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Oleoylethanolamide exerts partial and dose-dependent neuroprotection of substantia nigra dopamine neurons

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ABSTRACT

Oleoylethanolamide (OEA), agonist of nuclear PPAR-α receptors and antagonist of vanilloid TRPV1 receptors, has been reported to show cytoprotective properties. In this study, OEA-induced neuroprotection has been tested in vitro and in vivo models of 6-OHDA-induced degeneration of substantia nigra dopamine neurons. First, PPAR-α receptors were confirmed to be located in the nigrostriatal circuit, these receptors being expressed by dopamine neurons of the substantia nigra, and intrinsic neurons and fibers bundles of the dorsal striatum. In the substantia nigra, their location was confined to the ventral tier. The in vitro study showed that 1 μM OEA exerted a significantly neuroprotective effect on cultured nigral dopamine neurons, effects following U-shaped dose–response curves. Regarding the in vivo study, rats were locally injected with OEA into the right striatum and vehicle into the left striatum 30 min before 6-OHDA-induced striatal lesion. In the short term, signals of heme oxygenase-1 (oxidation marker, 24 and 48 h post-lesion) and OX6 (reactive microglia marker, 96 h post-lesion) were found to be significantly less intense in the striatum pretreated with 5 μM OEA. In the long term (1 month), reduction in striatal TH and synaptophysin was less intense whether the right striatum was pretreated with 5 μM OEA, and nigral TH+ neuron death was significantly reduced after pretreatment with 1 and 5 μM OEA. In vivo effects also followed U-shaped dose–response curves. In conclusion, OEA shows U-shaped partial and dose-dependent neuroprotective properties both in vitro and in vivo models of substantia nigra dopamine neuron degeneration. The occurrence of U-shaped dose–response relationships normally suggests toxicity due to high drug concentration or that opposing intracellular pathways are activated by different OEA doses.

1. Introduction

Fatty acid acylethanolamides are endogenous lipid modulators that include, among others, the endocannabinoid anandamide (AEA), which acts through cannabinoid receptors, and the lipids palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), considered as cannabinoid analogs (Pertwee, 1997; Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Lo Verme et al., 2005). OEA and PEA have recently been identified as endogenous ligands for peroxisome proliferator-activated receptor-α (PPAR-α) (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Lo Verme et al., 2005), a nuclear receptor for a wide family of compounds that includes sex steroids, glucocorticoids and thyroid hormone, among others (Fu et al., 2003). OEA also acts via other receptors, including the transient receptor potential vanilloid subtype 1 or TRPV1 (Wang et al., 2005; Thabuis et al., 2008) or the GPR119 receptor (Overton et al., 2006). Recently it has been demonstrated that OEA is antagonist of TRPV1 receptors (Almási et al., 2008). This acylethanolamide seems not act through cannabinoid CB1 or CB2 receptors (Rodriguez de Fonseca et al., 2001). These lipid ligands are locally synthesized upon demand through the action of a specific phospholipase D enzyme that acts on a membrane precursor, a lipid synthesized by an *N*-acyltransferase regulated by calcium and cAMP (Piomelli, 2003). Upon its release, they are actively up-taken by a specific transport system yet to be cloned, and degraded by a specific fatty acid amidohydrolase, a membrane-bound enzyme. Recent reports have identified OEA as a modulator of feeding, inflammation, glucose homeostasis and lipid metabolism, acting in adipose tissue, hepatocytes and neurons (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Bordet et al., 2006; Thabuis et al., 2008).

The main characteristic of endocannabinoids and related analogs like OEA is their enzymatic origin and their production upon demand following local stimuli. Since OEA is also released into the blood stream and transported attached to albumin it is

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reasonable to think that it can act as major hormone in targets distant from its site of production, regulating other major hormones through its effect on gene expression. These actions suggest a main role for OEA in metabolic diseases (Kersten et al., 2000). In vitro and microdialysis studies have reported that OEA and other acylethanolamides are produced in response to cellular stress (Berdyshev et al., 2000; Schabitz et al., 2002; Walter et al., 2002). OEA is produced by neurons and glial cells (Walter et al., 2002), after tissue damage such as ischemia (Schabitz et al., 2002). All together suggests that OEA can influence cytotoxicity as a homeostatic signal that regulates cell survival upon tissue damage. In fact, OEA has been reported to be cytoprotectant in an animal model of animal steatosis (Serrano et al., 2006), and several recent reports have described a neuroprotective effect of OEA, PEA and cannabinoid activation (Lombardi et al., 2007; Bisogno et al., 2008; Sun et al., 2007). This profile makes OEA an interesting candidate for cytoprotection that can mimic the recently described actions of PPAR-α agonists, such as the fibrates (Deplanque et al., 2003). Additionally, it is well know the regulation of apoptosis and inflammation by these nuclear receptors.

