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With Anandamide as Substrate Plant 5-Lipoxygenases Behave like 11-Lipoxygenases

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Anandamide, an endogenous ligand for cannabinoid receptors CB1 and CB2, was incubated with purified 5-lipoxygenases from barley and tomato. This yielded 11*S*-hydroperoxy-5,8,12,14-eicosatetraenoylethanolamide (11*S*-HPANA) as major product (about 70%). This is in contrast with the dioxygenation of arachidonic acid, where 5*S*-HPETE is the major product. This observation implies that the regiospecificity of the dioxygenation, catalyzed by nonmammalian 5-lipoxygenases, is altered by a modification at the carboxylic end of the substrate. Soybean 15-lipoxygenase forms 15*S*-HPANA (95%) and 11*S*-HPANA (5%), and in the second dioxygenation 5,15-diHPANA (45%) and 8,15-diHPANA (55%) are formed. Apparently, the regiospecificity of the soybean 15-lipoxygenase reaction is only slightly affected using anandamide as substrate.

In the early nineties anandamide (arachidonylethanolamide) was isolated from porcine brain lipids and found to act as a ligand for cannabinoid receptors CB1 and CB2 (1,2). Although structurally distinct from cannabinoids, it shows a similar receptor binding affinity. Anandamide is an arachidonic acid derivative and thereby a substrate for lipoxygenase (3-5) and for cyclooxygenase (6). Lipoxygenases (linoleate: oxygen oxidoreductase 1.13.11.12) are a group of non-heme iron-containing enzymes which catalyze the regio- and stereo-selective dioxygenation of polyunsaturated fatty acids with a (*Z*,*Z*)-1,4-pentadiene system. Porcine leukocyte 5-lipoxygenase was found inactive towards anandamide (4). In this study the potency of non-mammalian 5-lipoxygenases, like those from barley grains and tomato fruits, to use anandamide as substrate was investigated.

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Abbreviations used: sLOX, soybean lipoxygenase-1; bLOX, barley lipoxygenase-1; tLOX, tomato lipoxygenase; SPE, solid phase extraction; RP-HPLC, reversed-phase high performance liquid chromatography; CP-HPLC, chiral-phase high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; H(P)ANA, *N*-(2-hydroxyethyl)hydro(pero)xyarachidonylamide; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; BHT, 2,6-Di-*tert*-butyl-4-methyl phenol; NDGA, nordihydroguaiaretic acid; CD, circular dichroism.

EXPERIMENTAL PROCEDURES

Materials. Arachidonic acid, NDGA and sodiumborohydride were from Sigma. Anandamide was purchased from ICN biomedicals. Pyridine, 1,1,1,3,3,3-hexamethyldisilazane, chlorotrimethylsilane and BHT were of the highest quality from Aldrich. Palladium on calcium carbonate (5% Pd) was from Acros Organics. Tetrahydrofuran, hexane and methanol were from Biosolve. Barley grains were a gift from Ms. I. Kokkelink (Heineken, Zoeterwoude, The Netherlands).

Enzyme and substrate preparations. Lipoxygenases from barley grains (Triumph), tomato fruits (Trust) and soybeans (white Hilum) were purified as described (7-9). Anandamide and arachidonic acid were purified via solid phase extraction as follows: anandamide or arachidonic acid (5 mg) was dissolved in 2 ml methanol, and 50 ml sodium borate buffer (pH 9.0) was added under continuous stirring. After lowering the pH to 4, the solution was applied to a SPE (Bakerbond, 500 mg, J. T. Baker) column. Autooxidation products were eluted with 5 ml methanol/water (80/20 v/v). Anandamide or arachidonic acid was eluted with 5 ml methanol, concentrated to 2 ml under a stream of nitrogen and BHT was added to a final concentration of 40 μM.

