Pharmacological Research 91 (2015) 9–14

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Diacerein is a potent and selective inhibitor of palmitoylethanolamide inactivation with analgesic activity in a rat model of acute inflammatory pain

Stefania Petrosino[a,b], Akbar Ahmad[c], Gabriele Marcolongo[a], Emanuela Esposito[c], Marco Allarà[a,b], Roberta Verde[a,b], Salvatore Cuzzocrea[c], Vincenzo Di Marzo[b,*]

[a] Epitech Group S.r.l., Saccolongo, PD, Italy
[b] Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, C.N.R., Pozzuoli, NA, Italy
[c] Dipartimento di Scienze Biologiche ed Ambientali, Università di Messina, Italy

ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form 3 October 2014
Accepted 24 October 2014
Available online 1 November 2014

Keywords:
Diacerein
NAAA
Palmitoylethanolamide
Inflammation

ABSTRACT

Palmitoylethanolamide (PEA) is produced by mammalian cells from its biosynthetic precursor, *N*-palmitoyl-phosphatidyl-ethanolamine, and inactivated by enzymatic hydrolysis to palmitic acid and ethanolamine. Apart from fatty acid amide hydrolase (FAAH), the *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), a lysosomal enzyme, was also shown to catalyze the hydrolysis of PEA and to limit its analgesic and anti-inflammatory action. Here we report the finding of a new potential inhibitor of NAAA, EPT4900 (4,5-diacetyloxy-9,10-dioxo-anthracene-2-carboxylic acid, diacerein). EPT4900 exhibited a high inhibitory activity on human recombinant NAAA over-expressed in HEK293 cells (HEK-NAAA cells). EPT4900 selectively increased the levels of PEA in intact HEK-NAAA cells, and inhibited inflammation as well as hyperalgesia in rats treated with an intraplantar injection of carrageenan. This latter effect was accompanied by elevation of PEA endogenous levels in the paw skin.

Introduction

Palmitoylethanolamide (PEA) is a protective endogenous mediator produced "on demand" by mammalian cells [1,2] during inflammatory and neurodegenerative conditions to exert anti-inflammatory and analgesic properties [3–5]. PEA is biosynthesized from its phospholipid precursor, *N*-palmitoyl-phosphatidyl-ethanolamine, through the catalytic action of, among others, *N*-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) [6], and inactivated to palmitic acid and ethanolamine by fatty acid amide hydrolase (FAAH) [7] and, more specifically, by *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [8]. Several hypotheses have been proposed to explain the molecular mechanism of action of PEA, and these include: (1) an Autacoid Local Inflammation Antagonism (ALIA) mechanism through which PEA acts by down-regulating mast-cell degranulation [9]; (2) the direct stimulation of an as-yet uncharacterized cannabinoid CB_2 receptor-like target [3,10]; (3) an "entourage effect" [11–13] through which PEA is able to enhance the anti-inflammatory and anti-nociceptive effects exerted by another fatty acid ethanolamide, anandamide (AEA), which is often produced together with PEA, and activates cannabinoid CB_1 and CB_2 receptors or the transient receptor potential vanilloid receptor type 1 (TRPV1) channel [14–16]; and (4) the direct activation of the nuclear peroxisome proliferator-activated receptor-α (PPAR-α), which is known to mediate many of the anti-inflammatory, anti-hyperalgesic and neuroprotective effects of this compound [17,18]. Since it is reported that tissue concentrations of PEA are altered during several pathological conditions [19,20], research has been recently focused on the synthesis of pharmacological tools able to selectively modulate its tissue levels, and in particular a few specific inhibitors of the enzyme mainly responsible of PEA degradation, NAAA, have been discovered.

The aim of the present work was to find novel selective and effective NAAA inhibitors able to increase the endogenous levels of PEA. EPT4900 was selected among a group of molecules endowed of anti-inflammatory activity, but for which information on the molecular mechanism of action is still lacking [21–23]. NAAA activity was measured as the capability of preparations from human embryonic kidney (HEK)293 cells in which the cDNA encoding the human enzyme was stably overexpressed (HEK-NAAA cells), to hydrolyze [^{14}C]-PEA to palmitic acid and [1,2-^{14}C]-ethanolamine.

* Corresponding author at: Via Campi Flegrei 34, Comprensorio Olivetti, 80078 Pozzuoli, Napoli, Italy. Tel.: +39 081 8675018; fax: +39 081 8041770.
E-mail address: vdimarzo@icb.cnr.it (V. Di Marzo).

http://dx.doi.org/10.1016/j.phrs.2014.10.008
1043-6618/

Materials and methods

Chemicals and reagents

[1,2-^{14}C]-Palmitoylethanolamide was chemically synthesized as described previously [24]. HEK293 (human embryonic kidney) cells overexpressing human NAAA (HEK-NAAA) and HEK293 wild-type (HEK-WT) cells were provided from Allergan Inc. (Irvine, CA, USA).

Diacerein (EPT4900) was provided by Sigma-Aldrich and further purified by crystallization from acetic acid.

