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Palmitoylethanolamide counteracts reactive astrogliosis induced by β-amyloid peptide

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Abstract

Emerging evidence indicates that astrogliosis is involved in the pathogenesis of neurodegenerative disorders. Our previous findings suggested cannabinoids and Autacoid Local Injury Antagonism Amides (ALIAmides) attenuate glial response in models of neurodegeneration. The present study was aimed at exploring palmitoylethanolamide (PEA) ability to mitigate β-amyloid (Aβ)-induced astrogliosis. Experiments were carried out to investigate PEA's (10^{-7} M) effects upon the expression and release of pro-inflammatory molecules in rat primary astrocytes activated by soluble $A\beta_{1-42}$ (1 μg/ml) as well as to identify mechanisms responsible for such actions. The effects of Aβ and exogenous PEA on the astrocyte levels of the endocannabinoidsand of endogenous ALIAmides were also studied. The peroxisome proliferator-activated receptor (PPAR)-α (MK886, 3 μM) or PPAR-γ (GW9662, 9 nM) antagonists were co-administered with PEA. Aβ elevated endogenous PEA and d5–2-arachidonoylglycerol (2-AG) levels. Exogenous PEA blunted the Aβ-induced expression of pro-inflammatory molecules. This effect was reduced by PPAR-α antagonist. Moreover, this ALIAmide, like Aβ, increased 2-AG levels. These results indicate that PEA exhibits anti-inflammatory properties able to counteract Aβ-induced astrogliosis, and suggest novel treatment for neuroinflammatory/ neurodegenerative processes.

Keywords: astrocyte • β amyloid • palmitoylethanolamide • PPAR • neuroinflammation

Introduction

Once considered a marginal event, the crucial role of neuroinflammation in the onset and progression of many neurodegenerative diseases is now clear [1–3]. Acute neuroinflammatory responses are generally beneficial to the central nervous system (CNS), because they tend to minimize further injury and contribute to the repair of the damaged tissue. In contrast, when glial activation inappropriately persists long after the initial injury or insult, the prolonged condition of neuroinflammation induces profound changes in the pathophysiological significance of the glial reaction so that inflammation, from a defensive response, may inexorably turn into a detrimental process [4, 5]. Indeed, chronic neuroinflammation implicates protracted activation of both microglial and astroglial cells, with the consequent sustained release of pro-inflammatory molecules [6, 7]. In this context, the role of microglia has been extensively described; however, the astrocyte involvement remains to be better elucidated. After their release, pro-inflammatory signal molecules act in an autocrine way to self-perpetuate reactive gliosis and in a paracrine way to kill neighbouring neurons, thus expanding the neuropathological damage.

In neurodegenerative disorders, inflammation may be triggered by the accumulation of proteins with abnormal conformations or

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by signals emanating from injured neurons. Whatever causes it, the extracellular deposition of β-amyloid (Aβ) in the form of amyloid plaques in sporadic Alzheimer's disease (AD) cases is clearly among the most potent inducers of neuroinflammation in the CNS. Activated microglia and reactive astrocytes localize at fibrillar plaques, and their biochemical markers are elevated in the brain of AD patients [8–10]. Although phagocytic glia initially engulfs and degrades Aβ, it then, however, loses its capability and prolonged glial activation results in an increased release of chemokines and damaging cytokines [11]. Moreover, glial cells express receptors for advanced glycation end-products, which bind Aβ, thereby amplifying the generation of cytokines, glutamate and nitric oxide. In addition, recent evidence reported the capability of Aβ to induce cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) synthesis in astrocytes through a NF-κB dependent mechanism [12]. Finally, it is known that pro-inflammatory cytokines can, in turn, increase the expression of the amyloid precursor protein and its processing through amyloidogenic pathways [13]. In this way, Aβ accumulation may therefore establish a self-sustaining cycle whereby neuronal stress and glial activation foster an inflammatory response, which in turn promotes the synthesis and the accumulation of more Aβ, thus perpetuating glial cell activation [14, 15].

Furthermore, besides the sustained production of pathogenic substances, astrocytes in AD brains, changing from a basal to a reactive state, fail to provide their neuro-supportive functions, rendering neurons more vulnerable to toxic molecules. Therefore, because of their crucial role in the pathogenic processes responsible for AD progression, astroglial cells may provide a valuable therapeutic target for the treatment of AD. And any compound able to modulate astrocyte activation might be considered as a novel drug of potential therapeutic relevance [16]. Among these molecules, palmitoylethanolamide (PEA) has attracted much attention for its proven anti-inflammatory and neuroprotective properties, as observed in many neuropathological conditions, mainly in the peripheral nervous system [17, 18].

PEA, a naturally occurring amide of ethanolamine and palmitic acid, is a lipid messenger that mimics several endocannabinoid-driven actions, even though it does not bind to cannabinoid receptors [18, 19]. PEA has been extensively studied for its anti-inflammatory, analgesic, anti-epileptic and neuroprotective effects [20–27]. It has also been reported to inhibit food intake [28], to reduce gastrointestinal motility [29], to counteract cancer cell proliferation [30, 31] and to protect the vascular endothelium in the ischemic heart [32]. Some of these properties have been considered to be dependent on the expression of peroxisome proliferator-activated receptor-α (PPAR-α) [18, 33, 34]. PEA is abundant in the CNS and it is conspicuously produced by glial cells [35, 36]. However, at present, its physiological role and its pharmacological properties in the CNS remain, for the most part, unrevealed.

The present study was designed to investigate whether or not PEA is able to attenuate astrocyte activation in an *in vitro* model of Aβ-induced astrogliosis and it incorporates experiments aimed at exploring molecular mechanisms responsible for the effects induced by PEA in the model here examined.

Materials and methods

Cell culture and treatments

All experiments were carried out in cultured primary astrocytes freshly isolated from newborn Sprague-Dawley rats (2 days old) according to Vairano *et al.* [37]. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92). Brains homogenates were mechanically processed to obtain single cells that were seeded in 75 cm^2 flasks at a density of 3×10^6 cells/flask with 15 ml of culture medium [DMEM, 5% inactivated foetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin; all from Sigma-Aldrich, St. Louis, MO, USA] and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced after 24 hrs, and again twice a week until astrocytes were grown up to form a monolayer firmly attached to the bottom of the flask (7–8 days after dissection). Flasks were vigorously shaken in order to separate astrocytes from microglia and oligodendrocytes. The same process was repeated again after about another week of culture. Depending upon the experimental procedure, astrocytes were seeded on 10 cm Ø Petri dishes at a density of 1×10^6 cells/dish or on eight chambers polystyrene culture slides at a density of 3×10^4 cells/chamber. Purity of the cells in culture was tested with monoclonal anti-glial fibrillary acidic protein (GFAP) and only cultures with more than 95% $GFAP^+$ cells were employed for the experiments. The 5% of non-astrocyte cells were microglia, oligodendrocytes and fibroblasts.

