RESEARCH ARTICLE

Systemic Administration of Oleoylethanolamide Protects from Neuroinflammation and Anhedonia Induced by LPS in Rats

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Abstract

Background: The acylethanolamides oleoylethanolamide and palmitoylethanolamide are endogenous lipid mediators with proposed neuroprotectant properties in central nervous system (CNS) pathologies. The precise mechanisms remain partly unknown, but growing evidence suggests an antiinflammatory/antioxidant profile.

Methods: We tested whether oleoylethanolamide/palmitoylethanolamide (10 mg/kg, i.p.) attenuate neuroinflammation and acute phase responses (hypothalamus-pituitary-adrenal (HPA) stress axis activation, thermoregulation, and anhedonia) induced by lipopolysaccharide (0.5 mg/kg, i.p.) in rats.

Results: Lipopolysaccharide increased mRNA levels of the proinflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6, nuclear transcription factor-κB activity, and the expression of its inhibitory protein IκBα in cytoplasm, the inducible isoforms of nitric oxide synthase and cyclooxygenase-2, microsomal prostaglandin E2 synthase mRNA, and proinflammatory prostaglandin E2 content in frontal cortex 150 minutes after administration. As a result, the markers of nitrosative/oxidative stress nitrites (NO₂⁻) and malondialdehyde were increased. Pretreatment with oleoylethanolamide/palmitoylethanolamide reduced plasma tumor necrosis factor-α levels after lipopolysaccharide, but only oleoylethanolamide significantly reduced brain tumor necrosis factor-α mRNA. Oleoylethanolamide and palmitoylethanolamide prevented lipopolysaccharide-induced nuclear transcription factor-κB (NF-κB)/IκBα upregulation in nuclear and cytosolic extracts, respectively, the expression of inducible isoforms of nitric oxide synthase, cyclooxygenase-2, and microsomal prostaglandin E2 synthase and the levels of prostaglandin E2. Additionally, both acylethanolamides reduced lipopolysaccharide-induced...
oxidative/nitrosative stress. Neither oleoylethanolamide nor palmitoylethanolamide modified plasma corticosterone levels after lipopolysaccharide, but both acylethanolamides reduced the expression of hypothalamic markers of thermoregulation interleukin-1β, cyclooxygenase-2, and prostaglandin E2, and potentiated the hypothemic response after lipopolysaccharide. Interestingly, only oleoylethanolamide disrupted lipopolysaccharide-induced anhedonia in a saccharine preference test.

**Conclusions:** Results indicate that oleoylethanolamide and palmitoylethanolamide have antiinflammatory/neuroprotective properties and suggest a role for these acylethanolamides as modulators of CNS pathologies with a neuroinflammatory component.

**Keywords:** OEA, PEA, lipopolysaccharide, neuroinflammation, anhedonia

**Introduction**

Endogenous lipid transmitters derived from membrane precursors are a current focus of investigation due to the wide range of biological functions in which they participate, including modulation of neurotransmitter release, neuroplasticity, synaptogenesis, neurogenesis, brain information processing, and cellular energetic systems (Orio et al., 2013). Fatty acid acylethanolamides are endogenous lipid mediators with multiple physiological functions that include the endocannabinoid anandamide (arachidonylethanolamide [AEA]) and the noncannabinimimetic compounds N-oleoylethanolamide (OEA) and N-palmitoylethanolamide (PEA). Though involved in different functions, the acylethanolamides share biosynthetic and degradative mechanisms. They are synthesized on demand through a phospholipase D enzyme acting on a membrane phospholipid precursor, which is synthesized by a CAMP and Ca2+-dependent N-acetyltransferase (Piomelli, 2003).

Upon its release, they experience reuptake by a catalytically silent fatty acid amide hydrolase (FAAH)-1 variant (Fu et al., 2011) and are degraded through enzymatic hydrolysis by specific FAAH (Schmid et al., 1985; Cravatt et al., 1996).

OEA and PEA are structurally related compounds that act mainly thought the nuclear peroxisome proliferator-activated receptor-alpha (PPAR-α) (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Lo Verme et al., 2005; Di Cesare Mannelli et al., 2013), although they might bind the transient receptor potential vanilloid type-1 (Overt et al., 2006; Almasi et al., 2008; Godlewski et al., 2009), the G protein-coupled receptors GPR55 and GPR119 (Overt et al., 2006; Godlewski et al., 2009), or other PPAR isoforms (Paternity et al., 2013; but see Fu et al., 2003; LoVerme et al., 2006). OEA is known as a satiety factor (Rodriguez de Fonseca et al., 2001; Fu et al., 2003), and both PEA and OEA act as analgesics in inflammatory and neuropathic pain (Lo Verme et al., 2005; Suardiaz et al., 2007; Di Cesare Mannelli et al., 2013).

Growing evidence indicates that OEA and PEA may have neuroprotective properties in neurological disorders such as stroke (Sun et al., 2007; Zhou et al., 2012; Ahmad et al., 2012a), traumatic brain injury (Ahmad et al., 2012b), Parkinson’s disease (Gonzalez-Aparicio et al., 2013; Gonzalez-Aparicio and Moratalla, 2013), or addiction (Melis et al., 2008; Plaza-Zabala et al., 2010; Bilbao et al., 2013; Coppola and Mondola, 2013). Some of the mechanisms implicated are the modulation of antioxidant responses, neuroinflammation, glial cell proliferation/differentiation, neurogenesis, and neurotransmission.

