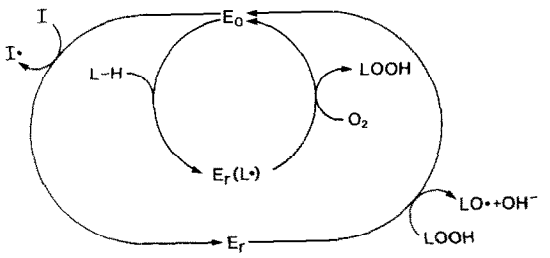


Fig. 1. Simplified scheme for the mechanism of lipoxygenase inhibition by reductive inactivation [8, 9]. The reduced, inactive form of the enzyme (E_r) is oxidized by fatty acyl hydroperoxide (LOOH) through a peroxidase reaction to form the oxidized active enzyme (E_o). E_o can then react with fatty acid substrate (L-H) and oxygen to form product hydroperoxide (inner cycle). Alternatively, reduction of E_o by inhibitor (I) leads to the formation of E_r (outer cycle). In the presence of LOOH, the enzyme cycles from E_r to E_o and back with the destruction of LOOH and inhibitor.

present study, we have investigated in detail the benzofuranol inhibitor L-656,224 [13], and its interaction with human leukocyte 5-lipoxygenase. We show that L-656,224 was metabolized rapidly by the enzyme in the presence of Ca^{2+}, ATP, and fatty acyl hydroperoxide and that it also stimulated the pseudoperoxidase activity of the 5-lipoxygenase. It was also found that 5-lipoxygenase can be covalently modified by a transformation product of the inhibitor, a process that may contribute to enzyme inactivation.

MATERIALS AND METHODS

Human 5-lipoxygenase preparations. Human leukocyte concentrates (buffy coat) were obtained from local blood collection centers. The isolation of leukocytes and the preparation of leukocyte homogenates have been described previously [14]. Homogenates were prepared by sonication from cells suspended at a concentration of 200×10^6/mL in 50 mM potassium phosphate buffer, pH 7.1, containing 0.1 M NaCl, 2 mM EDTA, 1 mM dithiothreitol, 60 μg/mL soybean trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride. Samples were then subjected to centrifugation at 10,000 *g* for 15 min, followed by 100,000 *g* for 1 hr. The resulting 100,000 *g* supernatants (approximately 6 mg protein/mL) were combined with an equal volume of glycerol, and stored at −70° until used. These preparations were stable for several months.

5-Lipoxygenase was purified as reported previously from 10,000 *g* supernatants of human leukocyte homogenates [14]. The purified enzyme in the final chromatography buffer was combined with glycerol to a final concentration of 50% (v/v), and stored at −70°. This preparation lost up to 50% of its activity as a result of freezing and thawing, but was otherwise stable over several months of storage.

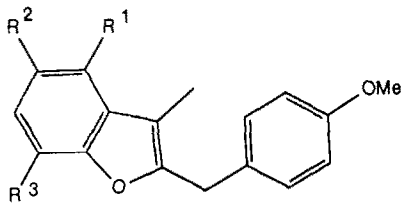
Measurement of 5-lipoxygenase activity. In some cases, enzyme samples were incubated for 10 min at 37° in 1 mL of a solution containing 0.1 M Tris–HCl, pH 7.5, 1.8 mM dithiothreitol, 1.6 mM EDTA, 3 mM $CaCl_2$, 2 mM ATP, 100 μM arachidonic acid and 2 μM 15-hydroperoxy-11,13-eicosadienoic acid (15-HPEDE). The samples were then placed on ice and combined with 1 mL of 1 μM 13-hydroxyoctadecadienoic acid in ethanol as internal standard. Following chloroform extraction, the samples were analyzed by HPLC for the quantitation of 5-HPETE and its reduction product 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). These are the major products of 5-lipoxygenase under these conditions and coelute with a retention time of 14 min after chromatography on Nova-Pak C_{18} columns (Waters) in methanol/water/trifluoroacetic acid/triethylamine (75:25:0.2:0.1) at 1.2 mL/min. One unit of 5-lipoxygenase activity is the amount of enzyme that produces 1 nmol of 5-HPETE + 5-HETE in this standard assay [14, 15]. In some cases, 5,8,14-eicosatrienoic acid (5,8,14-ETE) was used as the substrate. Under these circumstances, the assay was performed identically, but the products were 5-hydroperoxy-6,8,14-eicosatrienoic acid (5-HPETRE) or its reduction product 5-hydroxy-6,8,14-eicosatrienoic acid (5-HETRE) [16, 17], which coeluted at 20 min on HPLC.

