Dioxygenation of *N*-linoleoyl amides by soybean lipoxygenase-1

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Abstract Anandamide, a novel neurotransmitter, has been reported to be dioxygenated by brain lipoxygenase [1,11]. Anandamides constitute a new class of neuroregulatory fatty acid amides. However, little is known about the enzymatic dioxygenation of these lipids. Therefore, we have tested several members of the neuroactive fatty acid amide class containing a 1*Z*,4*Z*-pentadiene system whether they could be dioxygenated by soybean lipoxygenase-1, which is a model enzyme for mammalian lipoxygenases. In this study it was found that lipoxygenase-1 converts *N*-linoleoylethanolamide (ODNHEtOH), *N*-linoleoylamide ($ODNH_2$), *N*-linoleoylmethylamide (ODNHMe) and *N*,*N*-linoleoyldimethylamide ($ODN(Me)_2$ into 13-(*S*)-hydroperoxy-9*Z*,11*E*-octadeca-9,11-dienoyl amides derivatives. The apparent K_m values for ODNHEtOH (23.6 ± 3.7 μM), $ODNH_2$ (8.60 ± 0.65 μM) and linoleic acid (OD: 8.85 ± 0.74 μM) are not significantly different. The k_{cat} for $ODNH_2$ (32.4 ± 1.2 s^{-1}) is twice as small as compared to the turnover numbers of the other substrates, viz. ODNHEtOH (61.6 ± 5.0 s^{-1}) and OD (54.4 ± 2.0 s^{-1}). The results suggest that *N*-linoleoyl ethanolamide and *N*-linoleoyl amide can be readily converted by lipoxygenases in vivo.

Key words: Cannabinoid; Fatty acid amide; *N*-Linoleoyl amide; *N*-Linoleoyl ethanolamide; Lipoxygenase

1. Introduction

Over the last 4 years several findings have led to the assignment of fatty acid amides as a novel class of neuroregulatory molecules. These lipid derivatives may consist of a broad range of fatty acid amides, which vary in polar headgroup, alkyl chain length, degree and position of unsaturation. (1) *N*-Arachidonylethanolamide (anandamide, 20:4), a novel neurotransmitter [2], binds to the cannabinoid binding receptor 1 (CB1), mimicking the pharmacological effects of Δ^9-tetrahydrocannabinol, the active compound in marijuana [3]. (2) Palmitoylethanolamide (16:0) binds to the cannabinoid binding receptor 2 (CB2) and down-modulates mast cell activation [4]. (3) *cis*-9-Octadecanamide (oleamide, 18:1) induces sleep at nanomolar quantities in rats and humans [5].

N-Acylethanolamides, including *N*-linoleoylethanolamide (18:2), have been shown to be enzymatically synthesized by the Ca^{2+}- and THC-induced hydrolytic action of phospholipase D on *N*-acylphosphatidylethanolamides [6]. In addition, the hydrolysis of the amide function of anandamide and oleamide has been reported to occur by the same fatty acid amide hydrolase [7]. The hydrolysis of these fatty acid amides was competitively inhibited by the *N*-linoleoylethanol amide and *N*-linoleoylamide [8,9].

Though anandamide was shown to be oxidized by P_{450}-cytochrome oxidase [10] and brain lipoxygenases [1,11], little is known about the oxidative metabolism of these amides. The brain lipoxygenase product 12-hydroxy-eicosatetraenoyl ethanolamide was shown to bind to the CB1 receptor. [1]

Lipoxygenases are non-heme iron containing dioxygenases which catalyse the conversion of substrates containing one or more 1*Z*,4*Z*-pentadiene systems into 1-hydroperoxy-2*Z*,4*E*-pentadiene derivatives. This occurs in a regio- and stereospecific manner. Mammalian lipoxygenases convert archidonic acid into precursors for lipoxins and leukotrienes which are important molecules in inflammatory processes and immune response [12]. In plants, the role of lipoxygenases has not been unambiguously established. Soybean lipoxygenase-1 is easily obtained in quantities that allow extensive structural and mechanistic studies. Because of structural and functional similarities between lipoxygenases from various sources, soybean lipoxygenase-1 can serve in many respects as a model system for other lipoxygenases [13].