Given the significance and complexity of neuroinflammation in the physiopathology of central nervous system (CNS) diseases, we studied the role of OEA and PEA as modulators of the inflammatory/immune response after a lipopolysaccharide (LPS) challenge. LPS is a component of the outer membrane on gram-negative bacteria that is extensively used for neuroinflammation modeling. Systemic LPS injection to experimental animals elicits a multisystemic response that includes immune, endocrine, metabolic, and behavioral components known as the acute-phase response and sickness behavior (Hart, 1988; Konsman et al., 2002; Kushner and Rzewniki, 1997).

We tested the efficacy of OEA and PEA to modulate the canonical proinflammatory pathway triggered by the activation of the nuclear factor-κB (NF-κB) (Madrigal et al., 2001) after LPS and evaluated the acute-phase responses described as activation of hypothalamic-pituitary axis (HPA) (increases in plasma corticosterone), changes in hypothalamic markers of thermoregulation (interleukin [IL]-1β, cyclooxygenase [COX]-2, and prostaglandin [PGE]2), and behavioral malaise (by checking motivational behavior).

**Methods**

**Animals**

Ninety-four male outbred Wistar Hannover rats (HsdRccHan:Wist, from Harlan, Spain), weighing 350 to 400 g, were housed in groups (n=5–6) and maintained at a constant temperature of 24±2°C at a relative humidity of 70±5% in a 12-hour light-dark cycle (lights on at 8:00 AM). Animals were fed a standard pellet chow (A04 SAFE, Scientific Animal Food and Engineering, Augy, France) with fresh water and were maintained under constant conditions for 10 days prior to experiments. All experimental protocols were approved and followed the guidelines of the Animal Welfare Committee of the Universidad Complutense of Madrid according to European legislation (2010/63/UE).

**Drug Administration**

LPS (serotype O111:B4, ref. L2630 Sigma, Spain) was dissolved in saline and injected i.p. at 0.5 mg/kg. The dose was chosen according to previous reports to induce neuroinflammation (MacDowell et al., 2013). OEA (10 mg/kg, i.p.; synthesized in our laboratory; Giuffrida et al., 2000) and PEA (10 mg/kg, i.p.; Tocris, Spain) were dissolved in vehicle (5% Tween 80 in saline) and injected 10 minutes before LPS administration. The doses were chosen according to previous studies in rodents reporting anti-inflammatory/neuroprotective effects (Plaza-Zabala et al., 2010; Ahmad et al., 2012a, 2012b; Zhou et al., 2012).

**Tissue Samples and Plasma Collection**

Brain tissue samples were taken 150 minutes after LPS injection using a lethal dose of sodium pentobarbital (300 mg/kg, i.p., Dolophel, Spain). The timing of sacrifice after LPS was chosen on the basis of previous studies showing an NF-κB-dependent pro-inflammatory response in the frontal cortex of Wistar rats at this time point (Perez-Nieves et al., 2010; MacDowell et al., 2013). Brains were isolated from the skull, and meninges and blood vessels were carefully discarded. The frontal cortex and hypothalamus were excised and frozen at -80°C until assayed. Blood was collected by...
cardiac puncture using trisodium citrate (3.15% wt/vol) as anticoagulant. Plasma was obtained by blood centrifugation (2000 g) 15 minutes at 4°C and stored at -20°C until determinations.

Rat brain frontal cortex was chosen because of its high levels of proinflammatory/antiinflammatory mediators and its susceptibility to the neuroinflammatory process elicited by LPS (Garcia-Bueno et al., 2008) and because this brain area is an important neural substrate for the regulation of the HPA axis response to an immune/inflammatory challenge (Radley et al., 2006). Hypothalamus is the main brain area involved in thermoregulation and fever (Super, 1998).

Preparation of Nuclear and Cytosolic Extracts

A modified procedure based on the method of Schreiber and colleagues (Schreiber et al., 1989) was used. Briefly, brain frontal cortex and hypothalamus samples were homogenized in 300 μL buffer (10 mmol/L N-2-hydroxyethyl piperoxane-N-2-ethanesulfonic acid (pH 7.9); 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 10 mmol/L KCl, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL Nap-tosyl-lysine-chloromethyl ketone, 5 mmol/L NaF, 1 mmol/L NaVO₄, 0.5 mmol/L sucrose, and 10 mmol/L Na₂MoO₄). After 15 minutes, 0.5% Nonidet P-40 (Roche, Mannheim, Germany) was added.

The tubes were vortexed and nuclei were collected by centrifugation at 8000 g for 5 minutes. Supernatants were considered as the cytosolic fraction. The resuspended pellets were resuspended in 100 μL buffer supplemented with 20% glycerol and 0.4 mol/L KCl and shaken for 30 minutes at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 g for 5 minutes, and aliquots of the supernatant were stored at -80°C. All steps of the fractionation were carried out at 4°C.

Western-Blot Analyses

To determine the expression levels of the enzymes inducible nitric oxide synthase (iNOS) and COX-2, brain frontal cortices and hypothalamus were homogenized by sonication in 400 μL of phosphate-buffered saline (pH = 7) mixed with a protease inhibitor cocktail (Complete, Roche, Madrid, Spain) followed by centrifugation at 12,000 g for 10 minutes at 4°C. After adjusting protein levels in the supernatants, homogenates were mixed with Laemmli sample buffer (Bio Rad, CA) and 10 μL (1 mg/mL) was loaded into an electrophoresis gel.