Astrocytes were treated with 1 μg/ml Aβ (human fragment 1–42) (Tocris Bioscience, Bristol, UK) in the presence or absence of the following substances: PEA (10^{-7} M, Cayman Chemical, Ann Arbor, MI, USA), MK886 (3 μM), the selective PPAR-α antagonist and GW9662 (9 nM), the selective PPAR-γ antagonist (both purchased from Tocris Bioscience). The concentrations of the substances were chosen on the basis of their IC_{50} and according to the results of a series of pilot experiments aimed at identifying the lowest effective concentration (data not shown). In all set of experiments, no significant variation *versus* control was observed when PEA, MK886 or GW9662 were given alone (data not shown). All reagents were lipopolysaccharide free.

After 24 hrs of treatment astrocytes were processed for analyses. Experiments on mitogen-activated protein (MAP) kinases and nuclear transcription factors activation were carried out after 30 min. of treatment. RT-PCR experiments were performed after 12 hrs of treatment.

Measurement of nitrite release

Production of nitric oxide was determined by measuring the amount of nitrite (NO_2^-) accumulated in supernatants of primary rat astroglial cells after 24 hrs of treatment.

A spectrophotometer assay based on the Griess reaction was used [38]. The absorbance of controls and unknown samples was measured at 540 nm. The NO_2^- concentration was thus determined using a standard curve of sodium nitrite and referred to 1×10^6 cells.

Analysis of cytokine secretion

Quantitative determination of tumour necrosis factor (TNF)-α, interleukin 1β (IL-1β), S100B and PGE2 release was carried out performing ELISA assays (TNF-α and IL1 β kits purchased from Invitrogen, Milan, Italy; S100B kit from BioVendor; PGE2 kit from Cayman Chemical).

After 24 hrs of treatment, cell culture medium was collected and ELISA assay was performed according to kit instruction. The optical density of each sample was read at 450 nm using a microtiter plate reader (Bio-Rad Laboratories, Milan, Italy). Results are expressed as pg/ml.

Analysis of some proteins expression by Western blotting

Western blot analyses were performed on extracts of cell cultures challenged as previously described. Twenty-four hours after treatment, cells were detached from Petri dishes and each pellet was suspended in ice-cold hypotonic lysis buffer (Tris/HCl pH 7.5 50 mM; NaCl 150 mM; ethylenediaminetetraacetic acid [EDTA] 1 mM; Triton X-100 1%) supplemented with the proper protease inhibitor cocktail (Roche, Mannheim, Germany). Equivalent amounts (100 μg) of each sample underwent SDS-PAGE electrophoresis. Afterwards, proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories), the immunoblot was carried out blocking the membrane with one of the following primary antibodies: anti-GFAP 1:50,000, anti-iNOS 1:200, anti-COX-2 1:2000, anti-S100B 1:50, anti-p38 MAPK 1:200, anti-PPAR-α 1:1000, anti-PPAR-γ 1:400, anti β-actin 1:1000 (all purchased from Abcam, Cambridge, UK); anti-CB1 1:250, anti-CB2 1:250 (Santa Cruz Biotechnology, Heidelberg, Germany); anti-phosphorylated Jun N-terminal kinase (p-JNK) 10 μg/ml (Sigma Aldrich). After being extensively washed in Tris buffered saline (TBS) 1× with 0.1% Tween 20, the membrane was incubated for 2 hrs at room temperature with the proper secondary horseradish peroxidase (HRP)-conjugated antibodies antimouse (1:2000) or anti-rabbit (1:3000; both purchased from Abcam). Finally, the membrane was developed by using enhanced chemiluminescence substrate (Invitrogen). Bands were revealed through a Versadoc (Bio-Rad Laboratories) and the corresponded digital images were analysed with Quantity One Software (Bio-Rad Laboratories).

Analysis of protein expression by immunofluorescence

Astrocytes were plated onto glass slide chambers coated with poly-D-lysine (BD Bioscience, San Jose, CA, USA) with a density of 3×10^4 cells/chamber in DMEM supplemented with 5% inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma Aldrich), at 37°C in humidified atmosphere 5%CO_2/95% air. Confluent cells were treated as protocol schedule. The reaction was stopped 24 hrs later. Then, astrocytes were washed with PBS 1× and fixed with 4% paraformaldehyde in PBS 1×. Afterwards, cells were blocked in 10% albumin bovine serum 0.1% Triton-PBS solution for 90 min. and subsequently incubated for 1 hr with a 10% albumin bovine serum 0.1% Triton-PBS solution of one of the following antibodies: anti-GFAP 1:500; anti-iNOS 1:50; anti-S100B 1:100; anti-COX-2 1:100 (all purchased from Abcam). Finally, cells were incubated for 1 hr in the dark with the proper secondary antibody: Texas Red conjugated antimouse 1:64 or fluorescein isothiocyanate conjugated anti-rabbit 1:100 (both from Abcam). Nuclei were stained with Hoechst 1:5000 (Sigma Aldrich) added in the secondary antibody solution.

Pictures were taken using a camera (Nikon DIGITAL SIGHT DS-U1) connected with a microscope (Nikon ECLIPSE 80i by Nikon Instruments Europe B.V., Kingston, UK) provided of the proper fluorescent filters.