Alternatively, 5-lipoxygenase activity was measured spectrophotometrically. Reaction mixtures contained the enzyme sample in a total volume of 700 μL of 50 μM potassium phosphate buffer, pH 7.5, containing 0.5 mM $CaCl_2$, and 0.1 mM ATP. Inhibitors were added as stock solutions in ethanol, and the samples were incubated for 5 min at 25°. Reactions were initiated by the addition of 3.5 μL of an ethanolic solution of 4 mM 5,8,14-ETE plus 0.2 mM 13-HPOD. The ethanol concentration was maintained constant in all samples and did not exceed 1%. The reaction was monitored by the increase in absorbance at 235 nm during a 30-min incubation period. Under these conditions, the enzyme undergoes inactivation by a first order process. Therefore, the data were analysed by nonlinear regression analysis using the formula

$$y = v_f t + \frac{(v_o - v_f)(1 - e^{-k_{obs}t})}{k_{obs}} + y_o$$

where y is the amount of product formed at time t, y_o is the amount of product present at time t_o (fixed at 0), v_o is the initial velocity of the reaction, k_{obs} is the first order rate constant for enzyme inactivation, and v_f is the velocity of the reaction at infinite time (fixed at 0) [18]. It should be noted that 5,8,14-ETE was utilized as a substrate in these experiments because it is a very poor substrate for 12- and 15-lipoxygenase that contaminate 5-lipoxygenase in human leukocyte 100,000 *g* supernatants. Furthermore, 5-HPETRE is the single major 5-lipoxygenase product formed from this substrate and the reaction kinetics are not complicated by the LTA_4 synthase activity of the enzyme [16, 17].

Note that, when samples of purified 5-lipoxygenase were assayed, in some instances, phosphatidylcholine and a 60–90% saturation ammonium sulfate precipitate (60–90% ppt) protein fraction from leukocytes were also included as stimulatory factors in the reaction mixtures [4, 14, 19]. Phosphatidylcholine was added as a stock solution



L-656,224 R^1 = OH R^2 = propyl R^3 = Cl

L-655,325 R^1 = propyl R^2 = OH R^3 = H

Fig. 2. Structures of L-656,224 and L-655,325.

(2 mg/mL) in ethanol to give a final concentration of 2 μg/mL. The 60–90% ppt was prepared by ammonium sulfate fractionation of human leukocyte 10,000 *g* supernatants followed by dialysis as described [14]. Approximately 0.4 mg protein from this fraction was added per mL of assay reaction mixture.

Pseudoperoxidase assays with recombinant human 5-lipoxygenase and porcine 5-lipoxygenase. Escherichia coli expressing the cDNA for human 5-lipoxygenase [20] was obtained from T. Nguyen, Department of Molecular Biology, Merck Frosst Centre for Therapeutic Research. Pelletted bacteria were resuspended in homogeneization buffer [14] and lysed by sonication at 4°. The lysate was centrifuged at 100,000 *g* for 1 hr and the supernatant fraction was used as the source of enzyme. Immunoaffinity purified 5-lipoxygenase was prepared from porcine leukocytes as previously described [2].

For the pseudoperoxidase reactions, aliquots from these enzyme preparations were incubated at room temperature in reaction mixtures containing 10 μM L-656,224 and 10 μM 13-HPOD, as described [12]. The variations in the levels of 13-HPOD and inhibitor were followed by reverse-phase HPLC on a Nova-Pak C_{18} column using acetonitrile/water/acetic acid (65:35:0.01) as solvent system and a flow rate of 2 mL/min. The retention times of 13-HPOD and L-656,224 in this system were 2 and 3.7 min, respectively. The concentrations of these products were calculated by comparison of the absorbance area peak at 235 nm with known amounts of standards.

Degradation of L-656,224 by human 5-lipoxygenase. L-656,224 was synthesized as previously described by Lau *et al.* [21] (structure shown in Fig. 2). The compound was added as a stock solution (0.1 to 1 mM) in ethanol to incubation mixtures having the same composition as used for the spectrophotometric assay of human 5-lipoxygenase. The solutions were brought to 25° and the reactions were initiated by the addition of ethanolic stock solutions of 5,8,14-ETE and/or 13-HPOD to give the appropriate final concentration. At the desired times, the reactions were stopped by combining 2 mL of the reaction mixture with 1 mL of ice-cold L-655,325 (0.5 μM in ethanol) to serve as an internal standard. The samples were extracted twice with 2 mL of diethyl ether. The ether extracts were combined and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 100 μL of methanol, and 50 μL was subjected to reverse-phase HPLC on a column (3.9 mm × 15 cm) of Nova Pak C_{18} (Millipore) eluted isocratically at 1 mL/min with methanol/water (75:25). The effluent was monitored by absorbance at 254 nm. L-656,224 (retention time of 11 min) was quantitated from the area of the corresponding peak, relative to the area of the peak corresponding to L-655,325 (retention time of 6 min). In many experiments, 5-HPETRE + 5-HETRE formation was also measured. Samples (1 mL) of the reaction mixtures were combined with 1 mL of 1 μM 13-hydroxyoctadecadienoic acid, extracted with chloroform, and analysed for product content by HPLC as described above.