Membranes were blocked in 10 mM Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk/bovine serum albumin (BSA) and incubated with specific primary antibodies: IxBα (rabbit polyclonal antibody against an epitope mapping at the C-terminus of IxBα of human origin; dilution 1:1000 in 5% skimmed milk in BSA, Santa Cruz Biotechnology, CA); iNOS (rabbit polyclonal antibody against a peptide mapping at the amino terminus of iNOS of human origin; dilution 1:1000 in TBS-Tween, Santa Cruz Biotechnology, CA); COX-2 (goat polyclonal antibody against a peptide mapping at the C-terminus of COX-2 of human origin; dilution 1:750 in 5% BSA in TBS-Tween, Santa Cruz Biotechnology, CA). After washing with Tween 20, the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 90 minutes at room temperature. Blots were imaged using an Odyssey Fc System (Li-COR Biosciences), quantified by densitometry (NIH Image software), and expressed in arbitrary units of optical density. The housekeeping gene β-actin was used as loading control.

Real Time-Polymerase Chain Reaction Analysis

Total cytoplasmic RNA was prepared from samples of frontal cortex or hypothalamus using TRIZOL reagent (Invitrogen, Grand Island, NY); aliquots were converted to complementary DNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by real time-polymerase chain reaction (RT-PCR) using the following cycling conditions: 35 cycles of denaturation at 95°C for 10 seconds, annealing at 58–61°C for 15 seconds depending on the specific set of primers, and extension at 72°C for 20 seconds. Reactions were carried out in the presence of SYBR green (1:10,000 dilution, Molecular Probes, Eugene, OR) in a 20-L reaction in a Rotor-Gene (Corbett Research, Mortlake, Australia). The primers used were to detect IL-1β, IL-6, TNF-α, NF-kB p65 subunit, IxBα, iNOS, COX-2, and m-PGES-1 (sequence details in Table 1). Relative mRNA concentrations were obtained by comparing the take-off point of the different samples using the software provided in the unit. It establishes an inverse correlation between the number of cycles before take-off and the concentration of mRNA, while assigning arbitrary units to the results. Tubulin and GADPH primer levels were used to normalize data (results are shown using tubulin normalization).

Plasma Cytokine Determination

IL-1β and TNF-α plasma levels were determined using commercially available enzyme-linked immunosorbent assays (RayBiotech). Plasma samples were 1:2 diluted and assayed following the manufacturer’s guidelines. Quantification was performed using a standard curve of increasing cytokines’ concentrations. The optical density was measured

**Table 1. RT-PCR Primer Sequence Details**

| Forward Primers (3’-5’) | Reverse Primers (5’-3’) |
|------------------------|------------------------|
| IL-1β                  | ACGTTGCACTATGTGATGTTTCCCAG | ACGTTGAGAGCTTCTACATCATCACAT |
| IL-6                   | AAGCTGACGACGGGACTGACAAG | GAGCTGGCAGACGCTCTACG |
| TNF-α                  | CTGACGAGATCAGATCTGAAAA | ATGAAATGCGGAATGCTGGAGG |
| NFκB p65               | CATGCTGGCTTCAATACTGGGA | TGGTTGCTTCTGATGAGATGQTG |
| IκBα                   | TGGCTCTTCTCAACTTCCAGAACAA | TCAGATCAGACGACCGTCTTCCAGA |
| iNOS                   | GACACACACTCTTGACAGGA | CTCATGATAAACCTTCTGGC |
| COX-2                  | CTCTGGAGACCAACACAG | GCGAGTCCACGTGATAGAG |
| m-PGES-1               | GGTGAAGCGAATAAGCTGCAGCAG | TTATGCGCTTGCACAGCACAGCAGAG |
| Tubulin                | CTCGCCGGACGTTAATACAT | ACTGATGATGATCCTTGGTCT |
| GAPDH                  | TGACACACACAAGCCTTAAGC | GGCACTGGACATGTTAGCAG |

Abbreviations: COX, cyclooxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; m-PGES-1, microsomal prostaglandin E2 synthase; NF, nuclear factor; RT-PCR, real time-polymerase chain reaction; TNF, tumor necrosis factor.
using a microplate reader (Synergy 2; BioTek Instruments) set to 450 nm. The sensitivities of the assays were <80 pg/mL for IL-1β and <25 pg/mL for TNF-α. Intra-assay and inter-assay coefficients of variation were <10% and 12%, respectively, for both kits.

###Nitrites (NO₃⁻) Levels

As the stable metabolites of the free radical nitric oxide (NO), NO₃⁻ were measured by using the Griess method (Green et al., 1982). In an acidic solution with 1% sulphanilamide and 0.1% N-(1-Naphthyl)ethylenediamine (NEDA), nitrites convert into a pink compound that is photometrically calculated at 540 nm in a microplate reader (Synergy 2; BioTek).