Reverse transcriptase-PCR analysis

The mRNA level was determined using the semi-quantitative RT-PCR method (Invitrogen). Total mRNA was extracted from cells by use of an ultrapure TRIzol reagent (Gibco BRL, Milan, Italy) as directed by the manufacturer. The concentration and purity of total mRNA were determined from the A260/A280 ratio using a UV spectrophotometer (DU 40, Beckman, Fullerton, CA, USA). The primer sequences used for PCR amplification were: PPAR-α 5′-CTGGTCAAGCTCAGGACACA-3′ and antisense 5′-AAACGGATTGCATTGTGTGA-3′; PPAR-γ sense 5′-CTGACCCAATGGTTGCTGATTAC-3′ and antisense 5′-GGACGCAGGCTCTACTTTGATC-3′; GFAP sense 5′-GAAGCAGGGCAAGATGGAGC-3′ and antisense 5′-AGGTTGGTTTCATCTTGGAG-3′; GAPDH sense 5′-GAAGGTGAAGGTCGGAGT-3′ and antisense 5′-GAAGATGGTGATGGGATTTC-3′. 1 μg of total RNA from each specimen was subjected to RT-PCR, carried out using a SuperScript TM One-Step RT-PCR with Platinum Taq Kit (Invitrogen) in a total reaction volume of 25 μl, containing 2x reaction mix, 25 μM sense primer, 25 μM antisense primer, RT-PCR platinum Taq mix and autoclaved distilled water. Electrophoresis was performed on the amplification products using a 1% agarose gel and bands were visualized by staining with ethidium bromide. Integrated density values of bands representing amplified products were acquired and analysed by Versadoc (Bio-Rad Laboratories) and a computer programme (Quantity One Software, Bio-Rad Laboratories).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to detect AP-1 and NF-κB activation in primary astrocytes treated as above described. Double stranded oligonucleotides containing the AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) or NF-κB recognition sequence (5′-CAACGG CAGGGGAATCTCCCTCTCCTT-3′) were end-labelled with 32P-γ-ATP. Nuclear extracts were incubated for 15 min. with radiolabelled oligonucleotides (2.5–5.0 × 104 cpm) in 20 ml reaction buffer containing 2 mg poly dI-dC, 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dl-dithiothreitol, 1 mg/ml bovine serum albumin, 10% (v/v) glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× Tris Borate EDTA buffer at 150 V for 2 hrs at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 20 hrs. Subsequently, the relative bands were quantified by densitometric scanning with Versadoc (Bio-Rad Laboratories) and a computer programme (Quantity One Software, Bio-Rad Laboratories). 32P-γ-ATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim).

Measurement of PEA and endocannabinoid levels in cells

Cells were homogenized in chloroform:methanol:Tris-HCl 50 mM (2:1:1) containing 10 pmol of d8-anandamide (AEA), d4-PEA, d4-oleoylethanolamide (OEA) and d5–2-arachidonoylglycerol (2-AG) as internal standards. Homogenates were centrifuged at 13,000 × *g* for 16 min. (4°C), the aqueous phase plus debris were collected and extracted twice with 1 vol of chloroform. Organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform:methanol (99:1, v/v). Solutions were then purified by open bed chromatography on silica as previously described [38]. Fractions eluted with chloroform:methanol (9:1, v/v) and containing AEA, PEA, OEA and 2-AG, were collected, the excess solvent evaporated with a rotating evaporator and extracts analysed by