It is also relevant the relationships of PPAR-α with oxidant stress, a metabolic situation common to many toxic insults, such as those induced by neurotoxins like 6-OHDA (Beltowski et al., 2002). 6-OHDA is toxic for dopaminergic neurons in rats (Ungerstedt, 1998; Schwarting and Huston, 1996), and its toxicity is mostly due to the generation of reactive oxygen species (ROS) (Ben-Sharchar et al., 1991; Cadet et al., 1989; Cohen et al., 1976). After 6-OHDA administration there is also an associated inflammatory response in the affected tissue (Tatton and Kish, 1997). Interestingly oxidative stress and inflammation are neurodestructive phenomena linked to the pathogenesis of Parkinson's disease, a disorder where the nigrostriatal circuit is severely damaged (Jenner and Olanow, 1998; Fernandez-Espejo, 2004), and OEA could have a protective effect against these phenomena. PPAR-α are expressed by many nuclei of the brain including the nigrostriatal circuit (Kainu et al., 1994; Moreno et al., 2004) although, at the level of the substantia nigra, its regional distribution has not been established. Apart from those effects mediated by PPAR-α, OEA can also act through vanilloid TRPV1 receptors. The blockade of these receptors could mediate neuroprotective effects of OEA, because TRPV1 activation is known to induce cell death of mesencephalic dopaminergic neurons both in vivo and in vitro (Kim et al., 2005; Marinelli et al., 2006). The vanilloid TRPV1 receptor is widely distributed in the central nervous system, including the striatum and the substantia nigra, and OEA could act as neuroprotectant in these neural regions (Mezey et al., 2000).

Since the precise regional distribution of PPAR-α in the substantia nigra has not been established, and the potentially neuroprotective effects of OEA against 6-OHDA-induced dopamine neuron death in the nigrostriatal circuit have not been studied so far, the objectives of the study were i) to further discern the nigrostriatal location of PPAR-α in order to give anatomical support for the potentially neuroprotective effects of OEA after damaging the nigrostriatal circuit, and ii) to assess the neuroprotective effects of OEA against the dopaminergic toxin 6-OHDA, by using in vitro and in vivo models of 6-OHDA-induced damage of dopamine neurons of the nigrostriatal circuit.

2. Materials and methods

2.1. Animals

Male Wistar rats (275–325 g) and postnatal rat pups from the breeding colony of the University of Seville or Fundacion IMABIS, Malaga, were used. Laboratory temperature was kept at 22 ± 1 °C, and a 12-h light–dark cycle (lights on at 08:00 h) was maintained throughout the experiment. Food (lab chow) and water were available ad libitum.

2.2. Compounds and doses

Oleoylethanolamide (OEA) was synthesized in the IMABIS Foundation (Malaga, Spain) and gently provided by Dr. Rodriguez de Fonseca. Before use, OEA was kept in 100% DMSO until use, and the appropriate concentration was obtained at 20% DMSO and 80% double distilled water. For cell culture studies, 6-OHDA (MW = 205.64) was used at 0, 40 and 60 μM dissolved in 0.15% ascorbic acid and saline. 6-OHDA concentrations were hence 0, 8.22, and 12.34 μg/mL (volume per well = 200 μL). OEA (MW = 325.53) was used as 0, 0.5, 1, and 5 μM dissolved in 10% DMSO in Neurobasal. OEA concentrations were 0, 0.16, 0.32, and 1.6 μg/mL (volume per well = 200 μL). For in vivo studies, OEA was injected at 0, 0.5, 1, 5 and 15 μM that represents concentrations of 0, 0.16, 0.32, 1.6, and 4.8 μg/mL respectively (volume infusion = 2 μL). For inducing in vivo striatal lesion, 6-hydroxydopamine (6-OHDA, Sigma–Aldrich, Saint Louis, USA), a neurotoxin for dopamine neurons, was dissolved in saline solution (0.9% NaCl) and 0.2% ascorbic acid (Sigma–Aldrich), and injected at 5 μg/μL, free base (volume infusion = 2 μL).

