Effect of 4-Hydroxy-2(*E*)-nonenal on Soybean Lipoxygenase-1

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ABSTRACT: The oxidation of linoleic acid by soybean lipoxygenase-1 (LOX-1) was inhibited in a time-dependent manner by 4-hydroxy-2(*E*)-nonenal (HNE). Kinetic analysis indicated the effect was due to slow-binding inhibition conforming to an affinity labeling mechanism-based inhibition. After 25 min of preincubation of LOX-1 with and without HNE, Lineweaver-Burk reciprocal plots indicated mixed noncompetitive/competitive inhibition. Low concentrations of HNE influenced the electron paramagnetic resonance (EPR) signal of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPODE)-generated Fe^{3+}-LOX-1 slightly, but higher concentrations completely eliminated the EPR signal indicating an active site hindered from access by 13-HPODE. HNE may compete for the active site of LOX-1 because its precursor, 4-hydroperoxy-(2*E*)-nonenal, is a product of LOX-1 oxidation of (3*Z*)-nonenal. Also, it was an attractive hypothesis to suggest that HNE may disrupt the active site by forming a Michael adduct with one or more of the three histidines that ligate the iron active site of LOX-1.

Paper no. L8721 in *Lipids 36,* 623–628 (June 2001).

In the animal kingdom it is generally acknowledged that 4-hydroxy-2(*E*)-nonenal (HNE) originates from lipid peroxidation of n-6 polyunsaturated fatty acids (1). Recently, specific autoxidative pathways from both 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPODE) and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPODE) have been defined (2). Once formed, HNE has been documented to act as a lipid signal (3). Involvement of HNE in mammalian pathologies is diverse, including Alzheimer's disease (4), Parkinson's disease (5), cancer (6), atherosclerosis (7), sporadic amyotrophic lateral sclerosis (8), and alcoholic liver disease (9).

In plants, a metabolic pathway has been found in faba beans and soybeans that involves a sequence of 9-lipoxygenase oxidation of linoleic acid to 9-HPODE, hydroperoxide lyase cleavage of 9-HPODE to give 3(*Z*)-nonenal, and lipoxygenase and hydroperoxide peroxygenase action on 3(*Z*)-nonenal to produce HNE (10–12). However, there is evidence that the last step of oxidation of 3(*Z*)-nonenal readily occurs by autoxidation, even with plant enzymes (13). Since soybean lipoxygenase-1 (LOX-1) oxidized 3(*Z*)-nonenal stereospecifically to 4(*S*)-hydroperoxy-2(*E*)-nonenal, it is obvious that HNE can access the active site of LOX-1 and other lipoxygenase isozymes (12). Three essential histidines ligate the iron active site of LOX-1 (14). It has been shown that HNE forms a Michael adduct with histidine residues (15,16), thereby implying that HNE can react with and cause suicide inhibition of LOX-1 by compromising the active site. In addition, it has been shown that HNE can react with other amino acid residues, such as cysteine (17) and lysine (18), which also can contribute to the inactivation of LOX-1. The literature abounds with other examples of enzyme inactivation or activation by HNE. Those enzymes inactivated include glucose-6-phosphate dehydrogenase (19), microsomal cytochrome P450 (20), Na^+-K^+-ATPase (21), aldose reductase (22), cytochrome C oxidase (23), α-ketoglutarate dehydrogenase (24), pyruvate dehydrogenase (24), plasma membrane (Ca^{2+} + Mg^{2+})-ATPase (25), glutathione peroxidase (26), and glyceraldehyde-3-phosphate dehydrogenase (27). Interestingly, multicatalytic proteinase, which clears cells of oxidized protein, is inhibited by HNE-cross-linked protein (28), implying a mechanism for accumulation of age-related lipofuscin. Enzymes activated by HNE are those involved in lipid signaling, phospholipase D (29), and phosphoinositide-specific phospholipase C (30).