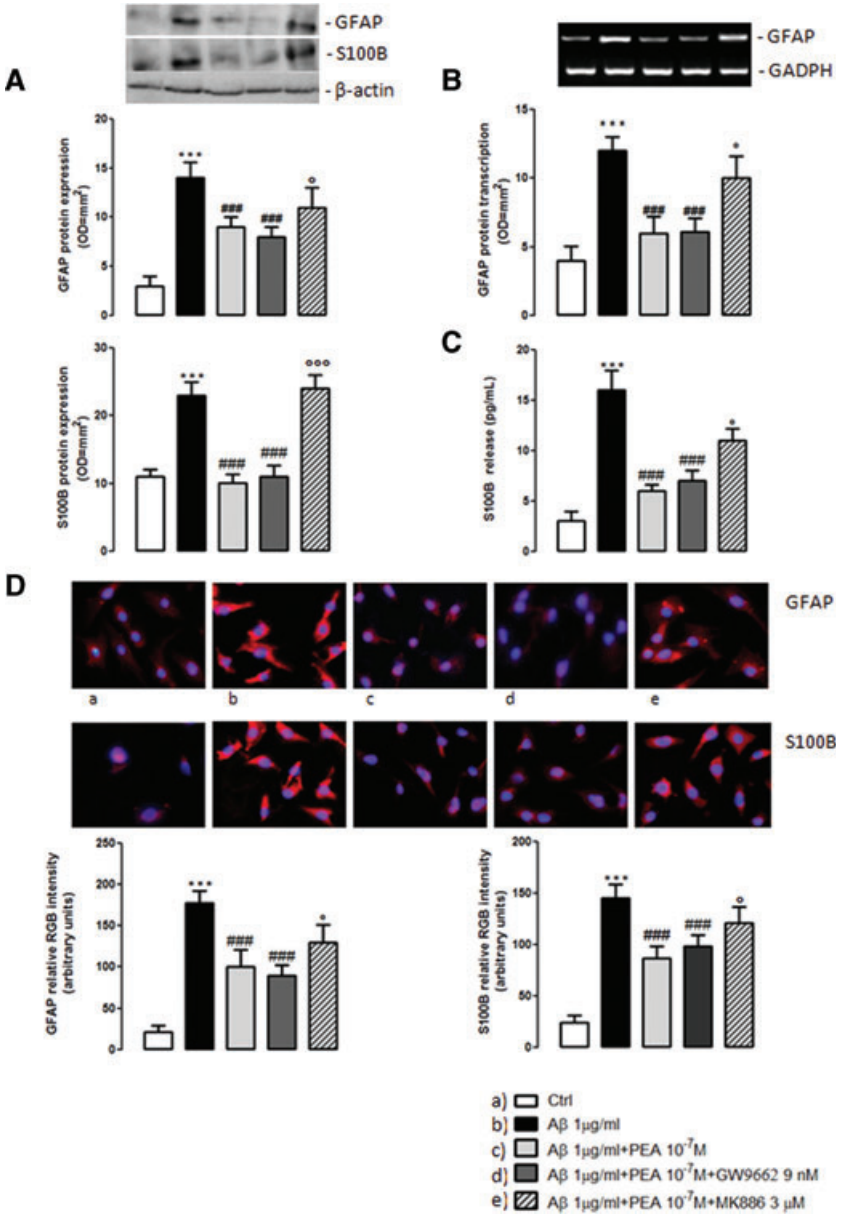

Fig. 1 PEA attenuates Aβ-induced astrocyte activation. Aβ-challenged (1 μg/ml) cells were treated with PEA (10^{-7} M) in the presence of the selective PPAR-γ antagonist (GW9662, 9 nM) or the selective PPAR-α antagonist (MK886, 3 μM). GFAP mRNA was evaluated 12 hrs following Aβ challenge; GFAP and S100B protein expression was evaluated after 24 hrs of treatments by Western blot and immunofluorescence analyses. S100B release in the cellular milieu was determined 24 hrs after Aβ challenge by ELISA assay. (**A**) Results of GFAP and S100B Western blot analysis and densitometric analysis of corresponding bands. β-actin was used as loading control. (**B**) Results of GFAP RT-PCR amplification and densitometric analysis of corresponding bands. GAPDH was used as standard control. (**C**) Measurement of S100B release by ELISA assay. (**D**) Analysis of GFAP and S100B protein expression by immunofluorescence (magnification 10×). Results are the mean ± S.E.M. of $n = 4$ separate experiments. $^{***}P <$ 0.001 *versus* control; $^{###}P < 0.01$ *versus* Aβ-challenged cells; $^{°°°}P < 0.001$ and $^{°}P < 0.05$ *versus* Aβ + PEA-challenged cells.

isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry carried out under conditions described previously [39] and allowing the separation of 2-AG, PEA, OEA and AEA. Results were expressed as pmol per dish, as each dish contained the same number of cells (1×10^6 cells/dish).

Statistical analysis

Results are expressed as mean ± S.E.M. of separate set of experiments. All statistical analyses were made using SigmaStat software. Effects of treatments were determined by one-way ANOVA. Multiple comparisons were performed with Bonferroni's test, whereas Neumann and Keuls' test was used for *post hoc* analyses. Value of $P < 0.05$ was considered significant.

Results

PEA attenuates Aβ-induced astrocyte activation

In order to test the effect of PEA on Aβ-induced astrogliosis, the expression of GFAP and S100B, specific markers of astrocyte activity, was explored. Reactive astrocytes display hypertrophied

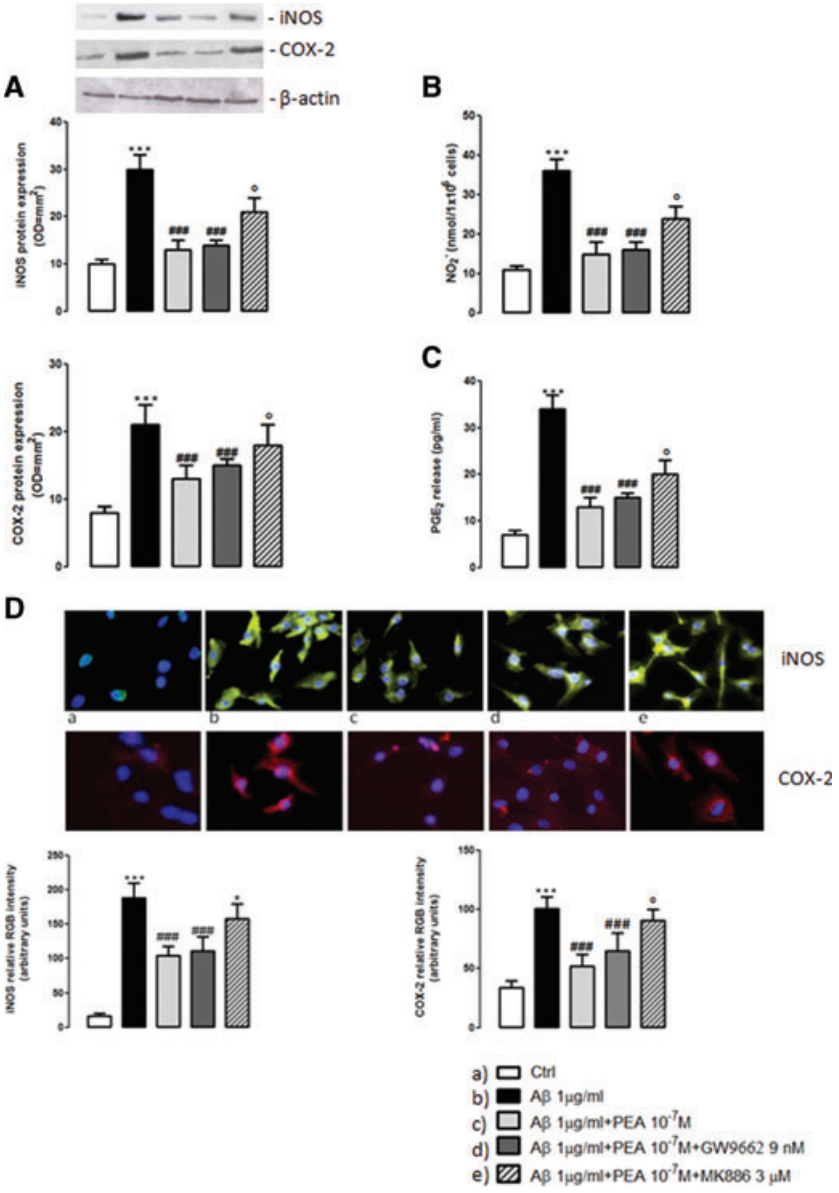

Fig. 2 PEA blunts Aβ-induced neuroinflammation. Aβ-challenged (1 μg/ml) cells were treated with PEA (10^{-7} M) in the presence of the selective PPAR-γ antagonist (GW9662, 9 nM) or the selective PPAR-α antagonist (MK886, 3 μM). iNOS and COX-2 protein expression was evaluated 24 hrs following treatments by Western blot and immunofluorescence analyses. After 24 hrs of Aβ-challenge, it was evaluated the release in the cellular milieu of nitric oxide, measured as its stable metabolite NO_2, and PGE2 by the Griess reaction and ELISA assay, respectively. (**A**) Results of iNOS and COX-2 Western blot analysis and densitometric analysis of corresponding bands. β-actin was used as loading control. (**B**) Determination of nitric oxide by the spectrophotometric assay based on the Griess reaction. (**C**) Measurement of PGE2 release by ELISA assay. (**D**) Analysis of iNOS and COX-2 protein expression by immunofluorescence (magnification 10×). Results from Western blot and immunofluorescence analyses are the mean ± S.E.M. of $n = 3$ separate experiments. Data from Griess reaction and ELISA assay are the mean ± S.E.M. of $n = 5$ independent experiments. $***P < 0.001$ *versus* control; $###P < 0.001$ *versus* Aβ-challenged cells; $°P < 0.05$ *versus* Aβ + PEA-challenged cells.

cell bodies and thickened processes exhibiting GFAP-immunoreactivity. Using immunofluorescence, Western blot and RT-PCR analyses, we observed a marked increase in both transcription and expression of GFAP after Aβ challenge. This enhancement was significantly attenuated by PEA treatment. Moreover, the PPAR-α antagonist MK886 was able to partly blunt the PEA-induced attenuation of GFAP transcription and expression. Such effect indicated a significant, although not exclusive, involvement of the PPAR-α receptor in mediating PEA action (Fig. 1).