###Lipid Peroxidation

Lipid peroxidation was measured by a modification of the method of Das and Ratty (1987), whereby the thiobarbituric acid reacting substances, predominantly malondialdehyde (MDA), produced as a secondary product were quantified by use of the 2-thiobarbituric acid (TBA) color reaction. Brain tissue was homogenized in 10 volumes (wt/vol) of sodium phosphate buffer (pH 7.4). Assays contained tissue homogenate, trichloroacetic acid (40% wt/vol), HCl (5 M), and TBA (2% wt/vol). Samples were heated for 15 minutes at 90°C and centrifuged at 12 000 g for 10 minutes. The MDA-TBA adduct (pink chromogen) of the supernatant was measured spectrophotometrically (532 nm) and the MDA concentration calculated by use of a standard curve prepared with MDA tetra-butylammonium salt. The results were expressed as nmol/mg protein.

###Plasma Corticosterone

Corticosterone was measured in plasma by using a commercially available kit by RIA Coat-a-Count (Siemens, Los Angeles, CA). A gamma counter (Wallac Wizard 1470, Perkin Elmer, Waltham, MA) was used to measure radioactivity of the samples. The time of blood extraction and plasma collection oscillated between 1:00 pm and 3:00 pm.

###NF-xB Transcription Factor Assay

NF-xB transcription factor activity was determined in nuclear extracts by using an enzyme-linked immunosorbent assay-based kit (Cayman Chemicals, Tallinn, Estonia). Nuclear extracts were incubated with specific NF-xB p65 subunit response element probes, and p65 bound to its response element probe was detected using a specific antibody against this subunit. Horseradish peroxidase-labeled secondary antibody was added and the binding was detected by spectrophotometry. Measurement was performed according to the manufacturer’s instructions. This assay is specific for p65 activation, and it does not cross-react with other NF-xB subunits, such as p50.

###PGE₂ Determination

PGE₂ levels were measured by commercially available enzyme immunoassay (PGE, EIA Kit-Monoclonal; Cayman Chemical, Tallinn, Estonia). Samples were sonicated in 400 mL homogenization buffer (0.1M phosphate buffer, pH=7.4, 1 mM EDTA, and 10 mM indomethacin), purified in 4 volumes ethanol for 5 minutes at 4°C, centrifuged at 3000 g for 10 minutes, and acidified with glacial extracted using SPE (C-18) acetic acid (pH = 3.5). PGE₂ was extracted using SPE (C-18) columns (Waters, MA) rinsed with methanol and water. After sample’s application, columns were washed with water and hexane and PGE₂ was eluted with ethyl acetate. Samples were evaporated to dryness under nitrogen and resuspended in enzyme immunoassay buffer. PGE₂ levels were measured in a 96-well plate and read at 405 nm following the manufacturer’s instructions (Synergy 2; BioTek Instruments). The sensitivity of the assay for PGE₂ was 15 pg/mL, intra- and interassay coefficients of variation were 6.6% and 15.5%, respectively.

###Protein Assay

Protein levels were measured using the Bradford method (Bradford, 1976).

###Measurement of Rectal Temperature

Rectal temperature was measured by the use of a digital readout thermocouple (BAT12 thermometer, Physitemp) with a resolution of 0.1°C accuracy of ±0.1°C attached to a RET-2 Rodent Sensor, which was inserted 2.5 cm into the rectum of the rat, the animal being lightly restrained by holding it in the hand of a trained individual to avoid stress-confounding factors. A steady readout was obtained within 10 seconds of probe insertion.

###Saccharine Preference Test

Rats fed ad libitum were housed individually and were offered a free choice between 2 bottles located in the cages in a random manner, one with a 0.1% saccharin solution and another with tap water, during the time of the experiment (30 hours). Separated groups of animals were used to test the thermic response and the preference for saccharine. The consumption of water and saccharin solution was recorded at specific time intervals after pharmacological treatments. The preference for saccharin was calculated as consumed saccharin solution/total fluid intake. No previous food or water deprivation was applied before the test.

###Statistical Analyses

Data in text and figures are expressed as mean ± SEM. Data were analyzed by 2-way ANOVA comparing 2 factors: inflammation (vehicle or LPS) and pretreatment (vehicle, OEA, PEA), followed by Bonferroni posthoc test when appropriate. Data on saccharine preference test, total fluid intake, and rectal temperatures were analyzed by 2-way repeated-measures ANOVA using treatment as a between-subjects factor and time as a repeated measure, followed by Bonferroni posthoc test. The behavioral experiments (rectal temperatures and saccharine preference test) and Western blots were performed independently for OEA and PEA, so the 2-way ANOVA were run accordingly (reported in results in this order: OEA and PEA). Additionally, in the behavioral experiments, we ran a 2-way ANOVA comparing the 2 factors (inflammation and pretreatment) at specific time points of the temporal curves: we chose 1 hour and 3 hours after LPS in the temperature curves, since the hypothalamic markers were studied at a time in between (2.5 hours); in the saccharine preference test, we chose the last time point of the test (30 hours), since it represents an accumulated measure over time. A P value ≤ .05 was considered statistically significant. Data were analyzed using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA).
Results

Effect of OEA and PEA on Proinflammatory Cytokines in Frontal Cortex

LPS administration increased mRNA expression of the proinflammatory cytokines TNF-α (Figure 1A; F(1,18) P = 5.97, P = .0245), IL-1β (Figure 1B; F(1,24) P = 10.34, P = .0037), and IL-6 (Figure 1C; F(1,19) P = 22.93, P = .0001) in frontal cortex 150 minutes after administration. Pretreatment with OEA significantly reduced the increase in TNF-α mRNA levels induced by LPS (interaction effect: F(2,19) P = 6.177) and had no significant effect on IL-1β and IL-6 in the presence or absence of LPS. Although PEA reduces LPS-induced increase in TNF-α and IL-1β (but not IL-6) mRNA, posthoc test revealed that these effects failed to reach statistical significance.

