The effect of hydroxylation of linoleoyl amides on their cannabinomimetic properties

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Abstract As yet, the physiological significance of hydroxylation of anandamide and linoleoyl amides is unknown. Therefore, we investigated whether hydroxylation of ODNHEtOH and $ODNH_2$ influences their binding abilities to the CB-1 receptor and whether it alters their reactivity towards a fatty acid amide hydrolase (FAAH) from rat brain. Neither the fatty acid amides nor their hydroxylated derivatives were able to displace the potent cannabinoid [^{3}H]CP 55.940 from the CB-1 receptor ($K_i > 1$ μM). Hydroxylation of ODNHEtOH resulted in a strong reduction of the maximum rate of hydrolysis by a FAAH, but the affinity of FAAH for the substrate remained of the same order of magnitude. Hydroxylation of $ODNH_2$ led to a decrease in the affinity of FAAH for the substrate, but its maximum rate of conversion was unaffected. Furthermore, hydroxylation of ODNHEtOH enhanced its capacity to inhibit competitively the hydrolysis of anandamide. The resulting prolonged lifetime of anandamide and other fatty acid amide derivatives may have a considerable impact on cellular signal transduction.

Key words: Anandamide; Lipoxygenase; Cannabinoid receptor; Fatty acid amide hydrolase

1. Introduction

Fatty acid amides constitute a new class of neuro- and immunoregulatory molecules [1]. Anandamide (arachidonyl ethanolamide) binds to the cannabinoid binding receptor 1 (CB-1), which is coupled to G_i-proteins [2]. As a consequence, several cellular signaling responses are induced, such as inhibition of adenylate cyclase [3] and of N-type Ca^{2+} channels [4]. In addition, palmitoyl ethanolamide binds to the CB-2 receptor and down-regulates mast cell activation [5]. Acyl ethanolamides, including linoleoyl ethanolamide, can be formed through enzymatic hydrolysis of the corresponding acyl phosphatidyl ethanolamides by phospolipase D [6].

Cellular concentrations of neuroregulatory molecules, like anandamide, are strictly regulated. Indeed, anandamide and other fatty acid amides have been reported to be rapidly degraded by a fatty acid amide hydrolase (FAAH). Recently, Cravatt et al. have isolated and cloned a FAAH from rat liver plasma membranes [7]. Linoleoyl ethanolamide and linoleoyl amide competitively inhibit the hydrolysis of anandamide [8,9].

It should be noted that anandamide was shown to be converted by mammalian lipoxygenases (LOX) into hydroxy-anandamide derivatives in vitro [10,11]. Lipoxygenases are non-heme iron containing dioxygenases which catalyze the conversion of substrates containing one or more *1Z*,4*Z*-pentadiene systems into 1-hydroperoxide-2*Z*,4*E*-pentadiene derivatives. These hydroperoxides can be toxic for biological systems. Therefore, the hydroperoxides are readily reduced by peroxidases, e.g. glutathione peroxidase [12], to hydroxyl compounds. 12-LOX metabolites, like 12-HETE, have been reported to modulate neurotransmitter activities [13]. Other LOX products, like leukotrienes and 15-HETE, have been shown to be largely neuroendocrine and vasoactive agents [14,15].

Hampson et al. demonstrated that 12-hydroxy arachidonyl ethanolamide, a brain LOX metabolite from anandamide, is capable of binding to the CB-1 receptor even better than anandamide [10]. However, 15-hydroxyarachidonyl ethanolamide is a much poorer ligand of the CB-1 receptor [10]. In contrast, Ueda et al. demonstrated that the 15-hydroxy derivative inhibits the electrically evoked contraction of mouse vas deferens, a typical response of cannabinoids, equally well as anandamide, but now the 12-hydroxy product is less effective [11].

Recently, we have shown that linoleoyl amides can be converted by soybean lipoxygenase-1 and we have suggested that this conversion can be exerted by brain lipoxygenases in vivo. [16] However, the physiological significance of the fatty acid amide lipoxygenase metabolism remains unclear. An interesting question is whether hydroxylation affects the bioactivities of the fatty acid amides. Therefore, we investigated whether hydroxylation of linoleoyl amides by the lipoxygenase pathway influences their binding abilities to the CB-1 receptor and whether it alters their reactivity towards a fatty acid amide hydrolase.