The finding that anandamide is converted in a similar fashion as arachidonic acid [1], prompted us to investigate whether other 1*Z*,4*Z*-pentadiene containing members of the fatty acid amide class may act as substrates of lipoxygenase. Therefore, we have conducted a study in which four *N*-linoleoyl amides with different amide headgroups were dioxygenated by soybean lipoxygenase-1. The substrates studied were *N*-linoleoyl ethanolamide, *N*-linoleoyl amide, *N*-linoleoyl methylamide and *N*,*N*-linoleoyl dimethylamide. Here, we present the characterisation of the products and the regio- and stereospecifity, as well as the kinetic parameters of the dioxygenation of these amides.

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Abbreviations: CB1/2, cannabinoid binding receptor 1 or 2; CD, circular dichroism; CP-HPLC, chiral-phase high-performance liquid chromatography; 2D-NMR, 2-dimensional nuclear magnetic resonance; FPLC, fast protein liquid chromatography; FT-IR, Fourier transform infrared (spectrometry); GC-MS, gas chromatography-mass spectrometry; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance; 13-HOD, 13-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoic acid; 13-HODNH_2, 13-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl-*N*-amide; 13-HODNHEtOH, 13-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl-*N*-ethanolamide; 13-HODNHMe, 13-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl-*N*-methylamide; 13-HODN(Me)_2, 13-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl-*N*,*N*-dimethylamide; LOX-1, lipoxygenase-1; OD, 9*Z*,12*Z*-octadecadienoicacid (linoleic acid); $ODNH_2$, 9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-amide; ODNHEtOH, 9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-ethanolamide; ODNHMe, 9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-methylamide; $ODN(Me)_2$, 9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*,*N*-dimethylamide; RP-HPLC, reversed-phase high-performance liquid chromatography; UV, ultraviolet (spectrophotometry); THC, tetrahydrocannabinol; TOCSY, **to**tal **c**orrelation **s**pectroscop**y**

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2. Materials and methods

2.1. Materials

Linoleic acid, (9*Z*,12*Z*-octadeca-9,12-dienoic acid, OD, 99% pure) was obtained from Sigma. *N*-linoleoylethanolamide, (9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-ethanolamide, ODNHEtOH), *N*-linoleoylamide (9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-amide, $ODNH_2$), *N*-linoleoyl methylamide (9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*-methylamide, ODNHMe) and *N*,*N*-linoleoyl dimethylamide (9*Z*,12*Z*-octadeca-9,12-dienoyl-*N*,*N*-dimethylamide, $ODN(Me)_2$) were synthesised, characterised (purity: >96% by gas–liquid chromatography, except for $ODN(Me)_2$: 86.5%) and kindly provided by Dr. G. Scheller (Bijvoet Center, Department of Bio-organic Chemistry, Utrecht University). Solutions of OD (215 and 4.0 mM), ODNHEtOH (387 and 4.0 mM), $ODNH_2$ (420 and 4.0 mM), ODNHMe (290 and 4.0 mM) and $ODN(Me)_2$ (380 and 4.0 mM) were stored in methanol (Merck, HPLC-grade) under N_2 at −25°C until use. All other reagents used were of the purest grade available.

2.2. Purification of soybean lipoxygenase-1

Lipoxygenase-1 was purified from soybean (Maple Glen) as described by Finazzi-Agrò et al. [14]. The enzyme preparation was submitted to an additional size exclusion chromatography step with a HiLoad Supradex G-200 prep. grade (60×2.6 cm i.d.; Pharmacia) column using a Pharmacia FPLC P-500 pump and Liquid Chromatography Controller LCC-501. The elution rate was 2.0 ml/min with 0.05 M sodium acetate buffer (pH 5.5) as the eluent, and UV-detection at 280 nm. This size-exclusion chromatography step was included to afford a homogeneous protein as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoreses [15]. The protein concentration was estimated from the absorbance at 280 nm ($\varepsilon_{280} = 1.6\times10^5$ $l\cdot mol^{-1}\cdot cm^{-1}$). The specific activity of the lipoxygenase preparation was 49.9 μmol (linoleic acid)$\cdot min^{-1}\cdot mg^{-1}$. The enzyme was stored at 4°C at a concentration of 1.9 mg/ml (20 mM) in 0.05 M sodium acetate buffer (pH 5.5) containing 134 g/l ammonium sulfate.