Metabolite generation. Each lipoxygenase was incubated with anandamide or arachidonic acid (final concentration 40 μM; 1 U lipoxygenase per 3 μmol substrate) in 100 mM sodium borate buffer (pH 9.0 for the 15-lipoxygenase and pH 7.0 for the 5-lipoxygenases). The reaction was followed spectrophotometrically at 236 nm. After completion, the pH was lowered to 4 and the products were purified with SPE (Bakerbond, 500 mg, J. T. Baker) and then analyzed by HPLC and/or GC/MS.

Spectrophotometric assay. Spectrophotometric assays were performed by continuously monitoring the change in absorbance at 236 nm on a Hewlett-Packard 8452A diode array spectrophotometer. A molar absorbance of 23000 M^{-1} cm^{-1} at 236 nm was used for H(P)ETEs and H(P)ANAs (3) and 25000 M^{-1} cm^{-1} at 234 nm for HPODs. For 8,15- and 5,15-diHPANA molar absorbances of 40000

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M^{-1} cm^{-1} at 269 nm and 33500 M^{-1} cm^{-1} at 243 nm were used respectively (10).

HPLC. Reaction products of anandamide and arachidonic acid with the various lipoxygenases were analyzed on a Hewlett-Packard 1090 LC HPLC system equipped with a HP 1040A diode array detector and an HP7994A analytical workstation. RP-HPLC was carried out on a Cosmosil 5C18 AR column (5 μm, 250 $\times$ 4.6 mm, Nacalai Tesque) with tetrahydrofuran/methanol/water/ acetic acid (25/40/35/0.1 v/v/v/v) as solvent at a flow rate of 1 ml/min. Chiral separations of the sodiumborohydride-reduced anandamide metabolites were carried out on a Chiralcel OD-R column (5 μm, 250 $\times$ 4.6 mm, Daicel) with methanol/water/acetic acid (75/25/0.1 v/v/v) as eluent at a flow rate of 0.5 ml/min. In separate experiments anandamide metabolites generated by the different lipoxygenases were isolated by RP-HPLC and reduced with an excess of sodiumborohydride at 0°C for 30 min. After lowering the pH to 4, the resulting hydroxy-anandamide metabolites were purified by SPE (Sep-Pak tC18 Vac, 50 mg, Waters), and analyzed by CP-HPLC.

Circular dichroism spectroscopy. CD-spectra of the RP-HPLC purified HANAs (about 40 μM HANA in methanol, optical pathway 1.0 cm) were recorded on a JASCO J-600 spectropolarimeter from 210 to 270 nm. Typically 10 spectra (resolution of 1 nm, scan speed 10 nm/min) were accumulated and corrected for an independently recorded baseline of 40 μM anandamide in methanol.

Gas chromatography-mass spectrometry. Anandamide metabolites prepared with bLOX and sLOX were reduced with an excess of sodiumborohydride at 0°C for 30 min and after lowering the pH to 4 purified by SPE (Sep-Pak tC18 Vac, 50 mg, Waters). The hydroxy-