Likewise, both expression and release of S100B were investigated. S100B is an astroglia-derived protein which acts as a neurotrophic factor and neuronal survival protein, even though the overproduction of S100B by activated glia can lead to exacerbation of neuroinflammation and neuronal dysfunction. S100B levels generally correlate with the severity of damage and elevated levels of S100B are closely associated with a sustained reactive gliosis. Results from cultured astrocytes showed a significant increase in both expression and release of S100B protein after Aβ exposure, and these effects were counteracted by PEA. Once again this effect of PEA was partly reduced by MK886, suggesting a PPAR-α involvement (Fig. 1).

PEA blunts Aβ-induced neuroinflammation

Another set of experiments was aimed at assessing the effect of PEA on the production of inflammatory factors induced by Aβ.

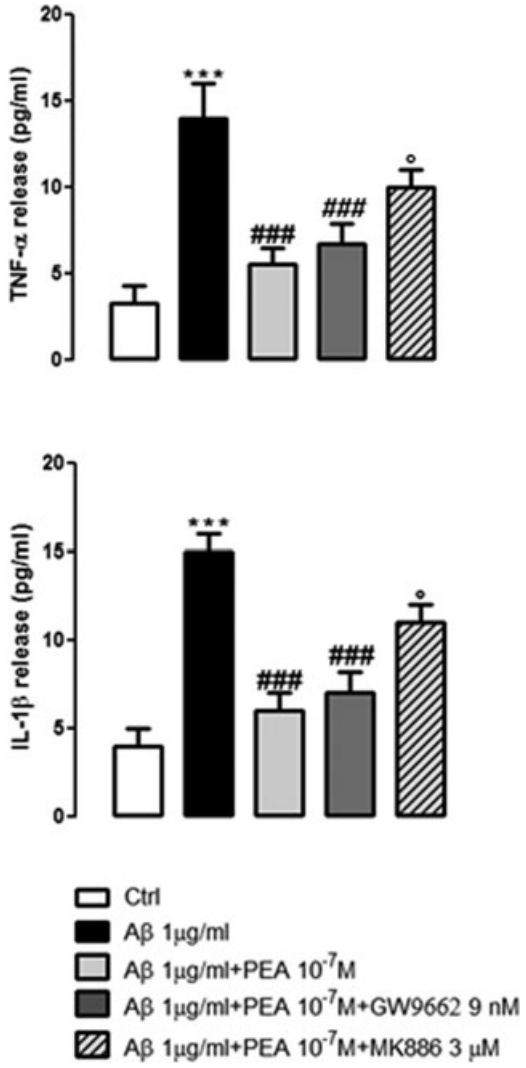

Fig. 3 PEA effects on Aβ-induced pro-inflammatory cytokine release. Aβ-challenged (1 μg/ml) cells were treated with PEA (10^{-7} M) in the presence of the selective PPAR-γ antagonist (GW9662, 9 nM) or the selective PPAR-α antagonist (MK886, 3 μM). TNF-α and IL-1β release was measured after 24 hrs of treatments by ELISA assay. Each bar shows the mean ± S.E.M. of $n = 3$ separate experiments. ***$P < 0.001$ *versus* control; ###$P < 0.001$ *versus* Aβ-challenged cells; °$P < 0.05$ *versus* Aβ + PEA-challenged cells.

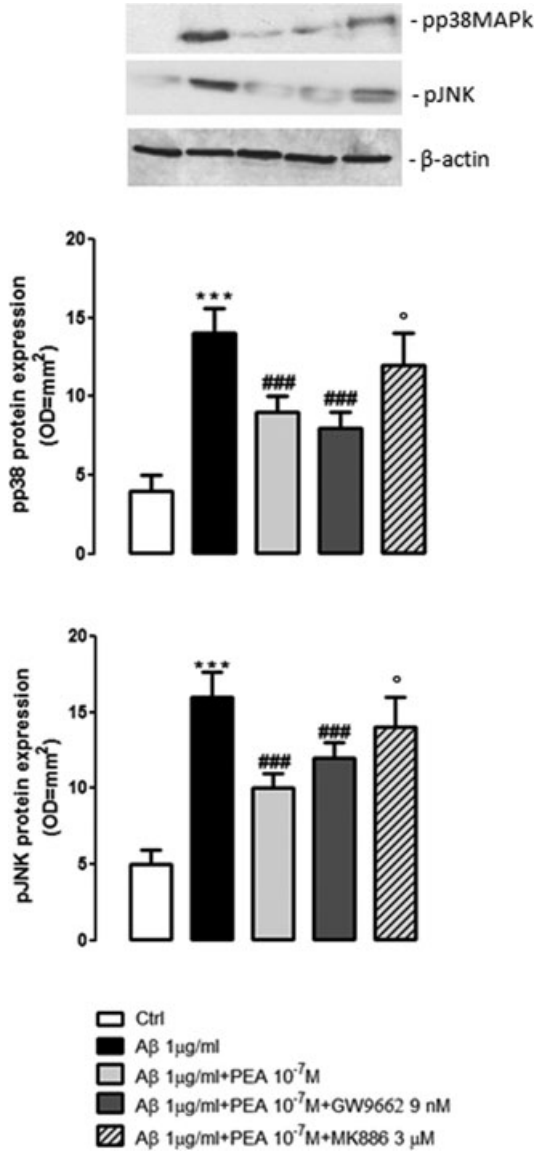

Fig. 4 Anti-inflammatory actions of PEA depend upon MAPK inhibition. Aβ-challenged (1 μg/ml) cells were treated with PEA (10^{-7} M) in the presence of the selective PPAR-γ antagonist (GW9662, 9 nM) or the selective PPAR-α antagonist (MK886, 3 μM). p38 and JNK phosphorylated protein expression was analysed after 30 min. of treatments by Western blot. β-actin was used as loading control. Each bar shows the mean ± S.E.M. of $n = 3$ independent experiments. ***$P < 0.001$ *versus* control; ###$P < 0.001$ *versus* Aβ-challenged cells; °$P < 0.05$ *versus* Aβ + PEA-challenged cells.