Characterization of the reaction products of L-656,224. [*Benzyl*-^{3}H]L-656,224 was prepared by New England Nuclear and was purified by reverse-phase HPLC. The exact specific activity of the purified material was not known; however, it could be estimated to be approximately 7.5×10^7 dpm/nmol. This material was combined with unlabelled L-656,224 to yield a solution (120 mM, 10^6 cpm/μL) in ethanol that was utilized in the experiments.

Reaction mixtures contained approximately 15 μg of purified 5-lipoxygenase, 1 μg/mL of phosphatidylcholine and 5×10^5 cpm of [^{3}H]L-656,224 in a total volume of 1 mL of 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM $CaCl_2$ and 0.1 mM ATP. The reaction mixtures were brought to 25° and 5 μL of ethanol or 0.2 mM 13-HPOD was added. After 15 min, the reaction was stopped by the addition of 1 mL of ice-cold methanol. After removal of precipitated protein by centrifugation at 10,000 *g* for 10 min, the samples (200 μL) were analyzed by HPLC using conditions described above for L-656,224. Fractions (1 mL) of the eluate were collected for the measurement of radioactivity by liquid scintillation counting.

Alkylation of 5-lipoxygenase by L-656,224. Reaction conditions were exactly as described above for the study of [^{3}H]L-656,224 degradation, except that, after the 15-min incubation period, 400 μL of 60% trichloroacetic acid was added. After a 2-hr incubation at 4°, the protein was collected by centrifugation and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) [22]. The gels were soaked for 20 min in Enlightning (New England Nuclear), dried, and exposed for 4 days to X-ray film (XAD, Kodak).

RESULTS

Inhibition of human leukocyte 5-lipoxygenase by L-656,224. In initial experiments, the effect of L-656,224 on the rate of the 5-lipoxygenase reaction was determined using human leukocyte 100,000 *g* supernatants as the source of enzyme. The reactions were initiated with substrate containing 13-HPOD to eliminate the kinetic lag phase, which allows the use of non-linear regression analysis to estimate the

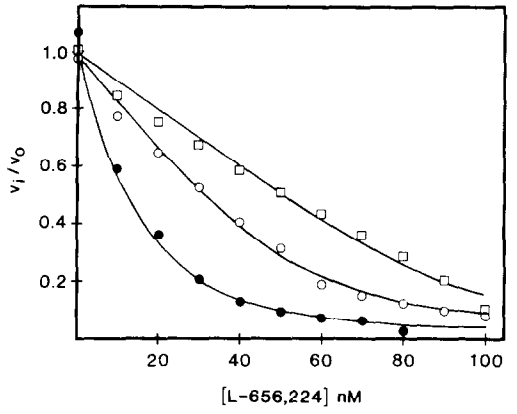


Fig. 3. Inhibition of human leukocyte 5-lipoxygenase by L-656,224. Human leukocyte 100,000 *g* supernatants were assayed for 5-lipoxygenase activity using the spectrophotometric method. Samples contained the indicated quantities of L-656,224 and three different enzyme concentrations: 30 μL (●), 60 μL (○) and 90 μL (□). The data are expressed as the ratio of initial velocity of the sample in the presence of the inhibitor (V_i) to the initial velocity of control samples assayed without inhibitor (V_o). Points are single determinations from a representative experiment that was performed three times with similar results.

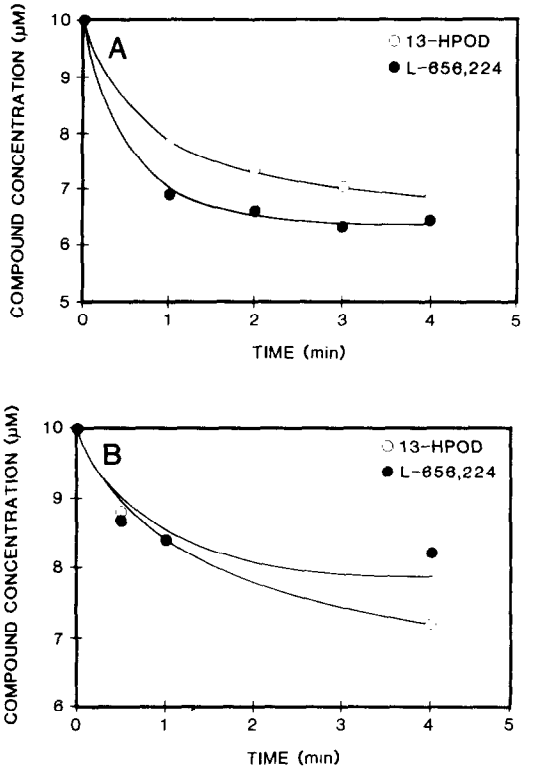


Fig. 4. Stimulation of the pseudoperoxidase activity of 5-lipoxygenase by L-656,224. Immunoaffinity purified porcine 5-lipoxygenase (A) and the supernatant fraction containing recombinant human 5-lipoxygenase (B) were incubated in the presence of 10 μM 13-HPOD and 10 μM L-656,224 under the conditions of the pseudoperoxidase assay. Aliquots were taken at different times and the levels of L-656,224 and 13-HPOD were determined by reverse-phase HPLC.