2.3. Methods of characterisation of the fatty acid amide derivatives

^{1}H-NMR spectra were recorded in $CDCl_3$ with a Bruker AC 300 (300 MHz) or with a Bruker AMX 500 (500 MHz) spectrometer at 27°C. A 2D-NMR TOCSY experiment was also performed on a Bruker AMX 500 instrument.

GC-MS analysis was performed by injecting the samples on column (25 m HT-5 SGE column, 0.1 μm film thickness). The column temperature was allowed to rise from 110 to 200°C at 40°C/min, and then to 300°C at 6°C/min. Mass spectrometry was performed with a JEOL JMS AX 505 spectrometer in positive ion electron impact mode over a mass range of 35–700 and electron ionisation at 70 eV.

IR spectra were measured on a Mattson Galaxy 5000 FTIR spectrometer. All spectra were recorded from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} and a co-addition of 60 scans in a nitrogen atmosphere at 20°C. The spectra were corrected with a co-addition of 60 blank scans and a manual baseline correction. UV absorption spectra were recorded from 200 to 250 nm on 40 μM hydroxy fatty acid amides in methanol with a Hewlett-Packard HP 8452A diode array spectrophotometer.

CD-spectra (resolution of 1 nm and 20 scans, 10 nm/min) were recorded on a Jasco J-600 spectrophotometer from 224 to 250 nm using 40 μM of pure product in methanol in a 1.0 cm cuvette at 20°C. Reversed- and chiral-phase HLPC chromatograms were obtained using a Hewlett Packard HP 1090 liquid chromatograph, equipped with a HP 1040A diode array detector with a detection limit <40 pmol (for conjugated dienes) and with a HP 79994A analytical workstation.

HPLC analysis and purification of the fatty acid amides were carried out on CP-Spher C18 (5 μm; 250×4.6 mm i.d.; Chrompack) or Cosmosil 5C18-AR (5 μm; 250×10 mm i.d.; Nacalai Tesque) columns, using a tetrahydrofuran/methanol/water/acetic acid mixture (25:30:45:0.01, v/v) as the eluent at flow rate of 1 ml/min (Chrompack column) or 3 ml/min (Nacalai Tesque column). Chiral analysis of the products was carried out on a Chiralcel OD-R (5 μm; 250×4.6 mm; Daicel) column using a methanol/water/acetic acid mixture (80:20:0.01, v/v) as the eluent for the enantiomers of the ODNHMe and $ODN(Me)_2$ and a methanol/water/acetic acid mixture (70:30:0.01, v/v) for the enantiomers of ODNHEtOH and $ODNH_2$ both at a flow rate of 0.5 ml/min.

2.4. Lipoxygenase assay: extraction and purification of reaction products

Typically, 40 μM of substrate in 30 ml rigorously stirred, air-saturated 0.1 M sodium borate buffer (pH 9.0) was incubated with 50 nM of lipoxygenase-1 for 60–90 min at 20°C. For the large scale production of oxygenated products from $ODNH_2$, ODNHMe, $ODN(Me)_2$ or ODNHEtOH reactions were performed in 300 or 600 ml borate buffer, respectively. The reactions were stopped by acidifying the reaction mixtures to pH 3 with 3 M HCl. The fatty acid amides were extracted with a BakerBond solid-state C18-column according to Van Aarle et al. [16]. The eluate was concentrated in vacuo (Rotavap) and residual water was azeotropically evaporated with methanol. The products were dissolved in 1 ml of methanol and stored at −25°C until use. The products were diluted to 2 ml of methanol and reduced with 32 or 16 mg (0.8 or 0.4 mmol; 3 equiv.) $NaBH_4$. This reaction mixture was stirred for 30 min under N_2 at 0°C. The reaction was terminated by adding 50 ml of water and acidifying the mixture to pH 3 with 3 M HCl. The reduced products were extracted and concentrated as described above. The hydroxy fatty acid amides were dissolved in 500 ml of methanol. Purification of these products was carried out with preparative HPLC (Nacalai Tesque column). The collected fractions were diluted with a 20-fold excess of water and then subjected to the C18 extraction and concentration procedure as described above. The purified compounds were dissolved in 1.0 ml of

Table 1
^{1}H-NMR data of the lipoxygenase products from ODNHEtOH, $ODNH_2$, ODNHMe and $ODN(Me)_2$