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Abbreviations: BSA, bovine serum albumin; CB-1/2, cannabinoid binding receptor-1/2; FAAH, fatty acid amide hydrolase; 12/15-HETE, 12/15-hydroxyeicosatetraenoic acid; 13-HODNHEtOH, 13-(*S*)-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl ethanolamide; 13-$HODNH_2$, 13-(*S*)-hydroxy-9*Z*,11*E*-octadeca-9,11-dienoyl amide; LCS, liquid scintillation cocktail; ODNHEtOH, 9*Z*,12*Z*-octadeca-9,12-dienoyl ethanolamide; $ODNH_2$, 9*Z*,12*Z*-octadeca-9,12-dienoyl amide; PEI, polyethylene imine; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reversed phase high performance liquid chromatography; TLC, thin layer chromatography

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2. Materials and methods

2.1. Materials

[^{3}H]CP 55.940 (126 Ci/mmol) was purchased from NEN Dupont (Boston, MA, USA). Anandamide was from Matreya Inc. (Pleasant Gap, USA) and the other fatty acid amide derivatives were synthesized and purified as described before by Van der Stelt et al. [16]. [1-^{14}C]Linoleic acid (55 mCi/mmol) and [1-^{14}C]linoleoyl ethanolamide (55 mCi/mmol) were purchased from ARC (St. Louis, MO), USA. [1-^{14}C]Arachidonic acid (52 mCi/mmol) was obtained from NEN DuPont. Ethanolamide and NH_3 (25%) were from Merck. All other reagents used were of the purest grade available.

The following procedure was used for the synthesis of [1-^{14}C]anandamide and [1-^{14}C]linoleoyl amide. Dry fatty acid (1 μmol) was dissolved in 1 ml dry toluene under nitrogen. To this solution 20 μmol oxalyl chloride was added and the mixture was allowed to react for 2 h at 50°C. The reaction mixture was cooled down to room temperature and the solvent with the excess of oxalyl chloride was evaporated with a nitrogen flow. The residue was dissolved in 0.5 ml dry dichloromethane and 10 μmol ethanolamine or NH_3 (25%) was added. After 1 h at room temperature, the residue was dried under a nitrogen flow. The products were dissolved in 100 μl methanol and subjected to preparative TLC (Silica F_{254}, 0.25 mm; 10×10 cm). The plates were developed in chloroform/methanol/ammonia (25%), 94/6/1 (v/v) for anandamide and 85/15/1 (v/v) for linoleoyl amide. The spots which corresponded to the fatty acid amides were scraped off and extracted with 5 ml chloroform. The silica was removed through filtration over cotton wool and the solvent was evaporated; the residue was dissolved in 500 μl methanol. Purity of the fatty acid amides (>95%) was checked with RP-HPLC as described below.

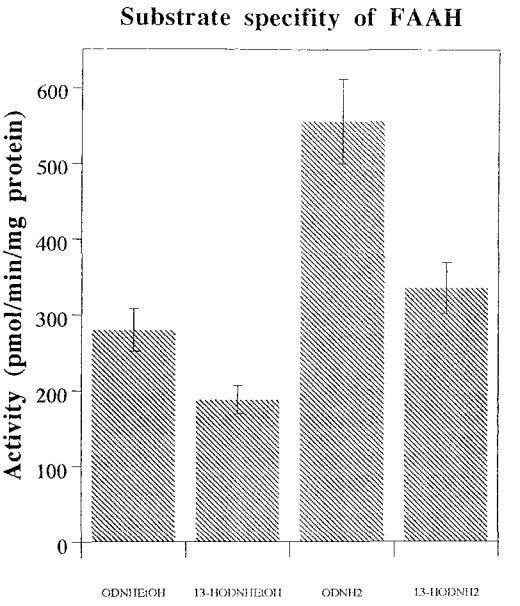

Fig. 2. Substrate specifity (±S.D.) of FAAH from the 11 300×*g* pellet fraction of rat brain. Incubations of 15 min were performed as described in Section 2.