Treatment with Aβ for 24 hrs resulted in a significant increase in iNOS and COX-2 expression, as determined by Western blot and immunofluorescence analyses. Such protein increase paralleled nitric oxide, IL-1β, TNF-α and PGE2 up-release, as determined by Griess reaction and ELISA experiments. Also in this case PEA antagonized the enhancement of both expression and release of all pro-inflammatory molecules detected, and the blockade of PPAR-α with MK886 partly attenuated these effects (Figs 2 and 3).

The anti-inflammatory actions of PEA depend upon MAPK inhibition

Further experiments were aimed at investigating whether the increase in the production of inflammatory factors induced by Aβ

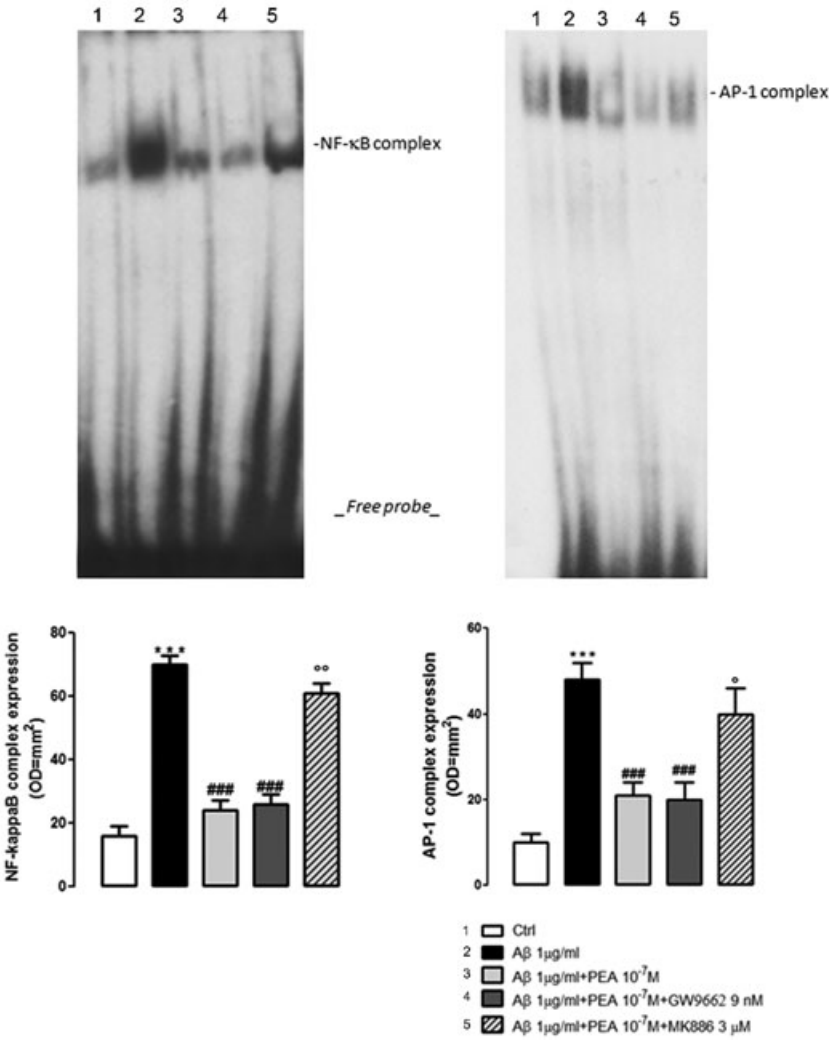

Fig. 5 PEA inhibits the Aβ-induced activation of NF-κB and AP-1 nuclear transcription factors. Aβ-challenged (1 μg/ml) cells were treated with PEA (10^{-7} M) in the presence of the selective PPAR-γ antagonist (GW9662, 9 nM) or the selective PPAR-α antagonist (MK886, 3 μM). Nuclear transcription factors activation was evaluated after 30 min. of treatments by EMSA analysis. Figure shows the results of NF-κB and AP-1 complex shifts (upper panel) and densitometric analysis of corresponding bands (lower panel). Treatment of astrocytes with Aβ induced the activation of the nuclear factors NF-κB and AP-1 (lines 2). PEA significantly inhibited this effect (lines 3). This inhibition was attenuated by the PPAR-α antagonist, MK886, in a partial, although significant, manner (lines 5). Each bar shows the mean ± S.E.M. of $n = 3$ separate experiments. $^{***}P < 0.001$ *versus* control; $^{\#\#\#}P < 0.001$ *versus* Aβ-challenged cells; $^{\circ\circ}P < 0.01$ and $^{\circ}P < 0.05$ *versus* Aβ + PEA-challenged cells.

was dependent on the activation of the MAPK pathway. Activation of the MAPKs, p38 MAPK and JNK, was measured by quantifying the level of phosphorylation of each kinase by Western blot 30 min. after the exposure to Aβ (Fig. 4). Treatment of astrocytes with Aβ resulted in an increase in the level of phosphorylation of the kinases, and it was accompanied with the activation of the nuclear factor NF-κB and AP-1, as shown by EMSA analysis (Fig. 5). PEA significantly inhibited MAPK phosphorylation and nuclear transcription factors, NF-κB and AP-1. This inhibition was attenuated by the PPAR-α antagonist, MK886, in a partly, although significant, manner.

PEA modulates the Aβ-induced alteration in PPAR transcription and expression

Additional experiments were aimed at investigating whether exposure to Aβ was able to affect PPAR transcription and expression. RT-PCR and Western blot analyses revealed that Aβ significantly down-regulated PPAR-α, but not PPAR-γ, transcription and expression. In the same experimental conditions, PEA was able to counteract the above reported alterations induced by Aβ (Fig. 6).