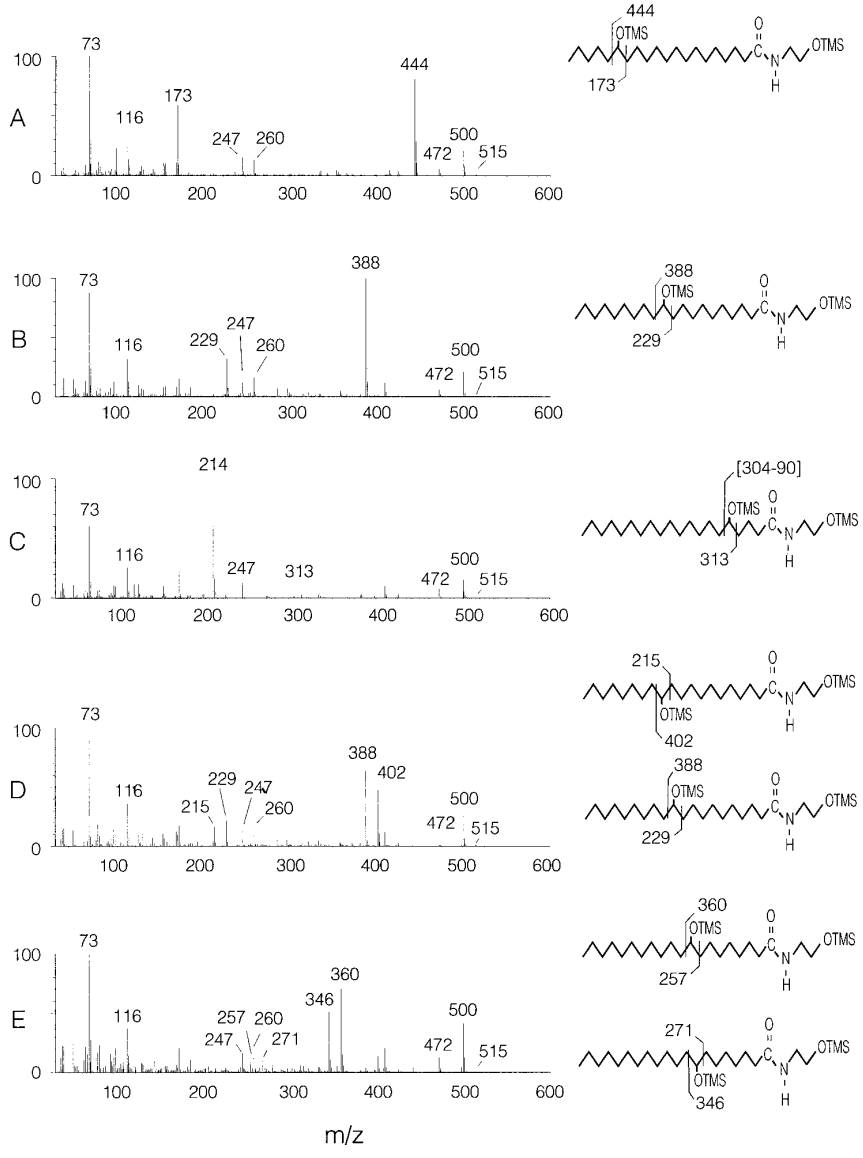

FIG. 1. Mass spectra and deduced structures of the monoHPANAs generated by the different lipoxygenases. Structures of 15-HPANA (A), 11-HPANA (B), 5-HPANA (C), 12-HPANA contaminated with 11-HPANA (D), and 8- and 9-HPANA (E) were deduced from the spectra of the reduced, hydrogenated and trimethylsilylated HPANA derivatives. Insets represent the deduced structures and their main fragmentation patterns.

TABLE 1

Relative Reaction Rates of sLOX, bLOX, and tLOX with Arachidonic Acid and Anandamide Compared with Linoleic Acid

	Enzyme		
Substrate	sLOX	bLOX	tLOX
Linoleic acid	100[a]	100	100
Arachidonic acid	160	15	12
Anandamide	88	33	15

[a] Reaction rates were measured spectrophotometrically by continuously monitoring the increase in absorbance at 234 nm for linoleic acid and at 236 nm for arachidonic acid and anandamide, with 40 μM substrate in 100 mM borate buffer (pH 9.0 for sLOX and pH 7.0 for bLOX and tLOX). The values indicate the reaction rates (linear parts of the progress curves) relative to linoleic acid.

anandamide metabolites were eluted with methanol, dried under a nitrogen stream, redissolved in 1 ml hexane and hydrogenated with a catalytic amount of palladium on calcium carbonate (5% Pd) in a hydrogen atmosphere. After 30 min, the catalyst was removed by filtration over a pre-washed (hexane) piece of cotton wool. Hexane was evaporated under a nitrogen stream and silylation reagent (50 μl; pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane (5/1/1 v/v/v)) was added. After 30 min at room temperature, the silylation reagent was evaporated under a stream of nitrogen and the residue was redissolved in 20 μl hexane. An aliquot was analyzed by GC/MS (Carlo Erba GC 8060 with a Fisons MD 800 mass detector) equipped with a CP-Sil 5 CB-MS column (25 m $\times$ 0.25 mm $\times$ 0.25 μm, Chrompack). The column temperature was held at 200°C for 1 min, increased in 13 min to 330°C and held at this temperature for 2 min. Mass spectra were recorded under electron impact with an ionization energy of 70 eV.