In this work we demonstrate that HNE inactivates LOX-1 by a mixed noncompetitive/competitive mechanism and that at least part of the competitive mechanism may be due to the decomposition of HNE in buffer.

MATERIALS AND METHODS

Materials. HNE was synthesized by a modification of a previously described method (31). Instead of oxidizing 3,4-epoxynonan-1-ol to the aldehyde by periodinane, the oxidation of Ratcliffe and Rodehorst was used (32), making certain to wash the ethyl ether solution of product HNE with NaOH, HCl, and $NaHCO_3$ solutions as prescribed by the method. The product HNE was somewhat less pure than that obtained with periodinane. Thus, HNE was purified by silicic acid (100 mesh; Mallinckrodt, Phillipsburg, NJ) column chromatography (40 g, column i.d. 2.5 cm) by sequentially eluting with 300 mL each of 7.5, and 10% acetone in hexane. HNE eluted between 200 and 300 mL in 95.5% purity. Linoleic acid was obtained from Nu-Chek-Prep (Elysian, MN). LOX-1 was isolated as previously described (33) and was stored at 3°C as a suspension in 2.3 M $(NH_4)_2SO_4$; protein concentration was 34.5 mg/mL. 13-HPODE was prepared by the method of Gardner (34).

Time-dependent inactivation of LOX-1 by HNE. The oxygen electrode assay (Gilson 5/6H oxygraph, Middleton, WI)

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Abbreviations: cmc, critical micelle concentration; EPR, electron paramagnetic resonance; HNE, 4-hydroxy-2(*E*)-nonenal; 9-HPODE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HPODE, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; LOX-1, soybean lipoxygenase-1.

was completed in a 2.4-mL cell held at 25˚C. The cell was filled with 0.1 M potassium borate buffer (pH 9.86) containing 0.36 μg LOX-1/mL. For zero-time measurement, 10 μL of HNE dissolved in methanol and 10 μL linoleic acid substrate (240 mM) in methanol were injected into the cell in rapid sequence. The final concentration of linoleic acid was 1 mM. Various concentrations of HNE were tested, 10 μL of 4.8, 15, 24, 30, 60, 120, and 240 mM in methanol per 2.4 mL cell, giving final concentrations of 0.02, 0.0625, 0.1, 0.125, 0.25, 0.5, and1 mM, respectively. For the measurements requiring precise timed incubation of HNE with the LOX-1 borate solution, a batch solution was prepared [24 mL 0.1 M potassium borate buffer (pH 9.86) containing 0.36 μg LOX-1/mL was stirred with 100 μL of methanolic HNE solution at the various concentrations described above]. The batch solution was incubated at 25°C for the prescribed time of 5.5, 11, 17, 30, 55, and 90 min, after which 2.4 mL was transferred to the oxygen electrode cell and 10 μL of 240 mM methanolic linoleic acid was injected into the cell to start the oxygen uptake measurement.

The control measurements were the same as described above, except the same volume of methanol replaced the methanolic HNE solution.

Lineweaver-Burk kinetic analyses of HNE inhibition. LOX-1 activity assays were measured by oxygen electrode without or with HNE inhibitor after preincubation for exactly 25 min at 25°C by adding either 0.417% methanol (10 μL methanol/2.4 mL LOX-1 solution) or 0.417% methanolic HNE solution (methanolic HNE tested were 30, 60, and 120 mM, giving 0.125, 0.25, and 0.5 mM final concentration, respectively). The LOX-1 solution was 0.97 μg LOX-1/mL in 0.1 M potassium borate (pH 9.86). After the 25-min preincubation, 2.4 mL was transferred to the oxygen electrode cell and 10 μL methanolic linoleic acid was injected to start the reaction. Methanolic linoleic acid was injected at concentrations of 24, 12, 8, 6, 4.8, or 4 mM giving final concentrations of 0.1, 0.05, 0.0334, 0.025, 0.02, or 0.01667 mM, respectively, all of which are below the critical micelle concentration (cmc) of linoleic acid at pH 9.86 (35). As many as 10 replicates were determined for the "without HNE" activities, and "with HNE" activities were replicated four to eight times. Lineweaver-Burk reciprocal data were plotted and interpreted according to Dixon and Webb (36).