Enzyme preparation and assay

HEK293 cells overexpressing human NAAA (HEK-NAAA) and HEK293 wild-type (HEK-WT) cells were suspended and homogenized in TRIS–HCl 20 mM (pH 7.4). The homogenate was centrifuged at 800 × *g* for 10 min and at 12000 × *g* for 30 min at 4 °C, successively. The 12000 × *g* pellet (membranes) was suspended in PBS (pH 7.4), subjected to two cycles of freezing and thawing and designated as enzyme to be used in the assay. The enzyme (50 μg/sample) was allowed to react at 37 °C for 30 min with 100 μM [1,2-^{14}C]-palmitoylethanolamide (10,000 c.p.m./sample) in citrate/sodium phosphate 50 mM (pH 5.2) and 0.1% Triton X-100, containing the test compound (EPT4900). The enzyme was also pre-incubated at 37 °C for 20 min with EPT4900 before the addition of substrate ([^{14}C]-PEA). The reaction was terminated by the addition of chloroform/methanol (1:1 by vol.) and quantification of [1,2-^{14}C]-ethanolamine was carry out by using Liquid Scintillation Analyzer (TRI-carb 2100TR).

Cell culture and treatment

HEK293 cells overexpressing human recombinant NAAA (HEK-NAAA cells) were cultured in EMEM (Eagle's Minimum Essential Medium) supplemented with blasticidin (2 mM), glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml) and 10% FBS in an humidified 5% CO_2 atmosphere at 37 °C. HEK293 wild-type (HEK-WT) cells were cultured in EMEM supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml) and 10% FBS in an humidified 5% CO_2 atmosphere at 37 °C. HEK-NAAA cells plated in culture plates were allowed to adhere and grow until 80% confluence and then treated with EPT4900 (1-10-50-100 μM) or vehicle (methanol 0.05%, Ctrl) for 40 min in an humidified 5% CO_2 atmosphere at 37 °C. The resultant cells and supernatants were collected and subjected to quantification of palmitoylethanolamide (PEA) levels.

PEA quantification in HEK-NAAA cells

Cells and supernatants were homogenized in a solution of chloroform/methanol/Tris–HCl 50 mM pH 7.4 (2:1:1 by volume) containing 5 pmol of [^{2}H]$_4$-PEA as internal standard [2,14]. The lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel. Fractions obtained by eluting the column with a solution of chloroform/methanol (90:10 by vol.) were analyzed by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole MS via a Shimadzu APCI interface. LC–APCI–MS analysis of PEA was carried out in the selected ion monitoring (SIM) mode, using *m*/*z* values of 304 and 300 (molecular ions +1 for deuterated and undeuterated PEA). PEA levels were calculated on the basis of their peak area ratios with the internal deuterated standard peak areas, and normalized per ml of supernatant volume.

Animals

The study was carried out on Sprague–Dawley male rats (200–230 g, Harlan, Nossan, Italy). Food and water were available ad libitum. The study was approved by the University of Messina Review Board for the care of animals. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Experimental groups

Rats were randomly allocated into the following groups:

(i) CAR + *saline group*: rats were subjected to carrageenan-induced paw edema (*N* = 10);
(ii) CAR + EPT4900 (10 mg/kg) dissolved in vehicle (10% ethanol): same as the CAR group + saline group but EPT 4900 (10 mg/kg, ip) was administered 30 min before CAR (*N* = 10);
(iii) CAR + EPT4900 (19 mg/kg) dissolved in vehicle (10% ethanol) same as the CAR group + saline group but EPT 4900 (19 mg/kg, ip) was administered 30 min before CAR (*N* = 10);
(iv) CAR + EPT4900 (25 mg/kg) dissolved in vehicle (10% ethanol) same as the CAR group + saline group but EPT 4900 (25 mg/kg, ip) was administered 30 min before CAR (*N* = 10);

The sham-operated group underwent the same identical surgical procedures as the CAR group, except that the saline or drugs were administered instead of carrageenan (*N* = 10).

Carrageenan-induced paw edema

Paw edema was induced as described previously [25] by a subplantar injection of 50 μl of sterile saline containing 1% (wt/vol) λ-carrageenan into the right hind paw, whereas controls were injected sterile saline alone. Paw volume was measured with a plethysmometer (Ugo Basile, Comerio, Varese, Italy) immediately prior to the injection of carrageenan and thereafter at hourly intervals for 6 h. Edema was expressed as the increase in paw volume (ml) after carrageenan injection relative to the pre-injection value for each animal. Results are expressed as paw volume change (ml).

Behavioral analysis

Behavioral testing was done with experimenter blinded to treatment conditions. Hyperalgesic responses to heat were determined by the Hargreaves' Method using a Basile Plantar Test [26] with a cut-off latency of 20 s employed to prevent tissue damage. Rats were individually confined to plexiglas chambers and allowed to habituate. A mobile unit consisting of a high intensity projector bulb was positioned to deliver a thermal stimulus directly to an individual hind paw from beneath the chamber. The withdrawal latency period of injected paws was determined with an electronic clock circuit and thermocouple. Results are expressed as paw-withdrawal latency(s).

PEA quantification in rat paw skin

After 6 h from the treatment, the paw skin of rats was subjected to extraction, purification and analysis of PEA levels as described above. PEA levels were calculated on the basis of their peak area ratios with the internal deuterated standard peak areas, and normalized per mg of wet tissue weight.