Proton[a]	ODNHEtOH(ppm, Hz)	$ODNH_2$(ppm, Hz)	ODNHMe(ppm, Hz)	$ODN(Me)_2$(ppm, Hz)
2	2.17; m; $J_{2,3}$ = 7.4	2.20; m; $J_{2,3}$ = 7.3	2.15; t; $J_{2,3}$ = 7.6	2.30; t; $J_{2,3}$ = 7.6
3–4	1.47–1.66; m	1.47–1.66; m	1.29–1.54; m	1.26–1.55; m
5–7	1.22–1.38; m	1.26–1.33; m	1.29–1.54; m	1.26–1.55; m
8	2.17; m	2.20; m	2.04; m	2.06; m
9	5.42; db.t; $J_{9,10}$ = 7.6	5.42; db.t; $J_{9,10}$ = 8.0	5.43; db.t; $J_{9,10}$ = 6.7	5.43; db.t; $J_{9,10}$ = 7.1
10	5.98; t; $J_{10,11}$ = 10.9	5.98; t; $J_{10,11}$ = 11.0	5.98; t; $J_{10,11}$ = 10.8	5.98; $J_{10,11}$ = 10.8
11	6.49; db.d.; $J_{11,10}$ = 11.0 $J_{11,12}$ = 15.0	6.49; db.d.; $J_{11,10}$ = 11.1 $J_{11,12}$ = 15.0	6.47; db.d.; $J_{11,10}$ = 11.0 $J_{11,12}$ = 14.9	6.49; db.d.; $J_{11,10}$ = 10.8 $J_{11,12}$ = 15.1
12	5.67; db.d; $J_{12,13}$ = 6.8 $J_{12,11}$ = 15.2	5.67; db.d; $J_{12,13}$ = 6.8 $J_{12,11}$ = 15.2	5.67; db.d; $J_{12,13}$ = 6.7 $J_{12,11}$ = 15.1	5.67; db.d;$J_{12,13}$ = 6.7 $J_{12,11}$ = 15.2
13	4.16; q; $J_{13,14}$ = 6.5	4.16; m; $J_{13,14}$ = 6.6	4.16; q; $J_{13,14}$ = 6.1	4.16; m; $J_{13,14}$ = 6.1
14	1.47–1.66; m	1.47–1.66; m	1.29–1.54; m	1.26–1.55; m
15–17	1.22–1.38; m	1.26–1.33; m	1.29–1.54; m	1.26–1.55; m
18	0.89; t; $J_{17,18}$ = 6.5	0.89; t; $J_{17,18}$ = 6.6	0.92	0.92; t
1'	3.42; q	5.4; broad	2.81; s	5.97; d
2'	3.72; t			
NH	5.9	5.4	5.8; broad	

[a]Assignment on the basis of a 2D-TOCSY spectrum. Structures are given in Fig. 1.
(ppm, m = multiplet, db = double, d = doublet, t = triplet, q = quintet, Hz).

Table 2
Characteristic mass fragments of TMS ethers of fully reduced lipoxygenase products

	13-HODNHEtOH	13-HODNH$_2$	13-HODNHMe	13-HODN(Me)$_2$	Molecule/radical
MW$^+$	487	371	385	399	
M$^+$-15	472	356	370	384	·CH$_3$
M$^+$-71	416	300	314	328	·C$_5$H$_{11}$
M$^+$-90	397		295		TMSOH
M$^+$-133	354				
M$^+$-161	326				
m/z	173	173	173	173	C$_6$H$_{12}$OTMS
m/z				87	
m/z	73	73	73	73	TMS
m/z		59			

methanol and stored under N_2 at −25°C. The main products were analysed by ^{1}H-NMR-, FTIR-, UV-, CD spectroscopy, GC-MS and CP-HPLC. A 2D-NMR TOCSY experiment was carried out with the main regio-isomeric product from ODNHEtOH.

The hydroxy fatty acid amides were dried under a nitrogen stream and dissolved in $CHCl_3$ or $CDCl_3$ to perform FTIR- or ^{1}H-NMR/2D-NMR measurements, respectively. For GC-MS analysis aliquots of the products were hydrogenated for 30 min at 0°C with H_2 and Pd (5%)/$CaCO_3$ in 2 ml of methanol and then silylated with a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilazane (20:4:4, v/v) according to Van Aarle et al. [16].