Electron paramagnetic resonance (EPR) analysis of Fe^{3+}-LOX-1 in presence of HNE. A LOX-1 stock solution (109 μM) in potassium borate buffer (0.1 M, pH 9.9) was prepared. To 120 μL aliquots of this enzyme stock, dilute solutions of HNE in ethanol (20 μL) were added such that the final concentrations of HNE were 0, 0.21, and 1.57 mM. These solutions were incubated for 1 h (25°C), at which point the enzyme was oxidized through addition of a 37 μL aliquot of 13-HPODE (2 mM) in methanol. Samples were immediately frozen in liquid nitrogen and EPR spectra acquired at 77 K in a quartz finger Dewar flask. The samples (177 μL) analyzed as follows: LOX-1 (74 μM), 13-HPODE (418 μM), and HNE = 0, 0.17, and 1.24 mM. All spectra were acquired on a Bruker ESP-300E X-band spectrometer. Key parameters were center field 130 mT, sweep width 100 mT, microwave power 63 mW, modulation amplitude 1.2 mT, and sweep time 80 s.

Analyses of HNE decrease in buffered solutions. HNE was trapped as the benzoxime and analyzed by flame-ionization detection gas chromatography as the trimethylsilyloxy ether as described (10). Methanolic HNE (15 μL of 60 mM HNE) was added to 3 mL 0.1 M potassium borate buffer (pH 9.86) or 3 mL potassium borate buffer containing 1.04 mg LOX-1. These solutions were incubated at 25°C, and 0.5 mL aliquots were taken at 0, 10, 40, 90, and 120 min to make the *O*-benzoxime derivative of HNE. Aliquots were added to 0.1 mL reagent containing 50 mM *O*-benzylhydroxylamine-HCl in 100 mM Na piperazine-*N,N′*-bis-(2-ethanesulfonate) (pH 6.5) and 0.4 mL methanol. After permitting the reagent to react for 10 min, 27.5 μg methyl nonadecanoate was added as an internal standard, and the derivative was extracted with 1 mL chloroform. The chloroform extract was evaporated with a stream of nitrogen and reacted with trimethylchlorosilane/hexamethyldisilazane/dry pyridine (3:2:2, by vol). After 10 min, the reagent was evaporated with a stream of nitrogen and taken up in 100 μL hexane for gas chromatographic analysis as reported previously (11). Values were relative based on 100% HNE content at zero time.

RESULTS AND DISCUSSION

An examination of the persistence of HNE in 0.1 M borate buffer (pH 9.86) revealed the loss of HNE (Table 1) with the appearance of seven unidentified compounds having a greater retention time by gas chromatography. The four main compounds had related mass spectra indicating that they were isomeric. The disappearance of HNE was not markedly affected by the presence of LOX-1, which was not too surprising as the concentration of LOX-1 was many orders of magnitude less than HNE. That is, HNE was present at 0.3 mM, and LOX-1 was only at 0.011 μM. Since HNE was present at only 57% of its original concentration after 40 min, the potential effect of HNE on LOX-1 was diminished at longer times. However, during short incubation times, when the rate of inhibition was greatest (Fig. 1), the amount of HNE was only slightly depleted. At 5.5 and 11 min, the amount of HNE was projected to be about 95 and 91%, respectively, at which time the rate of inhibition was the greatest.

TABLE 1
Loss of HNE with Time When Incubated in 0.1 M Potassium Borate Buffer (pH 9.86) at 25°C With or Without LOX-1[a]

	Percentage, relative to zero time	
Time (min)	Without LOX-1	With LOX-1
0	100	100
10	86.4	92.8
40	57.3	55.7
90	27.3	25.1
120	26.3	18.8

[a]The concentrations of 4-hydroxy-2(*E*)-nonenal (HNE) and soybean lipoxygenase-1 (LOX-1) were 0.3 mM and 0.35 mg/mL, respectively.