The endocannabinoid system in astrocytes treated with Aβ or PEA

When astrocytes were treated under the same conditions which lead to inflammation, an important increase in CB2 expression and a significant decrease in CB1 expression were observed (Fig. 7). In addition, Aβ exposure induced a marked increase in astrocyte levels of the endocannabinoid 2-AG, the preferential agonist at CB2 receptor. Moreover, the peptide challenge resulted in a clear enhancement of PEA and OEA, both able to bind to PPPAR-α.

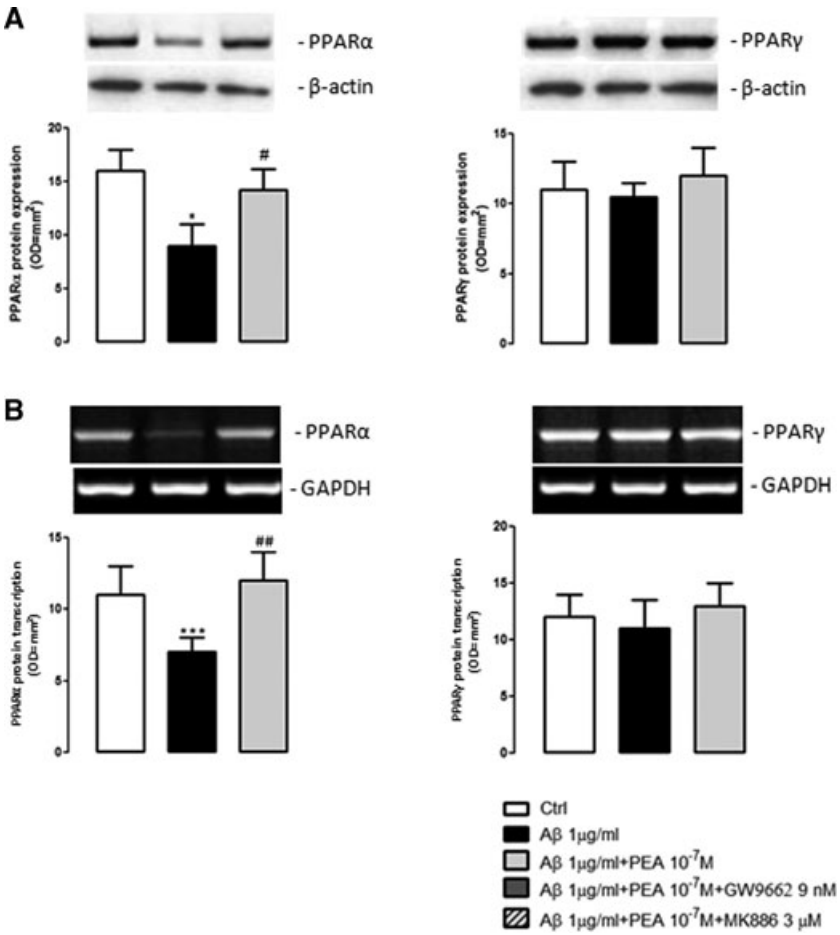

Fig. 6 PEA effect on PPARs transcription and expression. PPAR-α and PPAR-γ transcription and expression were evaluated in primary astrocytes after 24 hrs of exposure to Aβ (1 μg/ml), in the presence or absence of PEA (10^{-7} M). (**A**) Results of PPAR-α and PPAR-γ Western blot analysis and densitometric analysis of corresponding bands. β-actin was used as loading control. (**B**) Results of PPAR-α and PPAR-γ RT-PCR amplification and densitometric analysis of corresponding bands. GAPDH was used as standard control. Each bar shows the mean ± S.E.M. of $n = 4$ separate experiments. $***P <$ 0.001 and $*P < 0.05$ *versus* control; $\#\#P <$ 0.01 and $\#P < 0.05$ *versus* Aβ-challenged cells.

Treatment of astrocytes with PEA (10^{-7} M) alone resulted in an elevation of 2-AG levels. The effects of Aβ and PEA were not additive. In no other case were levels of the other endocannabinoid, AEA, significantly altered (Table 1).

Discussion

If a chronic state of astrocyte activation is believed to contribute substantially to the onset and progression of AD, then these cells may be reasonably considered to be a new target for novel and promising strategies helpful in the treatment of the disease [40]. Therefore, on the basis of this assumption, it become important to identify compounds which are able to mitigate astrogliosis and, consequently, to attenuate the detrimental over-expression of pro-inflammatory mediators.

To this purpose, the present study was aimed at investigating whether PEA would exhibit these properties in an *in vitro* model of Aβ neurotoxicity, and at exploring these possible effects. The results demonstrated that this ALIAmide is able to mitigate astrocyte reactivity and to blunt production of pro-inflammatory signals, suggesting a therapeutic potential for this compound.

Indeed the present findings show the capacity of PEA to significantly diminish either the altered expression of both COX-2 and iNOS, and the enhanced release of PGE2, nitric oxide, IL-1β and TNF-α. With regard to the intracellular signalling involved in Aβ-induced neuroinflammatory processes, it is well known that increases in the production of inflammatory factors are associated with the phosphorylation of MAP kinases, such as p-38 and JNK [41, 42], as well as with the activation of the nuclear transcription factors NF-kB and AP-1. All these factors are responsible for the expression of many inflammatory mediators, thus fostering a detrimental cycle in which neuroinflammation induces a self-sustained process [43–48]. In this context, our results provide evidence that PEA critically diminishes the Aβ-induced activation of p38 and JNK, as well as demonstrating that PEA reduces the subsequent activation of nuclear transcription factors, NF-kB and AP-1. This evidence helps to identify the molecular apparatus by which PEA contributes to down-regulate both astroglial reaction and pro-inflammatory signal overproduction.