To test whether the CNS effects of acylethanolamides may be affected by peripheral modulation of circulating cytokines, we measured TNF-α and IL-1β in plasma after treatments. Figure 1D (graph box) shows that both OEA and PEA modified the increase in plasma TNF-α observed after LPS injection (interaction effect F(2,47) P = 4.566; P = .0097). Levels of plasma IL-1β were not affected by the treatments (data not shown).

Effect of OEA and PEA in the Activation of Proinflammatory NF-κB

The release of proinflammatory cytokines TNF-α, IL-1β, and IL-6 after LPS may account for NF-κB activation, so we studied the mRNA expression and activity of NF-κB proinflammatory subunit p65 (Figure 2A-B) and its inhibitory protein IκBα (Figure 2C-E). LPS increased p65 subunit (F (1,18) P = 13.37, P = .0011) in nuclear extracts of frontal cortex, which is inhibited by OEA pretreatment at the level of mRNA (Figure 2A; F(2,18) P = 12.54, P = .0009) and activity (Figure 2B; interaction effect F(2,15) P = 5.313, P = .0180). PEA administration reduced the p65 mRNA expression (Figure 2A; F(2,18) P = 5.313, P = .018) but had no significant effect in the activity assay (Figure 2B; F(2,16) P = .7028, P = .5108). LPS also induced an upregulation of IκBα mRNA (Figure 2C; F(2,16) P = 25.60, P < .0001) and

Figure 1. Proinflammatory cytokines in frontal cortex (and plasma). Real time-polymerase chain reaction (RT-PCR) analysis of tumor necrosis factor (TNF)-α (A), interleukin (IL-)1β (B), and IL-6 (C) mRNAs in frontal cortex 150 minutes after lipopolysaccharide (LPS) administration. Data (n = 4–7 per group) are normalized by tubulin and are presented as means ± SEM. Data in D (graph box) represents plasma levels of TNF-α measured by ELISA. Different from control group: *P < .05, **P < .01, different from vehicle + LPS rats: #P < .05 (2-way ANOVA followed by Bonferroni posthoc test).
protein expression (F\(_{1,11}\)) = 22.79, P = .0008) in cytosolic extracts that was prevented by PEA (F\(_{1,19}\)) = 4.943, P = .0187) at the level of mRNA. Pretreatment with OEA reduced ixB\(_B\) protein expression in LPS-treated animals (interaction effect (F\(_{1,13}\)) = 39.48, P < .0001), whereas PEA had no significant effect at protein level.

**Proinflammatory Enzymes (COX-2 and iNOS): Effect of OEA and PEA**

NF-xB regulates the expression of genes involved in the accumulation of oxidative/nitrosative and inflammatory mediators after LPS exposure. Among others, 2 main sources of these mediators dependent on NF-xB are iNOS and COX-2. LPS induced an increase in iNOS protein expression (F\(_{1,19}\)) = 5.221, P = .0482) that was prevented by OEA (Figure 3A; interaction effect: F\(_{1,19}\) = 6.112, P = .0354) and by PEA (Figure 3B; interaction effect: F\(_{1,19}\) = 14.68, P = .004). Both acylethanolamides reduced iNOS mRNA expression in the LPS-treated condition (Figure 3C; interaction effect (F\(_{2,20}\)) = 10.44, P = .0013). Similarly, LPS-induced COX-2 upregulation (F\(_{1,19}\) = 13.97, P = .0353) was blocked by the respective preadministration of OEA and PEA (Figure 3D-E; interaction effects: F\(_{2,20}\)) = 61.66, P < .0001 and F\(_{1,11}\) = 9.388, P = .0135). COX-2 mRNA levels remain unchanged in all treatments at this time point (data not shown).

**Brain COX-2 and iNOS Main Products: PGE\(_2\) Synthesis and NO\(_2^-\) Accumulation. Effect of OEA and PEA**

The presumed major iNOS and COX-2 brain products, NO and PGE\(_2\), respectively, are potent oxidant/proinflammatory molecules that have been directly related to cellular damage/death in multiple CNS pathologies.

PGE\(_2\) is synthesized by a multienzymatic pathway in which the specific enzyme microsomal prostaglandin E\(_2\) synthase (mPGES-1) is the last step (Ivanov and Romanovsky, 2004). LPS increased mPGES-1 mRNA (Figure 4A; F\(_{1,20}\)) = 70.19, P < .0001) and PGE\(_2\) production (Figure 4B; F\(_{1,20}\)) = 9.574, P = .063) in cortical tissue. As can be observed in Figure 4A-B, both OEA and PEA prevented the LPS-induced upregulation of mPGES-1 and PGE\(_2\) (interaction effects: F\(_{1,20}\)) = 13.20, P = .0004 and (F\(_{2,20}\)) = 3.074, P = .0711).

Figure 4C shows the accumulation of the main NO metabolite, NO\(_2^-\), after LPS (F\(_{1,19}\)) = 5.692, P = .0276) that was prevented by pretreatment with both OEA and PEA (main effect of pretreatment (F\(_{1,19}\)) = 6.203, P = .0084; interaction F\(_{2,19}\) = 4.733, P = .63).