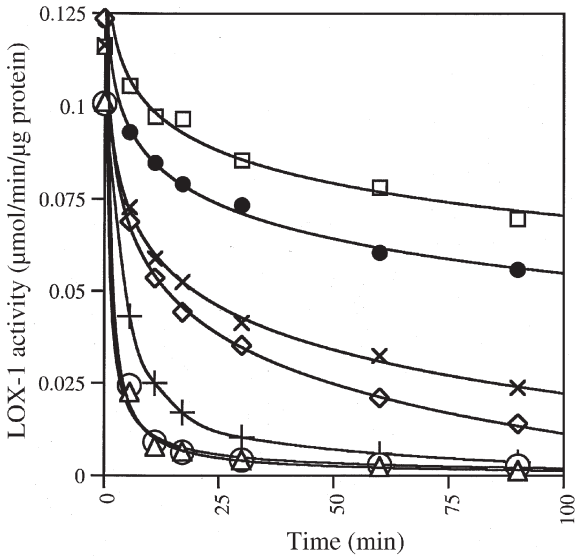

FIG. 1. Time-dependent inactivation of soybean lipoxygenase-1 (LOX-1) activity by various concentrations of 4-hydroxy-2(*E*)-nonenal (HNE). □, Control containing methanol only in place of methanolic HNE; ●, 0.02 mM HNE; ×, 0.0625 mM HNE; ◇, 0.125 mM HNE; +, 0.25 mM HNE; ○, 0.5 mM HNE; △, 1 mM HNE. All points represent mean values of replicates. The range in coefficient of variation in percentages was as follows: control, 13 to 18 (n = 19); 0.02 mM HNE, 3 to 31 (n = 4); 0.0625 mM HNE, 5 to 18 (n = 3); 0.125 mM HNE, 9 to 36 (n = 3); 0.25 mM HNE, 10 to 26 (n = 3); 0.5 mM HNE, 0 to 28 (n = 3); and 1 mM HNE, 22 to 81 (n = 3).

It was determined that HNE in concentrations ranging between 20 μM and 1 mM was an effective inhibitor of LOX-1 activity as assayed with 1 mM linoleic acid (Fig. 1). Previous studies with other enzymes showed that the effective concentrations of HNE required for inhibition ranged from a low for cytochrome P450 (0.24 μM to 0.8 mM) (20) and Na^+-K^+-ATPase (1 μM to 1 mM) (21) to a high for glucose-6-phosphate dehydrogenase (1 to 8 mM) (19). Other enzymes were reported to be effective at HNE concentrations between the foregoing values; thus, the inhibition of LOX-1 by 20 μM to 1 mM was comparable to the effect on many other enzymes. Interestingly, we did not see inhibition when HNE and linoleic acid were added simultaneously; that is, no significant slowing of the rate was seen over the course of the assay of 4 to 5 min. This result indicated that linoleic acid was a more effective competitor for the active site than HNE. However, when HNE was incubated with LOX-1 for a period of time in the absence of linoleic acid, subsequent activity with linoleic acid substrate was markedly reduced. After the data shown in Figure 1 were compensated for the control, the loss of activity was found to be complete after about 17 min of incubation with HNE.

The data presented in Figure 1 are indicative of a slow-binding inhibitor. As outlined by Copeland (37), slow-binding inhibitors are categorized into three types, which can be distinguished by kinetic analysis. The data derived from Figure 1 best fit the "affinity labeling and mechanism-based inhibition" according to Scheme 1, where E, enzyme; k, rate constant; and [I], inhibitor.

$$E \underset{k_4}{\overset{k_3[I]}{\rightleftharpoons}} E[I] \xrightarrow{k_5} E\text{-}I$$

SCHEME 1

For this type of inhibitor the rate equation is:

$$k_{obs} = \frac{k_5[I]}{K_i + [I]} \quad [1]$$

where k_{obs}, apparent (observed) rate constant; K_i, apparent concentration of inhibitor required to reach the half-maximal rate of inactivation, and [I], inhibitor concentration.