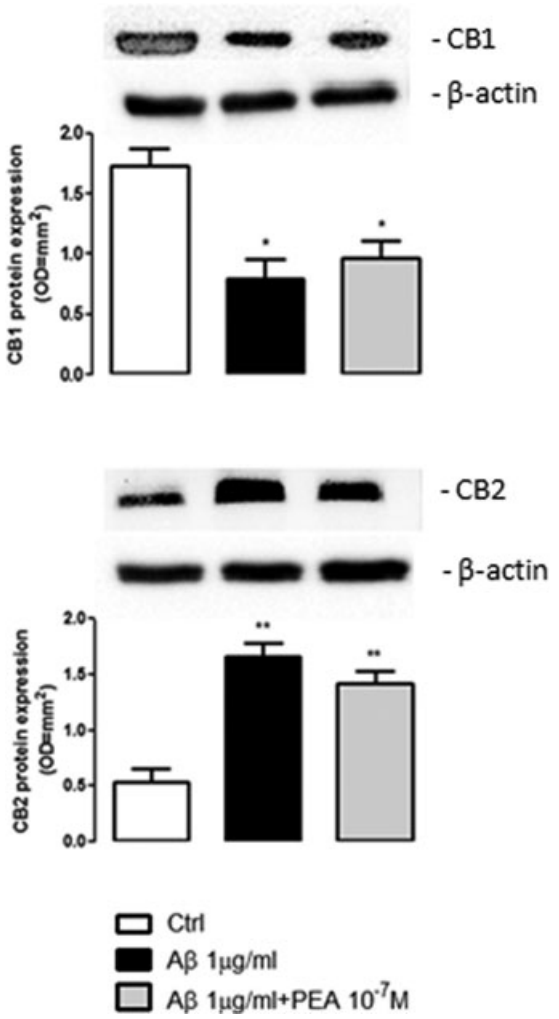

Fig. 7 PEA effect on cannabinoid receptors expression. The expression of CB1 and CB2 receptors was evaluated in primary astrocytes after 24 hrs of exposure to Aβ (1 μg/ml), in the presence or absence of PEA (10^{-7} M). Figure shows the results of Western blot and densitometric analysis of corresponding bands. β-actin was used as loading control. Each bar shows the mean ± S.E.M. of $n = 4$ independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ *versus* control.

Recent evidence suggests PEA works as an endogenous ligand at the PPAR-α nuclear site, where it binds with relatively high affinity to regulate gene expression [49]. The notion that PPAR-α is an important site at which PEA induces its effects is strongly supported by the reported failure of PEA to exert several of its pharmacological actions in mutant PPAR-$\alpha^{-/-}$ mice [18, 40]. Both PPAR-α and PEA have been detected in the CNS and their expression may change during pathological conditions [50]. To verify the PPAR-α involvement, further experiments were carried out using selective PPAR antagonists. Interestingly, findings clearly indicate the involvement of PPAR-α, because all effects reported in this study were significantly attenuated when PPAR-α was blocked by its selective antagonist, MK886. Taken together the results of the present study, in line with previous literature, recognize PPAR-α as a crucial, although not exclusive, site at which PEA generates its actions. To this regard both transcription and expression of PPARs were investigated. Findings demonstrate the ability of Aβ to significantly blunt PPAR-α expression in cultured primary rat astrocytes, thus raising the possibility that the down-regulation of PPAR-α may represent one of the molecular mechanism by which Aβ peptide exerts its toxicity. On the other hand, the results of these experiments also show the ability of PEA to restore the attenuated transcription and expression of PPAR-α induced by Aβ, implying that this property might in part contribute to its neuroprotective effects.

Table 1 Effect of Aβ and PEA on endocannabinoid, PEA and OEA levels in cultured astrocytes

Treatment	AEA	2-AG	PEA	OEA
Vehicle	0.8 ± 0.2	40.9 ± 6.9	8.6 ± 0.2	9.5 ± 0.9
PEA (10^{-7} M)	0.8 ± 0.3	69.2 ± 5.7*	12.6 ± 0.9**	11.3 ± 2.3
Aβ	0.8 ± 0.2	139.4 ± 15.1***	10.4 ± 0.4*	17.1 ± 2.4*
PEA + Aβ	0.9 ± 0.2	123.1 ± 25.6*	13.2 ± 1.1*	17.3 ± 2.4*

Data are expressed as pmol per dish and are means ± S.E.M. of $n = 4$ separate measurements.
Means were compared by ANOVA followed by the Bonferroni's test.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ *versus* vehicle.

Furthermore, PEA is an endogenously produced endocannabinoid-like molecule that is thought to affect the endocannabinoid system [50]. Moreover, a growing body of evidence shows a marked perturbation of this system both in AD patients and in animal models of the disease. On the basis of these considerations, additional experiments were performed in order to investigate the endocannabinoid levels and the expression of their receptors in the model here used and to better elucidate the role of PEA. In agreement with our previous data [51], the present investigation demonstrates a marked elevation of both 2AG and CB2 receptors induced by the exposure to Aβ peptide, confirming that changes in the endocannabinoid system occur on activated astrocytes following Aβ challenge. Interestingly, treatment of resting astrocytes with exogenous PEA also results in a slight, although significant, enhancement of 2AG level, suggesting that this ALIAmide is able to influence endocannabinoid signals. However, because PEA does not up-regulate 2-AG in Aβ-exposed astrocytes, probably because the concentration of this endocannabinoid already results maximally stimulated by the peptide, therefore, it is unlikely that, under the experimental conditions used here, PEA would counteract Aβ-induced astrogliosis even through the elevation of 2-AG. Nevertheless, future research should better elucidate the mutual relationships between PEA and the endocannabinoid system implicated in the modulation of astrogliosis.

In addition, Aβ enhances the levels of PEA and OEA, its congener and the more potent PPAR-α ligand, indicating that these compounds are up-regulated in activated astrocytes, possibly to antagonize the effects of PPAR-α down-regulation caused by Aβ exposure.

In conclusion, the current study describes the ability of PEA to mitigate astrocyte activation and neuroinflammatory process induced in primary astrocytes exposed to Aβ. The relevance of these findings resides in the assumption that pharmacological attenuation of excessive and prolonged reactive gliosis may serve as innovative strategies for therapies aimed at antagonizing the course of AD. This is particularly true considering that, in addition to the release of pro-inflammatory molecules, activated astrocytes neglect both homeostatic functions and modulation of microglia reactivity. Indeed, in a chronic inflammatory state a sustained astrocyte activation fails to attenuate microglia response to Aβ, contributing to perpetuate neuropathological processes. To summarize, this investigation expands our knowledge of the marked anti-inflammatory properties of this ALIAmide and also provides evidence for the molecular mechanisms possibly underlying the effects here observed.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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