initial velocity of the reaction [18]. As shown in Fig. 3, L-656,224 strongly inhibited the 5-lipoxygenase reaction, but its apparent potency depended upon the amount of enzyme used in the assay. The IC_{50} values ranged from 13 to 50 nM over a 3-fold change in enzyme concentration. These results implied that L-656,224 does not behave as a classical, reversible inhibitor of 5-lipoxygenase. Furthermore, during the course of these studies, we noted that if 13-HPOD was omitted from the reaction mixture, excessively long lag phases preceded product formation in the presence of L-656,224. These results suggested that L-656,224 may act by interfering with the oxidative activation of the enzyme.

L-656,224 Stimulation of the pseudoperoxidase activity of 5-lipoxygenase. The requirement for a 4-hydroxy substituent on the benzofuran ring of L-656,224 [13] suggests that this inhibitor could act by reducing the oxidized active form of 5-lipoxygenase to a reduced inactive form as shown for the inhibition of soybean lipoxygenase by nordihydroguaiaretic acid [8] and as illustrated in Fig. 1. In this case, addition of hydroperoxy fatty acid should reoxidize the enzyme, through the pseudoperoxidase activity. Therefore, in the presence of both L-656,224 and fatty acyl hydroperoxide, one should observe progressive destruction of the hydroperoxide as the enzyme is converted from oxidized to reduced form by L-656,224 and then back to the oxidized form again through the reaction with the hydroperoxide.

Figure 4 shows that such a pseudoperoxidase activity can be observed with L-656,224 using recombinant human 5-lipoxygenase from bacterial extracts or immunoaffinity purified 5-lipoxygenase from porcine leukocytes. After incubation with either enzyme preparation, the levels of both 13-HPOD and inhibitor (10 μM) decreased with time until 20–35% of the compounds were degraded. The reaction required active enzyme and no 13-HPOD degradation was detected when L-656,224 was omitted from the incubation mixture. Similar effects were also observed using the soybean lipoxygenase (data not shown) but in this case the stoichiometry of L-656,224 to 13-HPOD degradation was higher than for the 5-lipoxygenase (~2.5). These results indicate that L-656,224 can reduce 5-lipoxygenase and that the inhibitor is degraded during this reaction. The variations in the ratio of hydroperoxide to inhibitor consumption suggest that the first oxidation product of L-656,224 may undergo different reactions after its formation, depending on the source of enzyme or assay conditions (see below).

Degradation of L-656,224 during the 5-lipoxygenase reaction. The data presented above indicate that 5-lipoxygenase can cause the degradation of L-656,224 in the presence of relatively high concentrations of 13-HPOD. It was then determined whether the compound could also be destroyed in the presence of substrate during the 5-lipoxygenase reaction. The 100,000 *g* supernatant from human leukocyte was