Since this rate equation is reminiscent of the Michaelis-Menton equation, a reciprocal plot of k_{obs} vs. [I] ideally should give a linear plot. After correcting for the control, exponential extrapolation of the rate of inactivation obtained during the first 11 min (Fig. 1) k_{obs} was derived from $0.693/t_{1/2}$ min. The rate for 1 mM HNE was omitted as being too rapid to obtain reasonable data. The linear reciprocal plot shown in Figure 2 with a nonzero intercept conforms to the inhibitor type shown in Scheme 1. The intercept with the y-axis gave the 1/maximal rate of k_5, or the maximal rate of inactivation, $k_{inact.}$, that is, $1/k_{inact.} = 5$ or $k_{inact.} = 0.2$ min^{-1}. Intersection of the plot with the x-axis gave $-1/K_i = -1/3.4$ or $K_i = 0.294$ mM. This type of inhibition is reasonable in view of the fact that HNE is known to form Michael adducts with cysteine (17), lysine (18), and histidine (15,16). It is noted that LOX-1 possesses three essential histidines that ligate the iron active site. It is known from the literature that 4(*S*)-hydroperoxy-2(*E*)-nonenal is a LOX-1 product of 3(*Z*)-nonenal oxidation (12); thus, it seems reasonable that the smaller and structurally related HNE should be capable of accessing the active site.

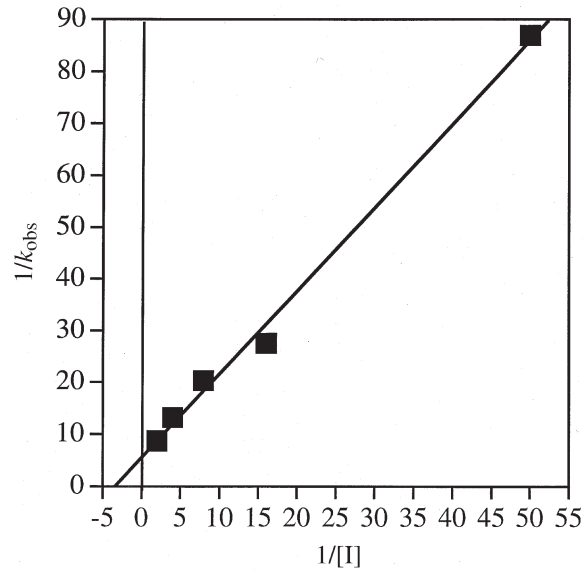

FIG. 2. Reciprocal plot of apparent (observed) rate constant, k_{obs} (min^{-1}), as a function of inhibitor [I] concentration (mM). Intercept with the x-axis is equivalent to $-1/K_i$, and the intercept with the y-axis is $1/k_{inact}$ (reciprocal of the maximal rate of inactivation). Derived from the data shown in Figure 1.

For this reason, Lineweaver-Burk kinetic analyses of the inhibitor effect of HNE were completed to confirm the foregoing assessment. In order to determine these data, LOX-1 was preincubated with HNE for 25 min, a time determined to be safely beyond the effective time required for inhibition; however, after 25 min the concentration of HNE was reduced to about 75% of its original value by decomposition in buffer. Also, all activity assays had to be completed below the cmc concentration of linoleic acid, because the true substrate of LOX-1 has been determined to be monomeric linoleic acid, not substrate trapped in micelles (38). This severely restricts the usable range of substrate concentrations, but reasonable data can be collected by the use of replicates. Thus, the K_M of LOX-1 was determined to be 23 μM (Fig. 3), which compares with 15 ± 3 μM reported by Schilstra *et al*. (39). Reciprocal plot analyses in the presence of various concentrations of HNE revealed mixed competitive/noncompetitive inhibition at the two lowest concentrations of HNE tested. Since 3(*Z*)-nonenal served as a substrate of LOX-1 and 4(*S*)-hydroperoxy-2(*E*)-nonenal was a product (12), it was expected that HNE would occupy the active site of LOX-1. The competitive aspect of HNE can be readily explained. The noncompetitive nature of the inhibition was expected due to the slow-binding kinetic analysis outlined above. Surprisingly, at the highest inhibitor concentration tested (0.5 mM), where one would expect the greatest degree of noncompetitive inhibition, the plot showed an almost classical competitive inhibition. In fact, the trend toward competitive inhibition occurred as the inhibitor concentration progressively increased.