RESULTS

Anandamide was incubated with three plant lipoxygenases and the reactions were followed spectrophotometrically. In comparison to arachidonic acid the reaction rates of sLOX, bLOX and tLOX with anandamide were halved doubled or equal, respectively (Table 1). All reactions were inhibited in the presence of NDGA, a known LOX inhibitor (11).

The reaction products were reduced, hydrogenated and trimethylsilylated, without further purification. With GC/MS the monoHPANA derivatives are separated into three peaks which can selectively be monitored at *m/z* 500, $[M\text{-}CH_3]^+$. The first peak contains 5-HPANA, the second peak 12-, 11-, 9- and 8-HPANA, and the third peak 15-HPANA (data not shown). The mass spectra of the anandamide metabolites are given in Fig. 1 and the most abundant fragmentations are listed in Table 2. The reduced, hydrogenated and trimethylsilylated monoHPANA derivatives typically show four main ion peaks, namely, *m/z* 73, the TMS fragment, *m/z* 500, the $[M\text{-}CH_3]^+$ fragment, and two ion peaks around the dioxygenated C-atom, where the fragmentation towards the *N*-2-ethyl-OTMS part is the most abundant. The only exception is 5-HPANA, where the fragmentation towards the *N*-2-ethyl-OTMS part yields *m/z* 214 instead of *m/z* 304, due to the loss of HOTMS. The hydrogenation step in the derivatisation scheme has the advantage that the structures show characteristic ion peaks that via selective ion monitoring the different anandamide metabolites can easily be recognized.

Surprisingly, the major product of the anandamide dioxygenation by plant 5-lipoxygenases is 11-HPANA, whereas with arachidonic acid as substrate 5-HPETE is formed. Reaction products were quantified by RP-HPLC (Fig. 2, Table 3) and identified by GC/MS.

The assignment of the absolute configuration was based on CD-spectroscopy in combination with chiral separations. The CD-spectra of 15-HPANA (sLOX), 11-HPANA (bLOX), and 5-HPANA (bLOX) showed a positive Cotton effect, so they have predominantly *S*-configuration (12). On this basis chiral separations were used to obtain the accurate *R*/*S* ratios of the anandamide metabolites (Table 3). The regiospecificity of the reaction of bLOX with anandamide was not pH dependent, as incubations between pH 5.2 and 9 gave identical RP-HPLC patterns.

Soybean lipoxygenase, at relatively high enzyme concentrations, is capable of converting arachidonic acid into 8,15- and 5,15-diHPETE (10). For anandamide, comparable results have been reported based on absorbance measurements (5). Here, the two doubly dioxygenated anandamide metabolites were separated by RP-HPLC, analyzed by GC/MS and the mass spectra are shown in Fig. 3. These compounds were identified on the basis of the $[M\text{-}CH_3]^+$ fragment (*m/z* 588), and show also the characteristic ion peaks around the dioxygenated C-atoms (Table 4). The compounds were identified as 8,15- and 5,15-diHPANA and are formed in a molar ratio of 55:45 as determined by RP-HPLC (data not shown).

DISCUSSION

Fatty acid ethanolamides, containing one or more 1*Z*,4*Z*-pentadiene systems, have been described as

TABLE 2

Characteristic Mass Fragments of the Reduced, Hydrogenated, and Trimethylsilylated Anandamide Derivatives (*cf.* Fig. 1)

	Characteristic fragment ions (*m/z*)		
Anandamide metabolite	$[M\text{-}CH_3]^+$	Dioxygenated C-atom	TMS
15-HPANA	500	444; 173	73
12-HPANA	500	402; 215	73
11-HPANA	500	388; 229	73
9-HPANA	500	360; 257	73
8-HPANA	500	346; 271	73
5-HPANA	500	214; 313	73