2.5. Spectrophotometric assay of lipoxygenase kinetics

Kinetic studies were performed according to the method of Hampson et al. [1]. Reaction rates were measured with a Hewlett-Packard 8452A diode array spectrophotometer equipped with a thermostated cuvette holder at 25°C. Absorbance changes at 234 nm due to the formation of conjugated hydroperoxides ($\varepsilon = 25\,000$ l·mol^{-1}·cm^{-1}) were recorded from 20 s until 120 s after the reaction was initiated. The assays were performed in a 1 ml cuvette. In order to achieve a substrate solution of 40 μM the 4.0 mM substrate stock solutions were diluted with 0.1 M borate buffer (pH 9). The 40 μM solution was then diluted further in the cuvette with the borate buffer and a glass bead to achieve homogenous solutions between 0 and 30 μM. The reaction was initiated by addition of 6 μl of a 0.4 μM enzyme solution (final concentration 2.4 nM). Apparent K_m and k_{cat} values were determined from the means of triplicate determinations with a coefficient of variation of 5%. Data were directly fitted to the standard Michaelis-Menten kinetic equation. Statistical significance of mean values was determined by the two-tailed Student t-test ($P > 0.05$, $n = 3$).

3. Results

In each reaction with lipoxygenase one major hydroperoxide regio-isomer with a maximal absorbance (λ_{max}) at 234 nm was formed according to the RP-HPLC analyses (data not shown). The UV spectra of the products were identical. After reduction with sodium borohydride and additional purification by RP-HPLC, FTIR spectra were recorded to verify the presence of various functional groups in the fatty acid amides. The FTIR spectra of the hydroxy-fatty acid amides exhibited absorbances at 3350 cm^{-1} (medium, broad) indicating a secondary hydroxyl group, 3007 cm^{-1} (weak) an olefin, 2928 and 2856 cm^{-1} (strong) alkane stretches, around 1650 and 1560 cm^{-1} (strong or medium) the amide I and amide II bands. To establish the geometry of a 1-hydroxy-2,4-diene-moiety in the products ^{1}H-NMR spectra were recorded. The data are summarised in Table 1. The geometric configurations of the conjugated dienes at positions 9–12 were determined as *cis–trans* on the basis of the coupling constants ($J_{9,10} = 7.0$ Hz, $J_{10,11} = 11.0$ Hz, $J_{11,12} = 15.1$ Hz). The position of the secondary hydroxyl group in the main product of the *N*-linoleoyl ethanolamide was located at the 13 position on the basis of the 2D-TOCSY spectrum, in particular the spin coupling between H_{13} (4.16 ppm) and H_{18} (0.89 ppm). The spin coupling between H_9 (5.98 ppm) and H_{18} was less, due to the large distance over which the magnetisation had to be transferred (Table 1).

The lipoxygenase products were further identified by mass spectrometry to establish the formation of 13-regio-isomers as already indicated by the 2D-NMR experiment for the 13-hydroxy *N*-linoleoyl ethanolamide. The mass spectra confirmed the presence of 13-hydroperoxides, in view of the prominent fragment at *m/z* 173 in all spectra, which is produced by cleavage at C_{13}–C_{12}. The most characteristic fragments are summarised in Table 2.

In order to determine the configuration of the chiral centre, the pure components were analysed by CP-HPLC. These CP-HPLC chromatograms show an excess of one of the two enantiomers. Furthermore, minor amounts of the *trans–trans* isomer are present, based on the UV spectrum of these components (data not shown). The ratios of enantiomeric hydroxy fatty acid amides are given in Table 3. The assignment of the absolute configuration of the products was based on CD spectroscopy [17]. Since the CD spectra of the products showed a positive Cotton effect, like 13-(*S*)-HOD, the dioxygenation of the substrates, with the exception of ODN(Me)$_2$, yields stereoselectively the *S*-enantiomer [18,19].

Incubations were performed in triplicate to determine the ratios between 13- and 9-regio-isomers from the peak areas in the RP-HPLC chromatograms (Table 3). Control incubations (without enzyme) did not show detectable non-enzymatic oxidation of the amide substrates, except for ODN(Me)$_2$. It was estimated that 20.2 ± 0.2% of this amide was autoxidized. However, before the products were characterised, they were purified with RP-HPLC and the autoxidation products (*E,E*-regio-isomers) were not included in the analysis.

The amide products were identified as the 13-(*S*)-hydroperoxide-9*Z*,11*E*-octadeca-9,11-dieneoylamides (Fig. 1).