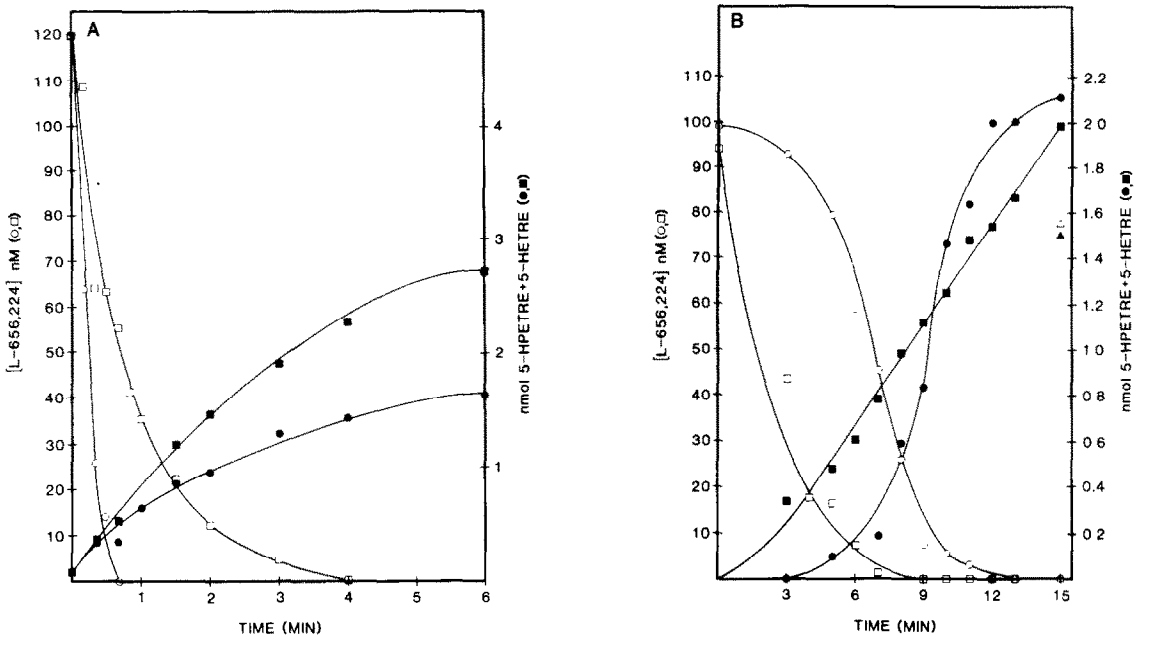


Fig. 5. Degradation of L-656,224 by 5-lipoxygenase. Human leukocyte supernatants (100 μL/mL of reaction mixture) were incubated under spectrophotometric assay conditions with 100 nM L-656,224. (A) Reactions were initiated by the addition of 1 μM 13-HPOD plus 10 μM (○, ●) or 20 μM (□, ■) 5,18,14-ETE. At the indicated times, samples were removed for the measurement of L-656,224 (open symbols) and 5-HPETRE + 5-HETRE (closed symbols) concentrations by HPLC. (B) The experiment was performed exactly as in A except that 13-HPOD was omitted. The L-656,224 concentrations in samples incubated with the reaction mixture for 15 min in the absence of substrate (△) or in the absence of Ca^{2+} and ATP (▲) are also shown. No lipoxygenase reaction product was formed in these samples. Data show the results of a single determination from a representative experiment that was repeated three times with similar conclusions.

used as the enzyme source and incubated under the conditions utilized for the spectrophotometric assay. The incubation mixtures contained 100 nM L-656,224, which resulted in an approximately 70% inhibition of product formation. The reactions were initiated with 10 or 20 μM 5,8,14-eicosatrienoic acid and a low concentration of 13-HPOD (1 μM) to eliminate lag phases. At various times, samples were removed for the measurement of L-656,224 and 5-HPETRE + HETRE levels by HPLC. The results (Fig. 5A) show that L-656,224 was degraded rapidly after the initiation of the enzymatic reaction. The degradation was more rapid in the presence of 10 μM than 20 μM substrate, suggesting that, in terms of the model shown in Fig. 1, L-656,224 and substrate may compete for reaction at E_o. It is interesting to note that, because the destruction of L-656,224 was so rapid under these conditions, little inhibitor was actually present during the time that the bulk of the product formation occurred.

The data in Fig. 4 indicated that fatty acyl hydroperoxide may be important in the reaction that leads to L-656,224 degradation by 5-lipoxygenase. We therefore repeated the experiment shown in Fig. 5A with the omission of the 13-HPOD. The results (Fig. 5B) showed that, in the absence of 1 μM 13-HPOD, the destruction of L-656,224 occurred much more slowly. Furthermore, under these conditions, the reaction was faster in the presence of 20 μM than 10 μM, 5,8,14-eicosatetraenoic acid. The explanation for this may lie in the observation that destruction of L-656,224 appeared to occur concomitantly with the formation of 5-HPETRE, which occurred more rapidly at the higher substrate concentration. This is consistent with a role for fatty acyl hydroperoxide in the L-656,224 degradation reaction. Note also that, as shown in Fig. 5B, little destruction of L-656,224 occurred in the absence of either the substrate or Ca^{2+} and ATP, conditions under which product formation did not occur.

Degradation of L-656,224 by purified human 5-lipoxygenase. The experiments in Fig. 5 were performed using 5-lipoxygenase from the soluble fraction of human leukocytes. To verify that the effects were not due to contaminating protein, the stability of L-656,224 in the presence of the purified enzyme from human leukocytes was also studied. As shown in Fig. 6A, incubation of purified 5-lipoxygenase with 100 nM L-656,224 in the presence of Ca^{2+} and ATP caused little to no destruction of the inhibitor whether or not substrate was added. However, purified 5-lipoxygenase synthesized little product under these conditions (Fig. 6B), due to a requirement for various other stimulatory factors [14]. Enzyme activity could be increased by low levels of phosphatidylcholine [4, 19] and by a protein fraction obtained from human leukocytes by precipitation of the 100,000 *g* supernatant at 60–90%