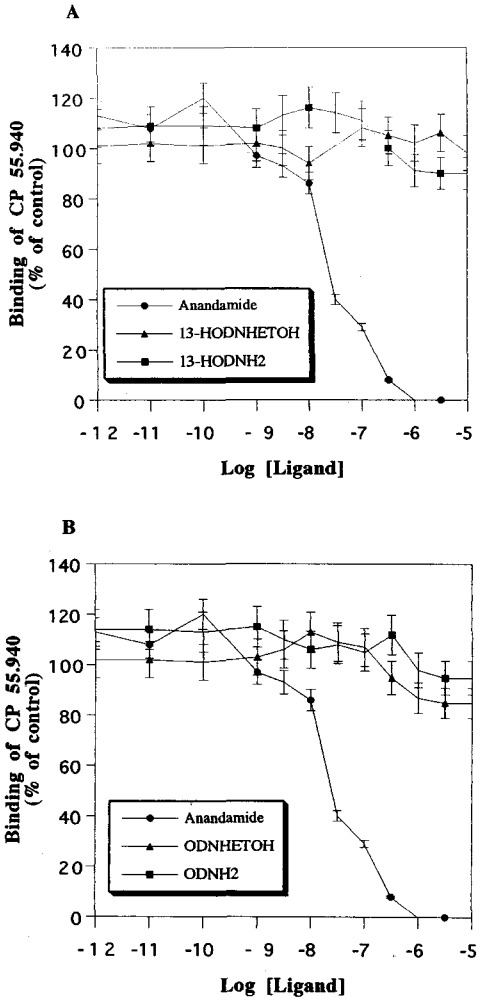

Fig. 1. Displacement curves of (A) linoleoyl amides (ODNHEtOH, $ODNH_2$) and (B) their hydroxylated products (13-HODNHEtOH, 13-$HODNH_2$). Error bars represent the S.D.

The hydroxylation of the [1-^{14}C]fatty acid amides was performed as described by Van der Stelt et al. [16]. The dioxygenation reaction was led to completion by the addition of an extra 50 nM lipoxygenase-1, 30 min after the reaction started. Soybean lipoxygenase-1 was purified as described by Finazzi Agrò [17]. Purity of the reduced products (>95%) was checked with RP-HPLC as described below.

2.2. Membrane preparation for binding assay

Male Wistar rats (250–280 g) were maintained on a 12 h light/dark schedule, before they were killed. Food and water were ad libitum. The experimental protocol and procedures used met the guidelines of the Ministry of Health (G.U. No. 40, February 18, 1992) and were approved by the Animal Care Committee (University of Rome 'Tor Vergata').

Membrane preparations were obtained using the method of Devane et al. [18] and were stored at a concentration of 1 mg/ml protein in buffer A (50 mM Tris-HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, pH 7.4) at −80°C for no longer than 1 week. The protein concentration was determined according to Bradford [19], using bovine serum albumin (BSA) as a standard.

2.3. Binding assay

Unless other wise stated, a rapid filtration assay was performed with ^{3}H-labeled CP 55.940, according to Compton et al. [20]. Incubations were performed in a final volume of 0.5 ml buffer B (50 mM Tris-HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, 5 mg/ml BSA, pH 7.4). PMSF (final concentration 50 μM) was added fresh each time just before the incubations started. The binding was initiated by the addition of 48 μg of protein of the membrane preparation and stopped after 1 h at 30°C. The washed filters were transferred to vials, which contained 0.5 ml 0.1% Triton X-100 and 3.5 ml liquid scintillation cocktail (LSC, OpticFluor, Canberra Packard). The vials were incubated overnight before counting. Unspecific binding was determined in the presence of 10 μM anandamide. Binding data were analyzed with the program GraphPad, which performs weighted non-linear least squares curve fitting to the general model of Feldman [21]. Data reported in this article are the mean of two independent experiments, each performed in triplicate.

2.4. Hydrolase assay

The cortex of one rat was homogenized with an UltraTurrax T 25 in 12.5 ml buffer C (50 mM Tris-HCl, 1 mM EDTA, pH 7.4). The homogenate was centrifuged for 20 min at 11 300×*g*. The pellet was

resuspended in buffer C at a protein concentration of 1 mg/ml and stored at −80°C.

Unless stated otherwise, the incubations were made according to Maurelli et al. [8] and Hillard et al. [22]. [1-^{14}C]Fatty acid amide, in a final concentration of 3 μM, was added to a total volume of 0.2 ml hydrolase assay buffer D (50 mM Tris-HCl, pH 9.0). The reaction was initiated by the addition of 10 μg protein and incubated for 15 min at 37°C. The reaction was terminated by the addition of 0.8 ml ice-cold methanol/chloroform (2:1, v/v), with vortexing. The radioactive substrates and products were extracted from the mixture according to the extraction procedure of Bligh and Dyer [23]. The final residue was dissolved in 50 μl methanol and subjected to RP-HPLC analysis, using a Perkin Elmer Nelson Model 1022 Plus Chromatograph, equipped with a Perkin Elmer series 200 LC Pump and a Canberra Packard Flow Scintillation Analyzer 500 TR Series with a 0.5 ml flow cell. Absorbance values were recorded at 204 nm.