In order to compare *N*-linoleoyl ethanolamide, linoleoylamide and linoleic acid metabolism by lipoxygenase-1, the kinetics of the reaction with the three substrates were deter-

Table 3
Ratios of regio-isomers (13/9) and enantiomers (13*S*/13*R*) in lipoxygenase products

Substrate	13 ± SD	9 ± SD	13*S*	13*R*
ODNHEtOH	91.9 ± 2.6	8.1 ± 2.6	96.6	3.4
ODNH$_2$	93.1 ± 1.6	6.9 ± 1.6	94.0	6.0
ODNHMe	86.7 ± 2.1	13.3 ± 2.1	93.0	7.0
ODN(Me)$_2$	90.2 ± 2.5[a]	9.8 ± 2.5[a]	66.1	33.9
OD	95	5	99	1

[a]These data were corrected for non-enzymatic oxidation.

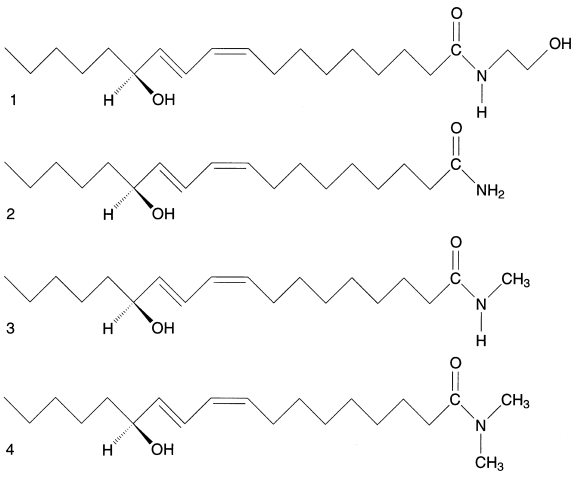

Fig. 1. Structures of the main products of the incubations of the amide substrates with lipoxygenase-1: 13-(*S*)-hydroxy-9*Z*,11*E*-octadecadienoyl-*N*-ethanolamide (1), 13-(*S*)-hydroxy-9*Z*,11*E*-octadecadienoylamide (2), 13-(*S*)-hydroxy-9*Z*,11*E*-octadecadienoyl-*N*-methylamide (3), 13-(*S*)-hydroxy-9*Z*,11*E*-octadecadienoyl-*N*,*N*-dimethylamide (4).

mined with a spectrophotometric assay measuring all reaction products absorbing at 234 nm (see Section 2.5). Apparent K_m ($\pm$S.D.) values of soybean lipoxygenase-1 with ODNHEtOH, $ODNH_2$ or OD as substrates were 23.6 ± 3.7 μM, 8.60 ± 0.65 μM and 8.85 ± 0.74 μM, respectively. These values are not significantly different from each other as determined with a two-tailed Student's *t*-test ($P > 0.05$; $n = 3$). The k_{cat} ($\pm$SD) values for lipoxygenase-1 for ODNHEtOH, $ODNH_2$ and OD were 61.6 ± 5.2 s^{-1}, 32.4 ± 1.2 s^{-1} and 54.4 ± 2.0 s^{-1}, respectively. The k_{cat} for $ODNH_2$ was significantly different from the k_{cat} values for ODNHEtOH and OD ($P > 0.05$; $n = 3$).

4. Discussion

Anandamide, a novel neurotransmitter, has been reported to be dioxygenated by lipoxygenases [1,11]. *N*-linoleoyl amides competitively inhibit anandamide breakdown, and they contain a 1*Z*,4*Z*-pentadiene system [8,9]. Therefore, they are also putative candidates to be dioxygenated by lipoxygenases. However, little is known about their metabolic pathway by lipoxygenases and their biological functions are unclear. Therefore, we conducted a study in which four *N*-linoleoyl amides with different amide headgroups, i.e. ethanolamide, amide, methyl amide and dimethyl amide, were dioxygenated by soybean lipoxygenase-1, a model enzyme for mammalian lipoxygenases.

The main products from all dioxygenation reactions were 13-(*S*)-hydroperoxy-9*Z*,11*E*-octadeca-9,11-dienoyl amides (Fig. 1). The regio- and stereoselectivities of the enzyme with the amide substrates (>90% 13-(*S*)-isomer) are similar to those observed with linoleic acid (95% 13-(*S*)-isomer). Because of the kinetics and the high regio- and stereoselectivities, the reaction closely resembles the one with linoleic acid.