2.3. In vitro study

2.3.1. Neuron culture

Primary cultures of substantia nigra neurons were established as previously described by Cardozo (1993), with some modifications suggested by others (Mena et al., 1997; Burke et al., 1998; Smeyne and Smeyne, 2002). Postnatal pups (P0) were killed by decapitation under aseptic conditions, and brains were removed and placed in cold HBSS (Hank's balanced salt solution, GIBCO, Invitrogen Corp., Carlsbad, CA, USA). Under a dissecting microscope, a 0.8–1.0 mm thick coronal section of the mesencephalon was made using a scalpel and the regions containing SN were isolated. The adjacent mesencephalic region including the ventral tegmental area was carefully excluded. Tissue was digested in a solution of papain (20 units/mL; Worthington, USA) with 0.2 mg/mL L-cysteine in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich) and 100 mM $CaCl_2$ and 50 mM EDTA for 40 min at 37 °C in an incubation bath. Afterwards tissue was mechanically dissociated and centrifuged (1000 rpm) during 5 min. The pellet was collected and layered on the top of inactivation medium containing 10% foetal calf serum (FCS), 1% trypsin inhibitor and 1% bovine serum albumin in DMEM and centrifuged for 5 min. The pellet was resuspended with feeding medium consisting on Neurobasal-A supplemented with 2% B27 and 1% Glutamax (Invitrogen Corp.) with adequate antibiotic, and cell counts were made. The cell suspension was adjusted at 30,000 cells per well on a standard 96-well plate. Each rat pup produced enough cells for 3–4 wells. Cultures were maintained at 37 °C in 5% CO_2 containing atmosphere.

2.3.2. Immunocytofluorescence procedure

For confirming the presence of cultured dopamine neurons of substantia nigra and cell integrity, some cell cultures were immunostained against tyrosine-hydroxylase (TH, limiting enzyme for dopamine synthesis) and V-GAT (vesicular GABA transporter located in the membrane of GABA synaptic vesicles) for visualizing GABAergic terminals. Thus cells were plated in clean slides coated with a solution containing, poly-D-lysine, collagen and acetic acid in 1:1:3. The cultures were fixed using cold 4% paraformaldehyde in PB, pH 7.4 for 10 min. Followed several washes of PBS, cultures were incubated in a 0.1% Triton X-100 in PBS solution during 20 min at room temperature. After that cultures were blocked for 45 min in a blocking solution containing 5% FBS and 0.1% Triton X-100 in 0.1 mM PBS (pH 7.4). Neurons were incubated overnight at 4 °C in blocking solution containing rabbit monoclonal anti-TH (1:1000, Sigma–Aldrich), and mouse monoclonal anti-V-GAT (1: 1000; Synaptic System, Germany). Following three washes with 0.1% Triton X-100–PBS, cultures were then incubated with secondary antibodies conjugated with FITC and rhodamine respectively in blocking solution for an hour at room temperature, followed by nuclei staining with DAPI (1:1000 in PBS, Molecular Probes, Invitrogen Corp.). The slides were coverslipped using Slowfade antifade kit (Molecular Probes, Invitrogen Corp.), and visualized by fluorescence microscopy. Controls without both primary antibodies were confirmed to be negative.

2.3.3. 6-OHDA/OEA cell culture treatment, and LDH and MTT assays

Exposure to 6-OHDA was initiated after 4–5 days in vitro. Medium was removed and changed to Neurobasal without B27 for 2 h (Molina-Holgado et al., 2005). OEA (0, 0.5, 1, and 5 μM in 10% DMSO in Neurobasal) was added to neurons. Time and dose are based on Su et al. (2006). After 2 h freshly prepared 6-OHDA (0, 40 and 60 μM) in 0.15% ascorbic acid and saline was added during 15 min to produce specific death of dopaminergic neurons (Ding et al., 2004). After 15 min, the medium was removed, the cultures were gently washed twice with Neurobasal and then further incubated for 24 h, in order to carry out the LDH and MTT assays.

Cytotoxicity was evaluated by release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium by dead and dying cells (Cytotoxicity Detection kit, Roche, Indianapolis, USA). Total LDH release was calculated by incubating untreated cells with 0.5% Triton X-100 for 1 h to induce maximal cell lysis. Basal death was calculated from untreated wells without B27. Treatment values were then expressed as percent of the maximal LDH release. Background LDH release (media alone) was subtracted from the experimental values. Cell viability was measured with the MTT assay. This assay is based in a water-soluble tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MMT, Sigma)