The HPLC analysis of the radioactive substrates and products was carried out on a C18 (5 μm, 30×3 mm i.d.: SGE) column, using a mixture of methanol/water/acetic acid (85/15/0.1, v/v) for the non-hydroxylated substrates and methanol/tetrahydrofuran/water/acetic acid (30/25/45/0.1, v/v) for the hydroxylated substrates as the eluent at a flow rate of 0.8 ml/min. LSC (UltimaFlo M, Canberra Packard) was mixed with the eluent at a ratio of 1:2 (eluent/LSC). The ratio substrate/product was calculated from the peak areas of the chromatograms, assessing peak identity by co-injection of authentic standards.

3. Results

In order to investigate the effect of hydroxylation on the bioactivities of linoleoyl amides, fatty acid amides and their hydroxylated derivatives were assessed as ligands to displace the potent cannabinoid [^{3}H]CP 55.940 from the CB-1 receptor of rat brain. From this radioligand displacement assay it is clear that neither the linoleoyl amides nor their hydroxylated products were capable of displacing the radioligand from the receptor (Fig. 1A,B). The K_i of all four ligands exceeded 1 μM, being much larger than the K_i of anandamide of 27 ± 4 nM.

To investigate if hydroxylation affects the hydrolysis of linoleoyl amide by a FAAH, the 1-^{14}C-labeled fatty acid amides (3 μM) and their hydroxylated derivatives (3 μM) were incubated with rat brain membrane preparation with a concentration of 50 μg/ml protein. Interestingly, hydroxylation slowed down the conversion of ODNHEtOH and $ODNH_2$ by FAAH (Fig. 2). The activity of FAAH towards the fatty acid amides was measured at several substrate concentrations and the obtained data were fitted to the standard

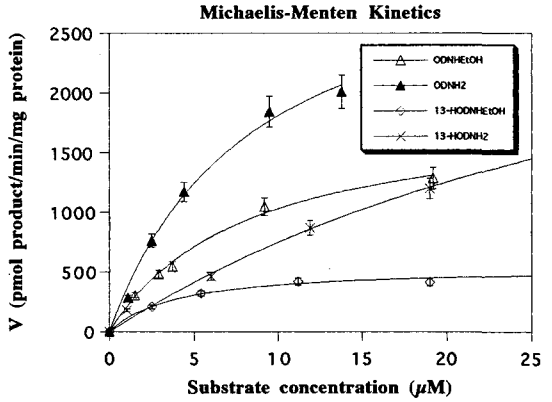

Fig. 3. Michaelis-Menten curves of the fatty acid amide hydrolysis by the 11 300×g pellet fraction of rat brain. Error bars represent S.D.

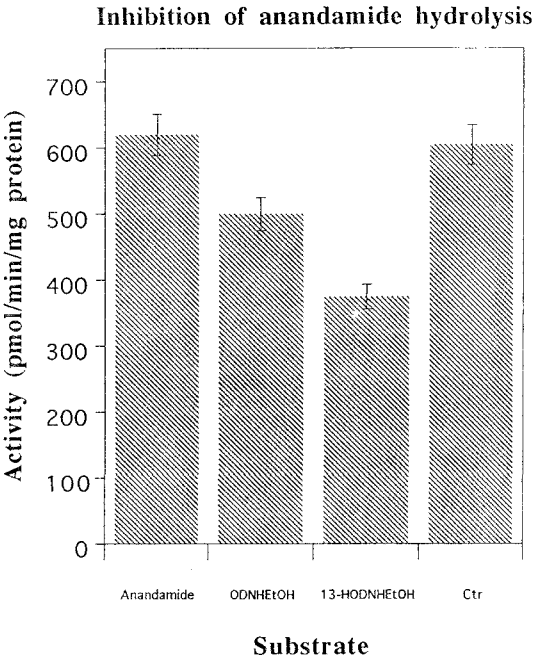

Fig. 4. Inhibition of anandamide hydrolysis by rat brain FAAH in the presence of linoleoyl ethanolamide (ODNHEtOH) and its hydroxylated derivative (13-HODNHEtOH). The error bars represent the S.D.