The comparable reactivities of the substrates suggest that ODNHEtOH may also be a lipoxygenase substrate in vivo. Although the turnover number is smaller for $ODNH_2$ than for linoleic acid, it is likely that this substrate can be utilised by lipoxygenases in biological systems. However, it is necessary to study the conversion of the amide substrates with other lipoxygenases.

It is equally important to take into account the activity of the fatty acid amide hydrolase. It is interesting to know whether the substrates are dioxygenated before or after hydrolysis. Finally, it is of importance to know whether these products are capable of inducing or reducing the pharmacological effects reported for the substrates and analogues, i.e. oleamide has been shown to induce sleep, probably because it has a 'horse shoe' conformation [21]. *N*-linoleoyl amide with two *cis* double bonds may adopt an even more pronounced 'horse shoe' conformation [20], and induce sleep as well. The oxygenated lipoxygenase product *N*-linoleoyl amide does not have the 'horse shoe' conformation, due to the presence of the *cis–trans*-conjugated diene, and therefore may not induce sleep. This kind of experiments are currently under investigation.

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References

[1] Hampson, A.J., Hill, W.A.G., Zan-Phillips, M., Makriyannis, A., Leung, E., Eylen, R.M. and Bornheim, L.M. (1995) Biochim. Biophys. Acta 1259, 173–179.
[2] Devane, W.A., Hannus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Science 258, 1946–1949A.
[3] Gaoni, Y. and Mechoulam, R. (1964) J. Am. Chem. Soc. 86, 1646–1647.
[4] Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S.D. and Leon, A. (1995) Proc. Natl. Acad. Sci. USA 92, 3376–3380.
[5] Cravatt, B.F., Prospero-Garcia, O., Siuzdak, G., Gilula, N.B., Henriksen, S.J., Boger, D.L. and Lerner, R.A. (1995) Science 268, 1506–1509.
[6] Di Marzo, V., De Petrocellis, L., Sepe, N. and Buono, A. (1996) Biochem. J. 316, 977–984.
[7] Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A. and Gilula, N.B. (1996) Nature 384, 83–87.
[8] Maurelli, S., Bisogno, T., DePetrocellis, L., DiLuccia, A., Marino, G. and DiMarzo, V. (1995) FEBS Lett. 377, 82–86.
[9] Di Tomasso, E., Beltsamo, M. and Piomelli, D. (1996) Nature 382, 677–678.
[10] Bornheim, L.M., Kim, K.Y., Chen, B.C. and Correia, M.A. (1995) Biochem. Pharmacol. 50, 677–686.
[11] Ueda, N., Yamamoto, K., Yamamoto, S., Tokunaga, T., Shirakawa, E., Shinkai, H., Ogawa, M., Sato, T., Kudo, F., Inoue, K., Takizawa, H., Nagano, T., Hirobe, M., Matsuki, N. and Saito, H. (1995) Biochim. Biophys. Acta 1254, 127–134.
[12] Yamamoto, S. (1992) Biochim. Biophys. Acta 1254, 127–134.
[13] Prigge, S.T., Boyington, J.C., Gafney, B.J. and Amzel, L.M. (1996) Prot. Struct. Funct. Genet. 24, 275–291.
[14] Finazzi-Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 1254, 127–134.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Van Aarle, P.G.M., De Barse, M.M.J., Veldink, G.A. and Vliegenthart, J.F.G. (1991) FEBS Lett. 280, 159–162.
[17] K. Nakanishi, N. Berova, and R.W. Woody (Eds.), Circular Dichroism: Principles and Applications, VCH Publ., New York, 1994.
[18] Kühn, H., Wiesner, R., Lankin, V.Z., Nekrasov, A., Alder, L. and Schewe, T. (1987) Anal. Biochem. 160, 24–34.
[19] Van Os, C.P.A., Rijke-Schilder, G.P.M. and Vliegenthart, J.F.G. (1979) Biochem. Biophys. Acta 575, 470–484.
[20] Scheller, G., Jager, E., Hoffmann, B., Schmitt, M. and Schreier, P. (1995) J. Agric. Food Chem. 43, 1768–1774.
[21] Patterson, J.E., Ollmans, I.R., Cravatt, B.F., Boger, D.L., Wong, C.H. and Lerner, R.A. (1996) J. Am. Chem. Soc. 86, 1646–1647.