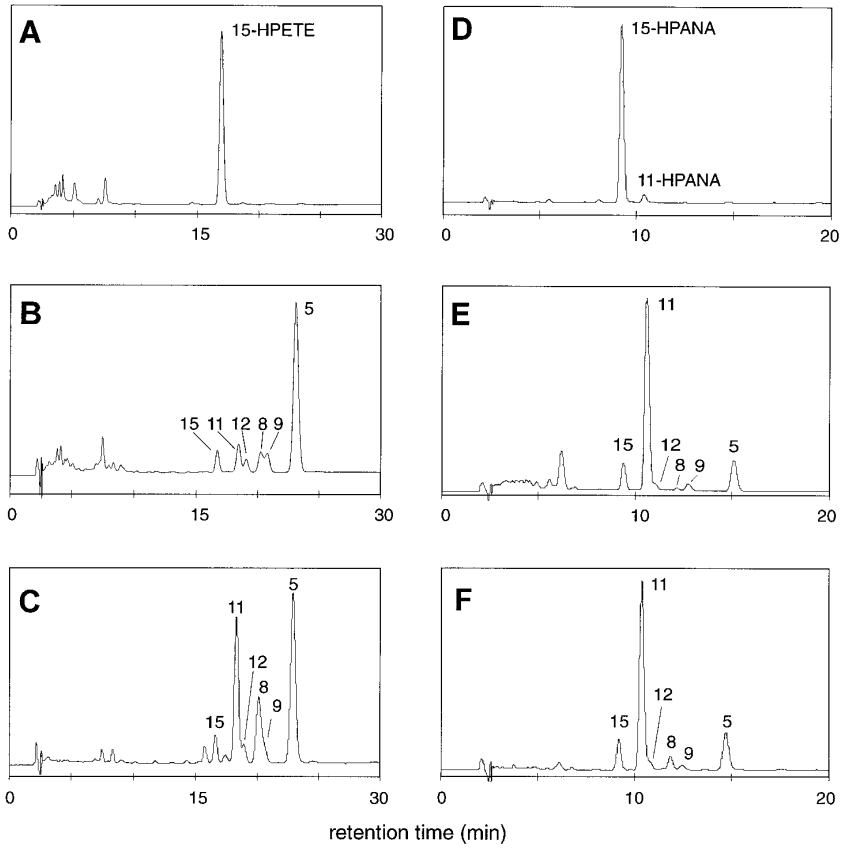

FIG. 2. Reversed-phase HPLC analysis of reaction products formed from arachidonic acid and anandamide with various lipoxygenases monitored at 236 nm. Arachidonic acid at 40 μM was incubated with sLOX (A), bLOX (B) and tLOX (C). Anandamide at 40 μM was incubated with sLOX (D), bLOX (E), and tLOX (F). The elution positions of 8- and 9-HPANA may be interchanged.

suitable substrates for lipoxygenases. Native 15-lipoxygenases from soybeans, rabbit reticulocyte, and porcine leukocyte 12-lipoxygenase show the same regiospecificity towards anandamide as with arachidonic acid (3,4). However, porcine leukocyte 5-lipoxygenase has been reported to show no activity towards anandamide

TABLE 3

The Product Specificity (Percentage) for Arachidonic Acid and Anandamide by Soybean Lipoxygenase-1 (sLOX), Barley Lipoxygenase-1 (bLOX), and Tomato Lipoxygenase (tLOX) with Enantiomeric Composition of the Major Anandamide Metabolites

Position OOH	sLOX		bLOX		tLOX	
	HPETE	HPANA	HPETE	HPANA	HPETE	HPANA
15-	100[a]	95 (95/5)[b]	6	9 (60/40)	5	10 (50/50)
12-	—	—	4	1	3	2
11-	—	5 (80/20)	10	72 (95/5)	30	65 (95/5)
9-	—	—	7	3[c]	3	2[c]
8-	—	—	7	1[c]	19	5[c]
5-	— (80/20)	—	66	14 (95/5)	40	16

[a] Isomeric ratio of the dioxygenation products by the three lipoxygenases, as determined by RP-HPLC.
[b] The enantiomeric ratios (*S*/*R*) of the main products, as determined by chiral separations in combination with CD spectroscopy.
[c] The distributions of 8- and 9-HPANA may be interchanged.