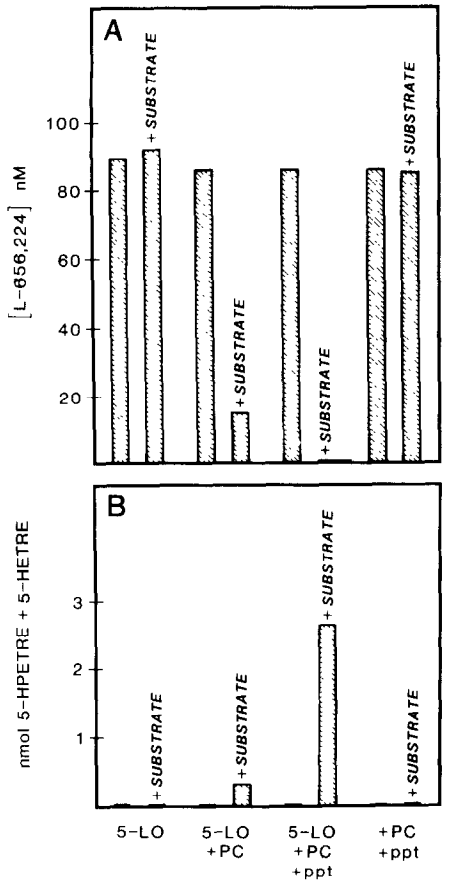


Fig. 6. Degradation of L-656,224 by purified 5-lipoxygenase. Purified human leukocyte 5-lipoxygenase (5-LO, approximately 3 μg/mL) was incubated under spectrophotometric assay conditions with 100 nM L-656,224. As indicated, some samples also contained 1 μg/mL phosphatidylcholine (PC), and/or 0.4 mg/mL of the 60–90% ppt stimulatory factor (ppt). One set of samples contained these factors, without the enzyme. Reactions were initiated with the addition of 5 μL/mL of 10 mM 5,8,14-eicosatrienoic acid in ethanol (+substrate) or 5 μL/mL of ethanol as a control. At the end of the 10-min reaction period, samples were assayed for L-656,224 concentration (A) and the total amount of 5-HPETRE + 5-HETRE formed in the 700 μL reaction mixture (B). Note that the labels under the bars apply to both A and B.

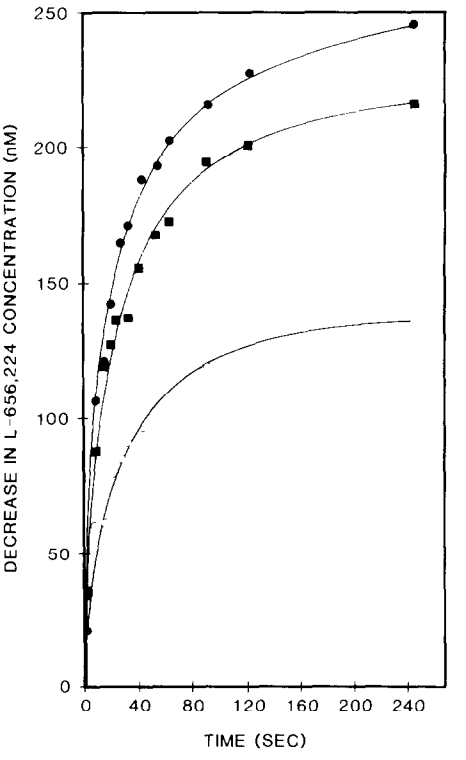


Fig. 7. Time course of L-656,224 degradation by human 5-lipoxygenase. Purified 5-lipoxygenase (3 μg/mL) was incubated under spectrophotometric assay conditions in the presence of 2 μg/mL phosphatidylcholine and 150 nM (○), 300 nM (■), or 450 nM (●) L-656,224. Reactions were initiated by the addition of 1 μM 13-HPOD, and at the indicated times, samples were removed for the measurement of L-656,224 concentration. Data represent the results of single determinations from a representative experiment that was repeated three times with similar findings.

saturation of ammonium sulfate [14]. As seen in Fig. 6, when phosphatidylcholine (2 μg/mL) was added to the reaction mixture, product formation occurred, and the purified 5-lipoxygenase caused an 85% destruction of L-656,224, but only in the presence of substrate. Product formation and inhibitor loss were augmented further when both phosphatidylcholine and the 60–90% ppt were included (Fig. 6, A and B). However, the destruction of L-656,224 was clearly dependent on the presence of 5-lipoxygenase, as phosphatidylcholine plus the 60–90% ammonium sulfate precipitate alone did not catalyse this reaction. Figure 7 shows the time course of the destruction of L-656,224 (150–450 nM) catalysed by purified 5-lipoxygenase in the presence of Ca^{2+}, ATP, phosphatidylcholine and 1 μM 13-HPOD. The initial rate of the reaction was dependent on inhibitor concentration and decreased rapidly with time up to conversion of 55–85% of L-656,224 to other species.