This observation of classical competitive inhibition at 0.5 mM HNE does not have a straightforward explanation. Some competitive inhibition could be caused by decomposition products of HNE, which would afford higher concentrations after 25 min preincubation with 0.5 mM HNE. To test this possibility, HNE was preincubated in buffer for 2 to 4 h before testing for its effect on LOX-1 activity. Pretreated HNE was then incubated with LOX-1 for 10 min prior to adding 1 mM linoleic acid. Preincubated HNE, 0.5 and 1 mM, inhibited LOX-1 at 17 and 58%, respectively, compared to controls. Little significant difference in activity could be seen with the time of preincubation between 2 and 4 h. By contrast, a 10 min exposure of LOX-1 to HNE (not preincubated in buffer) at 0.5 and 1 mM inhibited activity 73 and 85%, respectively. As seen in Table 1, the amount of HNE remaining after 2 h preincubation in buffer was less than 25%. It is conceivable that the inhibition observed with preincubation is partly due to residual HNE, but the lack of significant effect over time is suggestive of the contribution from degraded HNE. It is conceivable that the competitive aspect of the Lineweaver-Burk plots observed at higher HNE concentrations was due to degraded HNE from 25 min preincubation of HNE. Nevertheless, some degree of mixed competitive/noncompetitive inhibition should be expected.

Disruption of the active site was supported by EPR results. An EPR signal of LOX-1 was generated by oxidizing native Fe^{2+}-LOX-1 to Fe^{3+}-LOX-1 by exposing the enzyme to its product, 13-HPODE, followed by immediate liquid N_2 freezing (Fig. 4). In the presence of low HNE concentrations (0.21 mM incubation concentration, 0.17 mM after 13-HPODE addition) the EPR signal from oxidized LOX-1 was slightly affected. A 22% decrease in peak-to-peak width of the $g_\perp$ (g = 6) signal was observed: 11.3mT in the absence of HNE and 8.8 mT in the presence of HNE. In view of the half-maximal inhibitor concentration (K_i) for HNE of 0.294 mM (Fig. 2), this result was not unreasonable. When the concentration of HNE was increased (1.57 mM incubation concentration, 1.24 mM after 13-HPODE addition), the EPR signal completely disappeared. Since 1.24/1.57 mM HNE is substantially greater than K_i, complete inhibition was anticipated; however, the relatively low HNE/LOX-1 incubation ratio of 21 may have depleted the actual HNE concentration by noninhibitory reactions with LOX-1 to some extent. In the present EPR study, the HNE/LOX-1 incubation ratio of 21 compared to literature HNE/protein ratios of 15–143 that were utilized to modify histidine residues (15). From these observations it was concluded that HNE caused the active site to be sufficiently hindered to prevent oxidation of Fe^{2+}-LOX-1 to Fe^{3+}-LOX-1 by 13-HPODE.