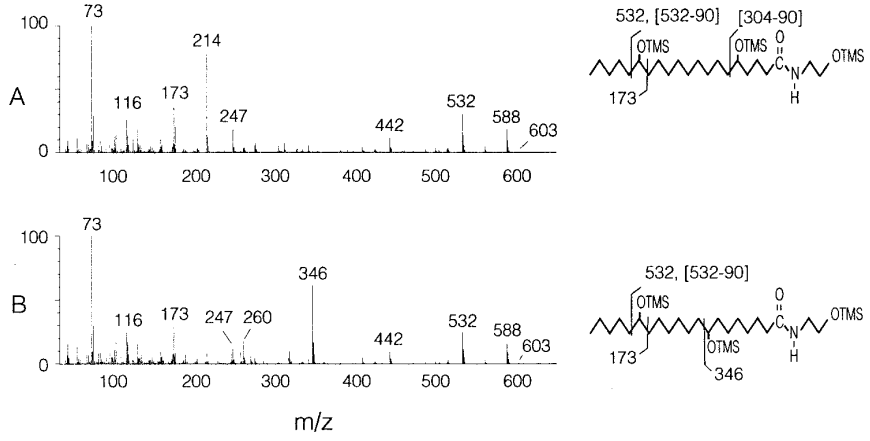

FIG. 3. Double dioxygenation of ANA by sLOX. Mass spectra of the reduced, hydrogenated and trimethylsilylated 5,15- (A) and 8,15-diHANA (B) derivatives. Insets represent the deduced structures and their main fragmentation patterns.

(4). Interestingly, we observed here that 5-lipoxygenases from barley grains and tomato fruits converted anandamide into 11*S*-hydroperoxy-5,8,12,14-eicosatetraenoylethanolamide (11*S*-HPANA). With arachidonic acid as substrate these 5-lipoxygenases yield 5*S*-HPETE as the major product, while they are inactive towards methyl arachidonate. This implies that with anandamide as substrate for plant 5-lipoxygenases hydrogen abstraction occurs at C-13, whereas with arachidonic acid it occurs at C-7.

In a study with four different *N*-linoleoyl amides, the dioxygenation by soybean 15-lipoxygenase showed similar regio- and stereoselectivities, compared to linoleic acid (13). The results reported here show that also the double dioxygenation of anandamide by soybean 15-lipoxygenase is similar to that of arachidonic acid.

Recently, the X-ray crystallographic data of a mammalian 15-lipoxygenase have been published together with a hypothesis to explain the positional specificity of mammalian lipoxygenases (14,15). In this model the substrate is oriented with its methyl end towards the active site for all different lipoxygenases. Our data show that anandamide enters the active site of the plant 5-lipoxygenases, but the observed regioselectivity suggest that the presence of the ethanolamide group prevents the substrate from penetrating deeper into the active site cavity, to allow the C-5 regioselectivity.

The observations reported here demonstrate that the regiospecificity of the 5-lipoxygenase reaction changes through modification of the carboxylic terminus of the substrate, while it has been shown earlier to change after site-directed mutagenesis (16).

TABLE 4

Characteristic Mass Fragments of the Reduced, Hydrogenated, and Trimethylsilylated Doubly Dioxygenated Anandamide Metabolites (*cf.* Fig. 3)

	Characteristic fragment ions (*m/z*)		
Anandamide metabolite	$[M\text{-}CH_3]^+$	Dioxygenated C-atoms	TMS
8,15-diHPANA	588	532; 442; 346; 173	73
5,15-diHPANA	588	532; 442; 214; 173	73

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