Attempt to identify a metabolite of L-656,224 from its reaction with 5-lipoxygenase. It was desirable to identify the reaction product of the 5-lipoxygenase-catalysed reaction of L-656,224 to ascertain the chemical mechanisms involved. Consequently, [^{3}H]L-656,224 was incubated with purified enzyme in the presence of Ca^{2+}, ATP, phosphatidylcholine and 1 μM 13-HPOD for 10 min at 25°, and the reaction mixture then immediately subjected to HPLC. The results showed a nearly total destruction of the [^{3}H]L-656,224 that occurred only when 13-HPOD was included in the reaction mixture. A major peak was detected after HPLC (eluting at 16 min, Fig. 8), suggesting the formation of a single prominent reaction product. However, attempts to isolate or characterize this compound were unsuccessful due to its extreme instability. Interestingly, if crude preparations of 5-lipoxygenase were used in this experiment, the destruction of

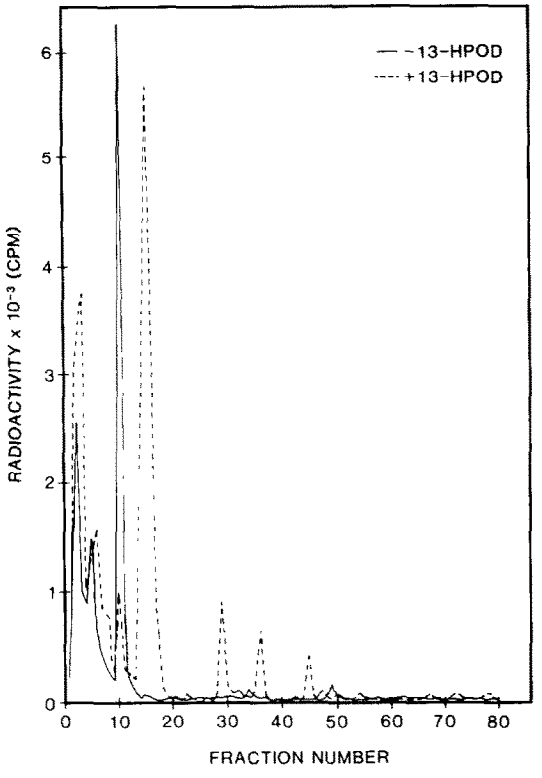


Fig. 8. Degradation products of L-656,224 from its reaction with 5-lipoxygenase. Purified 5-lipoxygenase (15 μg/mL) was incubated under spectrophotometric assay conditions with 2 μg/mL phosphatidylcholine and 5×10^5 cpm of [^{3}H]L-656,224 (approximately 17 nM). Reactions were initiated by the addition of 5 μL of 0.2 mM 13-HPOD or 5 μL ethanol as a control (final volume of 1 mL). After 15 min, the reaction mixtures were combined with 1 mL of methanol and subjected to HPLC analysis. Fractions of 1 mL were collected and measured for radioactivity by liquid scintillation counting. The data show the total cpm in each column fraction.

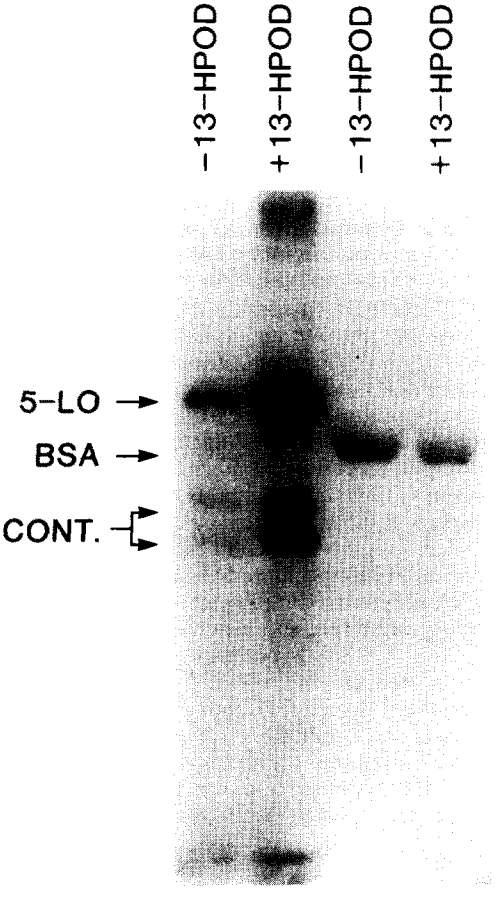


Fig. 9. Covalent modification of 5-lipoxygenase by L-656,224. Experiments were conducted exactly as described in the legend to Fig. 8 except that in some samples 5-lipoxygenase was replaced by an equal concentration of bovine serum albumin, and the reactions were terminated by the addition of 400 μL of 60% trichloroacetic acid. The precipitated protein was recovered by centrifugation and subjected to SDS–PAGE followed by autoradiography. The resulting autoradiogram is labelled to show the positions of 5-lipoxygenase (5-LO), bovine serum albumin (BSA), and contaminant proteins in the 5-lipoxygenase samples (CONT.). Samples incubated in the absence (−13-HPOD) and presence (+13-HPOD) of 13-HPOD are also designated.