A potential physiological function of HNE is suggested by the results. It is well known that pathogens activate the oxylipin pathway (e.g., Ref. 40). More specifically, Deighton *et al*. (41) found that HNE accumulates at levels as high as 19,000 pmol/g tissue at the site of fungal infection of sweet peppers. In certain experiments HNE was found at even higher levels of 30,000 pmol/g (Deighton, N., unpublished data). Because of HNE reaction with protein and/or glutathione, it is plausible that the amount produced was actually higher. The concentrations found by Deighton translate

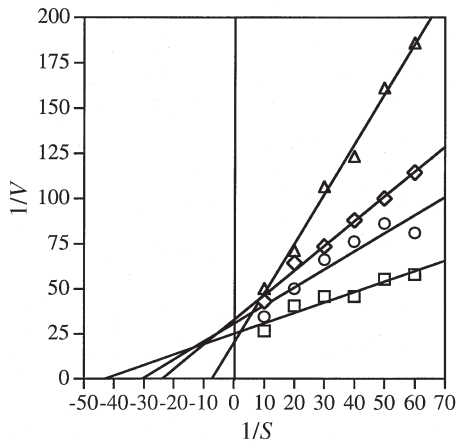

FIG. 3. Lineweaver-Burk reciprocal plots of LOX-1 activity with and without HNE inhibitor. Preincubation with or without HNE was 25 min at 25°C before measurement of activity with linoleic acid. Reciprocals are activity, *V*, in μmol/min/μg LOX-1, and linoleic acid substrate, *S*, in mM. □, Activity without HNE; ○, activity with 0.125 mM HNE ◇, activity with 0.25 mM HNE; and △, activity with 0.5 mM HNE. All points are mean values of replicates. The ranges in coefficient of variation in percentages were as follows: "without HNE," 14 to 35 (n = 10); 0.125 mM HNE, 9 to 22 (n = 4); 0.25 mM HNE, 11 to 24 (n = 6); 0.5 mM, 7 to 22 (n = 4). For abbreviations see Figure 1.

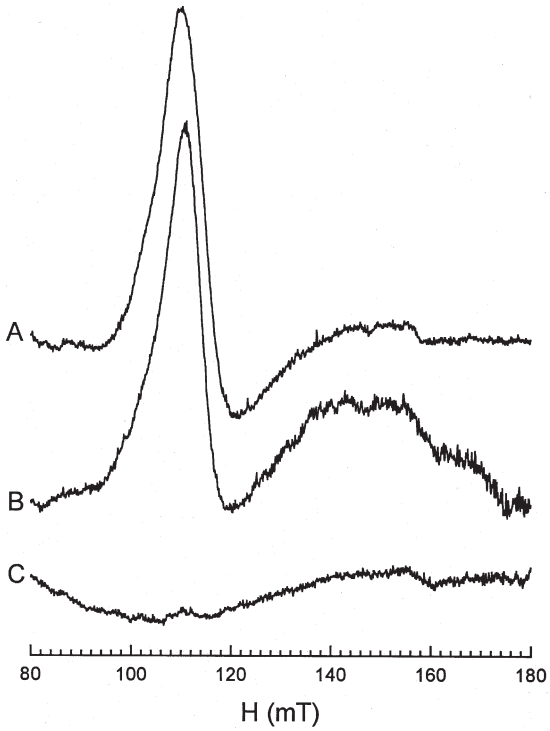

FIG. 4. Electron paramagnetic resonance spectra of oxidized LOX-1. The enzyme was oxidized by 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid in the absence (A) and presence of HNE at 0.17 (B) and 1.24 mM (C). For abbreviations see Figure 1.

to 0.019 to 0.03 mM, which brackets the lowest concentration of HNE tested in this study (Fig. 1). Thus, it seems possible that the downstream oxylipin HNE, and possibly related oxylipins, may serve to switch off the lipid signal pathway through inactivation of one of the initiating enzymes. Because linoleic acid effectively competes for the active site, this "feedback suicide" of LOX is suggested to occur only after substrate depletion.

Further research is planned to locate the specific site of LOX-1 inactivation by HNE.

ACKNOWLEDGMENT

The Scottish Crop Research Institute is grant-aided by the Scottish Executive Rural Affairs Department.

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[Received December 22, 2000, and in final revised form and accepted May 14, 2001]