**Lipid Peroxidation: Effect of OEA and PEA**

As a marker of cellular damage elicited by oxidative/nitrosative stress, lipid peroxidation was assessed by measuring MDA accumulation. Figure 4D shows that OEA and PEA pretreatments prevented the LPS-induced overaccumulation of MDA in frontal cortex (effect of pretreatment F\(_{1,19}\)) = 10.57, P = .0008 and interaction F\(_{2,19}\) = 4.009, P = .0353).

**Effects of OEA and PEA on Plasma Corticosterone Levels**

The quantification of plasma corticosterone levels at the time of blood extraction (1:00-3:00 pm) revealed an expected corticosterone increase in LPS-injected animals (F\(_{1,20}\)) = 18.29, P = .0003). The LPS-induced increase in corticosterone levels rose in 42% over control values (control: 244.10 ± 13.8 ng/mL). Interestingly, at that time point, neither OEA (51.3% over controls) nor PEA (57.57% over controls) prevented the increase in corticosterone induced by LPS, suggesting that the mechanism of these compounds modulating neuroinflammation is independent of systemic corticosterone levels (Table 2).
Hypothalamic Markers of Thermoregulation: Effects of OEA and PEA

As another acute-phase response after LPS administration, we studied the expression of molecular markers related with temperature regulation in the hypothalamus.

The pyrogenic and proinflammatory cytokine IL-1β increased its mRNA up to 6 times in hypothalamus after LPS administration ($F(1, 26) P = 14.62, P = .0007$), and this increase was blocked by OEA and PEA pretreatments (Figure 5A; interaction effect: $F(2, 26) P = 4.982, P = .0147$). Similarly, LPS induced an upregulation of COX-2 mRNA in hypothalamus (Figure 5B; $F (1, 25) P = 12.60, P = .0016$) that was prevented by OEA and PEA (interaction effect: $F(2, 25) P = 8.285, P = .0017$).

PGE2, one of the major COX-2 products, is presumably a mediator of temperature deregulation after LPS (Ivanov and Romanovsky, 2004). As represented in Figure 5C-D, OEA and PEA prevented the mRNA upregulation of its synthesis enzyme mPGES-1 (interaction effect: $F_{(2,28)} P = 5.950, P = .0070$) and the PGE2 accumulation (interaction: $F_{(2,20)} P = 6.132, P = .0113$) induced by LPS in hypothalamus, suggesting an involvement of both acylethanolamides in the acute-phase responses of LPS related with body temperature regulation.

Thermic Response

Figure 5E and F show the temperature deregulation after LPS injection. Three basal temperatures were recorded every 30 minutes before LPS administration. The media of the 2 first basal temperatures ($t = -1.0$ hour and $-0.5$ hour) was represented as “B” in the figures. Arrow indicates time of LPS injection. Basal temperatures immediately before LPS injection ($t = 0$) did not differ significantly between groups of treatments ($F_{(3,23)} P = .58, P = .65, n.s.$, and $F_{(3,20)} P = 1.15, P = .037, n.s.$, for OEA (Figure 5E) and PEA (Figure 5F) experiments, respectively) Analysis of the temperature temporal curves by repeated measures 2-way
ANOVA showed interactions between time and treatment ($F_{(15,100)} = 6.696, P < .0001$ and $F_{(1,100)} = 14.60, P < .0001$) and main effects of time ($F_{(5,100)} = 19.0, P < .0001$ and $F_{(1,100)} = 27.12, P < .0001$) and treatment ($F_{(3,100)} = 4.91, P = .0108$ and $F_{(3,100)} = 51.35, P < .0001$). Additional analyses revealed that LPS induced a hypothermic response immediately after the injection and up to 3 to 6 hours posttreatment. Two-way ANOVA at specific time points revealed that pretreatments with OEA and PEA potentiated the hypothermic response 60 minutes after LPS ($F_{(1,100)} = 4.01, P < .05$). Pretreatment with PEA was significantly more effective than OEA ($F = 10.25, P < .0001$) and main effects of time ($F_{(5,100)} = 51.35, P < .0001$) and treatment ($F_{(3,100)} = 3.83, P = .024$) and treatment ($F_{(3,100)} = 7.443, P = .0077$ and $F_{(3,100)} = 19.17, P < .0001$). Subsequent analyses revealed that levels of time and treatment are not significantly different from OEA pretreatment. OEA pretreatment had no effect in this motivational test. The comparison between the factors inflammation and pretreatment by 2-way ANOVA at the time point of 30 hours posttreatment revealed no effect of OEA pretreatment in the same condition ($F_{(1,100)} = 25.92, P < .0001$) and no effect of PEA in the same condition ($F_{(3,100)} = 0.12, P = .92, n.s.$). The total amount of liquid (water + saccharine solution) drunk by the animals in this test differs significantly between control and LPS-treated animals. OEA or PEA did not modify this LPS-induced effect (Table 3).

### Discussion

Recent studies have demonstrated that PEA and OEA endogenous levels are regulated in several CNS pathologies (Baker et al., 2001; Hansen et al., 2001; Schabitz et al., 2002; Berger et al., 2004; Degn et al., 2007; Bisogno et al., 2008; Hill et al., 2009; Hauer et al., 2013) and in acute inflammatory conditions induced by LPS (Balvers et al., 2012). Because of the proposed homeostatic protective role for both bioactive lipids, this acute response could be considered as part of an antiinflammatory protective homeostatic response regulating cell survival and damage (Fidaleo et al., 2014). Herewith, to further investigate the role of both acylethanolamides as a possible homeostatic mechanism in the brain, we decided to explore whether their exogenous administration might serve as a new neuroprotective pharmacologic manoeuvre.