Michaelis-Menten equation, without the use of any graphical method such as Lineweaver-Burk (Fig. 3). Rat brain FAAH had an equal affinity for the non-hydroxylated substrates ODNHEtOH (K_m = 8.0 ± 1.0 (S.D.) μM) and $ODNH_2$ (K_m = 8.3 ± 1.5 μM). The maximum hydrolysis rate for $ODNH_2$ (V_{max} = 3317 ± 289 pmol/min/mg protein) was twice as high as for ODNHEtOH (V_{max} = 1859 ± 105 pmol/min/mg protein). Hydroxylation of the substrates had a different effect on each of them. For 13-HODNHEtOH the K_m remained of the same order of magnitude (K_m = 3.9 ± 0.8 μM), but the maximum reaction rate was reduced to 27% (V_{max} = 543 ± 25 pmol/min/mg protein). The affinity of FAAH for 13-$HODNH_2$ was reduced to 20% (K_m = 40 ± 11 μM), but the V_{max} (3829 ± 674 pmol/min/mg protein) was unaltered.

To investigate the effect of hydroxylation on the competitive inhibition of the anandamide hydrolysis by linoleoyl ethanolamide, a 5 μM solution of [1-^{14}C]anandamide was incubated with 10 μM ODNHEtOH or 10 μM 13-HODNHEtOH and rat brain membrane preparation with a protein concentration of 50 μg/ml (Fig. 4). Interestingly, hydroxylation doubled the ability of ODNHEtOH to inhibit anandamide hydrolysis.

4. Discussion

Fatty acid amides such as linoleoyl ethanolamide and linoleoyl amide can be hydroxylated by the lipoxygenase pathway in vitro [16]. As yet, the physiological significance of this metabolic pathway is not established and it is unclear whether this hydroxylation alters the bioactivities of the fatty acid amides. Therefore, we investigated whether hydroxylation of linoleoyl amides by the lipoxygenase pathway influences their binding abilities to the CB-1 receptor and whether it affects their reactivity towards a FAAH.

Although several extensive structure-activity studies of CB-1 endo-cannabinoid ligands have been reported [24,25], only Sheskin et al. reported a K_i for ODNHEtOH of >25 μM [26]. Our results are consistent with theirs. Hydroxylation of the 1*Z*,4*Z*-pentadiene containing fatty acid amides does not result in a more enhanced binding with the CB-1 receptor, as observed for the 12-lipoxygenase product of anandamide [10]. At least three double bonds seem necessary for an endo-cannabinoid to show any affinity for the CB-1 receptor [26]. Therefore, hydroxylation of linoleoyl amides does not *directly* affect the cannabinomimetic properties of these fatty acid amides.

Hydroxylation of ODNHEtOH results in a strong reduction of the maximum rate of hydrolysis by a FAAH, but the affinity of FAAH remains of the same order of magnitude. Hydroxylation of $ODNH_2$ leads to a decrease in the affinity of FAAH for the substrate, but its maximum conversion rate is unaffected. Therefore, the activity of FAAH is reduced at low 13-$HODNH_2$ concentrations. Furthermore, hydroxylation of ODNHEtOH enhances its capacity to competitively inhibit the hydrolysis of anandamide. Thus, hydroxylation by the lipoxygenase pathway of linoleoyl amides impairs the hydrolysis of fatty acid amides by a FAAH. The resulting prolonged the lifetime of anandamide and other fatty acid amide derivatives may thus have a considerable impact on cellular signal transduction.

Dersarnaud et al. observed that replacing *cis* double bonds with *trans* double bonds reduced the inhibitory effects of fatty acid amides on the hydrolysis of [^{3}H]anandamide by rat brain microsomes [27]. Therefore, it is reasonable to suggest that the enhanced inhibitory effect we observed is mainly due to the introduction of the hydroxyl function at the 13-position of the fatty acid amide and not to the isomerisation of the two *cis* double bonds into a *cis-trans*-conjugated diene. Work is in progress to study whether other regio-isomeric lipoxygenase products of fatty acid amides exhibit a similar effect.

In conclusion, this study has shown that hydroxylation of linoleoyl amides by the lipoxygenase pathway via dioxygenation, followed by reduction of the hydroperoxides, does not alter their binding abilities to the CB-1 receptor. However, hydroxylation of 1*Z*,4*Z*-pentadiene containing fatty acid amides can affect their bioactivities by impairing their reactivity towards a fatty acid amide hydrolase. Hence, the prolonged lifetime of anandamide and other fatty acid amide derivatives can have a considerable impact on cellular signaling.

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