[^{3}H]L-656,224 also occurred, but no major metabolite could be detected after HPLC under the same conditions.

Reaction of a degradation product of L-656,224 with 5-lipoxygenase. The instability of the reaction product of L-656,224 with 5-lipoxygenase suggested the possibility that this product might react with the enzyme. To test this hypothesis, purified human 5-lipoxygenase was incubated with [^{3}H]L-656,224 in the presence and absence of 13-HPOD under the conditions utilized for the experiment in Fig. 8. After the reaction, the protein was concentrated by trichloroacetic acid precipitation and subjected to SDS–PAGE [22] and autoradiography. As seen in Fig. 9, 5-lipoxygenase became strongly labelled by [^{3}H]L-656,224 after incubation in the presence of 13-HPOD, in contrast to incubation in the absence of 13-HPOD. When 5-lipoxygenase was replaced by bovine serum albumin, a low level of labelling could be observed upon incubation with [^{3}H]L-656,224 but did not increase in the presence of 13-HPOD. Note that, in the case of purified 5-lipoxygenase incubated in the presence of [^{3}H]L-656,224 and 13-HPOD, not only the enzyme itself, but also some minor contaminant proteins of lower molecular weight [14] were labelled, indicating that not all of the reactive species remains associated with the enzyme.

DISCUSSION

In this series of experiments, we have attempted to gain some insight into the mechanism of action of L-656,224, a 5-lipoxygenase inhibitor of the 4-hydroxybenzofuran class. Kinetic studies revealed that this compound does not behave as a classical, reversible inhibitor, and its chemical structure suggested the possibility that it may act as a reducing

agent to interefere directly with the chemistry of the 5-lipoxygenase reaction.

Previous studies of the soybean lipoxygenase have shown that this enzyme can exist in an inactive, ferrous and an active, ferric form. The ferrous enzyme is oxidized to the ferric form through a reaction with 13-HPOD which results in destruction of the hydroperoxide molecule [23]. Recently, Kemal *et al.* [8] demonstrated that nordihydroguaiaretic acid, a catechol inhibitor of lipoxygenases, can directly reduce the ferric enzyme to the ferrous form. Thus in the presence of both 13-HPOD and the inhibitor, the enzyme cycles between two forms, catalysing the destruction of the 13-HPOD.

Our results with L-656,224 indicate that this inhibitor can stimulate the pseudoperoxidase activity of human 5-lipoxygenase, suggesting that it inhibits 5-lipoxygenase through a mechanism similar to that of nordihydroguaiaretic acid. If this is true, then the reduction of the enzyme by L-656,224 may be expected to be accompanied by the generation of a radical form of the inhibitor. The finding that L-656,224 is destroyed rapidly through its interaction with 5-lipoxygenase and fatty acyl hydroperoxide is consistent with this hypothesis.

The degradation of L-656,224 was dependent upon the presence of active 5-lipoxygenase. Thus under conditions such that the enzyme does not catalyze product formation appreciably (i.e. omission of Ca^{2+}, ATP, or stimulatory factors), L-656,224 degradation did not occur (Figs 5 and 6). The reaction also required the presence of fatty acyl hydroperoxide, either added exogenously, or generated *in situ* by the enzyme. The results are also consistent with the mechanism proposed for nordihydroguaiaretic acid, and illustrated in Fig. 1.

The finding that L-656,224 can reduce the active enzyme to its inactive form would be sufficient to explain its inhibitory effect on the 5-lipoxygenase. However, our data also suggest the possibility that L-656,224 may irreversibly inactivate the enzyme, or accelerate the inactivation process that normally occurs during the 5-lipoxygenase reaction, since some reactive species derived from the inhibitor can covalently modify 5-lipoxygenase (as well as other proteins). In this regard, it is interesting to note that the data in Fig. 5A suggest that, when the 5-lipoxygenase reaction was initiated in the presence of both fatty acyl hydroperoxide and L-656,224, most of the inhibitor was destroyed prior to the bulk of the product formation. Thus it is possible that, under these conditions, a rapid reaction with L-656,224 led to an inactivation of a portion of the 5-lipoxygenase, and that the product that was subsequently synthesized resulted through the action of the remaining active enzyme.

Obviously, these experiments were carried out using cell-free extracts or purified enzyme under conditions that would not occur in an intact cell. It is not known how 5-lipoxygenase becomes oxidatively activated intracellularly, but one might expect that high concentrations of hydroperoxy fatty acids would not accumulate. Thus we cannot say to what extent rapid degradation of L-656,224 and subsequent protein alkylation would occur *in vivo*, although considerations are of importance for the future design of 5-lipoxygenase inhibitors intended for clinical use.

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