Our study provides new evidence of the brain antiinflammatory properties of OEA and PEA in a model of neuroinflammation in vivo. Our previous data indicate that OEA crosses the

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**Proxstaglandin (PG)E2 synthesis and release, nitrite accumulation, and lipid peroxidation in frontal cortex.**

- **A** Real-time polymerase chain reaction (RT-PCR) analysis of the PGE2 synthesis enzyme microsomal prostaglandin E2 synthase (mPGES-1).
- **B** PGE2 levels measured by enzyme immunoassay.
- **C** NO synthesis.
- **D** Malondialdehyde accumulation as marker of lipid peroxidation.

Data (n=3–6) are represented as means ± SEM. Different from control group: *P < .05, **P < .01, ***P < .001; different from vehicle + lipopolysaccharide (LPS): $P < .05, *P < .01, **P < .001$; different from palmitoylethanolamide (PEA) + saline: $P < .05$ (2-way ANOVA followed by Bonferroni post hoc test).
blood-brain barrier and reaches the brain rapidly after i.p. administration. Specifically, peripheral administration of OEA (20 mg/kg, i.p.) induced an increase in the OEA dialysate concentration in the dorsal striatum 20 minutes after injection (Gonzalez-Aparicio et al., 2014). Other authors detected a sustained 2-fold increase in OEA striatal levels over baseline for more than 2 hours after a single i.p. administration of OEA (20 mg/kg), reaching the maximum peak concentration around 15 minutes postinjection (Plaza-Zabala et al., 2010). In both studies, the OEA concentration is within the range reported to produce stimulation of PPAR-α receptor-dependent transcription (120 nM) (Fu et al., 2003). PEA has been reported to cross modestly the blood brain barrier after an oral dose (Artamonov et al., 2005). Nevertheless, in the present study we observed that OEA and PEA prevented the LPS-induced increase in plasma TNF-α levels. These results, together with the studies mentioned above, indicate that the anti-inflammatory effects of OEA and PEA observed in the brain may be a consequence of the modulation of peripheral inflammation (ie, modulation of innate immune TLR4 receptors) by these acylethanolamides and/or the direct action in the CNS. Disregarding the mechanisms involved, the brain is deeply affected by OEA and PEA pretreatments.

Here, we observed that OEA prevented LPS-induced increase in cortical TNF-α mRNA levels and both acylethanolamides reduced NF-κB activation, the expression of iNOS and COX-2, accumulation of NO₂⁻, and lipid peroxidation in frontal cortex. We supply further confirmation of this anti-inflammatory mechanism by showing OEA and PEA reductions in LPS-induced increases in mPGES-1 and PGE₂ levels.
We also provide the first evidence to our knowledge supporting a differential role for OEA and PEA influencing the acute-phase responses after LPS. Thus, OEA and PEA did not modify the increase in plasma corticosterone levels elicited by LPS. In the hypothalamus, OEA and PEA potently altered the expression of IL-1β, COX-2, and PGE₂, which are presumably mediators of
body temperature regulation, and they enhanced the hypothermic response 60 minutes after LPS administration. Interestingly, at a behavioral level, only OEA affected the motivational state of the animals by inhibition of LPS-induced anhedonia, demonstrating that OEA might exert important roles in controlling motivational processes (hedonic responses) as described for fat-containing food (Rodríguez de Fonseca et al., 2001; Tellez et al., 2013).

These selective effects of both acylethanolamides on LPS-induced acute-phase responses might reflect differential mechanisms of action that need to be further explored. OEA/PEA binding to PPAR-α receptor may mediate these effects (Fidaleo et al., 2014), but PPAR-α independent actions of these acylethanolamides cannot be ruled out.

The antiinflammatory profile of both acylethanolamides has been previously described in vitro, where OEA was shown to reduce iNOS, COX-2, and the cytokines TNF-α and IL-6 in blood vessels after LPS-induced LDL modification and inflammation (Fan et al., 2014) and in animal models of inflammatory and neuropathic pain (Lo Verme et al., 2005; Guardiaz et al., 2007; Di Cesare Mannelli et al., 2013).

OEA and PEA blocked the expression and/or activity of the p65 subunit in cortical nuclear extracts, which mediates most of the NF-κB transcriptional activity. LPS also increased the expression of the NF-κB inhibitor protein IκBα in cytosolic extracts, which can be considered an autoregulatory mechanism switched on by NF-κB to block its stimulation, and was similarly prevented by OEA and PEA pretreatments. Our results are in agreement with other studies where PEA and OEA prevented IκBα degradation and p65 NF-κB nuclear translocation in peripheral hyperalgesia (D’Agestino et al., 2009) and stroke injury (Sun et al., 2007; Ahmad et al., 2012b).

Sickness behavior after LPS was evaluated by measurement of the following acute-phase responses: activation of HPA axis, body temperature regulation, and anhedonia. Activation of HPA axis was checked by measurement of plasma corticosterone levels. LPS induced an increase in plasma corticosterone that has been previously reported (Pérez-Nieves et al., 2010). However, neither OEA nor PEA prevented the rise in corticosterone induced by LPS. Our results are in agreement with a previous study in which the administration of URB597, a selective inhibitor of FAAH that enhances the level of endocannabinoids, did not alter an LPS-induced increase in plasma corticosterone (Kerr et al., 2012). However, the bidirectional relationship between endocannabinoids and plasma glucocorticoids released in the stress response is well documented (Gorzalka et al., 2008; Hill et al., 2010). It is necessary to develop more detailed neuroendocrine studies regarding the time course of synthesis and release of corticosterone and other stress hormones after LPS to completely discard a role of noncannabinoid acylethanolamines in the regulation of HPA axis activation.

Regarding temperature regulation, we observed a marked hypothemia induced by LPS immediately after its administration and lasting between 3 and 6 hours. Our results are in agreement with other studies reporting dose- and serotype-specific effects of LPS: high doses (0.25 -0.5 mg/kg, i.p.) of E.coli O111:B4 induced a monophasic hypothemic response in rodents (Akarsu and Mamuk, 2007). It is important to note that, although fever is a most predicted response, hypothermia occurs in the most severe cases of sepsis (Clemmer et al., 1992; Arons et al., 1999). It has been suggested that the hypothermia in response to LPS is caused by reduced thermogenesis, involves antipyretic products released from peripheral macrophages, and is mediated by prostaglandins (Derijck RH et al., 1994). In our study, the onset of this hypothermic response caused by LPS is around the time of sacrifice of the animals (150 minutes). Biochemical determinations revealed that, at this time point, LPS induced a marked increase in pyretic molecules, such as IL-1β, COX-2, and PGE2, in the hypothalamus, probably as a homeostatic mechanism to recover normal temperature. Interestingly, OEA and PEA pretreatments potentiated the hypothermic response 60 minutes after LPS. Body temperature regulation is a highly preserved homeostatic response that is probably difficult to maintain altered by a single dose of these endogenous components. We observed robust effects of both acylethanolamides preventing the LPS-induced high increases in IL-1β, COX-2, mPGES-1 mRNAs, and PGE2, levels, which strengthens our hypothesis of OEA and PEA attempting to maintain the hypothemia induced by LPS. Hypothemia can be understood as an adaptive response that enhances recovery by conserving energy to combat acute inflammation and enhance survival (Leon, 2004; Maes et al., 2012). Recently, another N-ethanolamide derived from fatty acids, commonly known as the endocannabinoid AEA, has been involved in the LPS-induced thermic response through action on CB1 receptors (Steiner et al., 2011), and a role for COX-1 and not COX-2 has been suggested for LPS-induced hypothermia (Steiner et al., 2009). Interestingly, peripheral and brain AEA levels are elevated during the systemic inflammatory response to LPS (Liu et al., 2003; Fernandez-Solari et al., 2006). However, Kerr and colleagues (2012) reported that LPS failed to alter AEA, OEA, and PEA levels in the hypothalamus.

The sickness behavior is also characterized by a behavioral inhibition, physio-somatic disturbances such as fatigue and malaise, and an inability to feel pleasure or anhedonia (Maes et al., 2012). In our study, the influence of OEA and PEA in motivational behavior was tested by checking anhedonia in a saccharine preference test. LPS-injected animals pretreated with OEA, but not PEA, showed a preference for the natural reward saccharine similar to control animals, which is interpreted as a disruption of LPS-induced anhedonia. Anhedonia is a prolonged effect of LPS that persists beyond the acute sickness response, and this behavioral change is thought to reflect a depressive-like phenotype (Willner et al., 1987). Modulation of LPS acute neuroinflammatory responses by OEA can therefore elicit long-lasting motivational behavioral effects and possibly antidepressant-like effects. Total amount of liquid (water plus saccharine solution) was, however, reduced in LPS-injected rats independently of any pretreatment. A decrease in total drinking could be indicative of behavioral inhibition or fatigue during LPS-induced sickness behavior. Despite the fact it is a satiety factor (Romano et al., 2014), OEA at a single dose did not modify the preference for fluids or the total drinking in control animals. However, interestingly, OEA affected the saccharine preference in LPS rats and modified the anhedonic state after LPS, inducing a positive motivational state similar to control animals. Elimination of motivational deficits by OEA could be linked with a role of this lipid mediator on the control of dopamine release in the reward system. This effect has been clearly demonstrated for high-fat-containing foods (Tellez et al., 2013) or nicotine-mediated reward (Mascia et al., 2011; Bucznyski et al., 2012). Alternatively, anhedonia has been directly related with lasting lipid peroxidation alterations in the prefrontal cortex in a chronic stress depression paradigm (Cline et al., 2014). In our study, OEA prevented both lipid peroxidation in frontal cortex and anhedonia after LPS. Further studies will be necessary to ascertain whether inhibition of LPS-induced lipid peroxidation by OEA is long lasting and may be related with the OEA antianhedonic effect.

The proposed neuroprotective effects of OEA and PEA may derive in part from their antiinflammatory and antioxidative functions, as well as their modulation of neuronal activity (Melis et al., 2013). Given the importance of neuroinflammation in the physiopathology of neuropsychiatric diseases, our results...
suggest that OEA and PEA might help delay the onset of neurodegenerative and neuropsychiatric diseases by reducing the insults to brain functions. Finally, from a translational point of view, OEA might also have a beneficial profile as a therapeutic agent, since it may ameliorate the motivational state of individuals with neuroinflammatory or immune related neuropsychiatric conditions.

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