Abstract

Some agonists of the peroxisome proliferator-activated receptor γ (PPARγ) belonging to the thiazolidinedione (TZD) family, as well as the cyclopentenone prostaglandin 15-dPGJ2, have been shown to cause neuroprotection in animal models of stroke. We have tested whether the TZD-unrelated PPARγ agonist L-796,449 is neuroprotective after permanent middle cerebral artery occlusion (MCAO) in the rat brain. Our results show that L-796,449 decreases MCAO-induced infarct size and improves neurologic scores. This protection is concomitant to inhibition of MCAO-induced brain expression of inducible NO synthase (iNOS) and the matrix metalloproteinase MMP-9 and to upregulation of the cytoprotective stress protein heme oxygenase-1 (HO-1). Analysis of the NF-κB signaling, and that it may be recruiting both PPARγ-dependent and independent pathways. In summary, our results provide new insights for stroke treatment.

Key Words: COX-2, HO-1, iNOS, Middle cerebral artery occlusion (MCAO), MMP-9, NF-κB.

INTRODUCTION

The inflammatory cascade triggered by the ischemic injury, in both the occluded blood vessels and brain parenchyma, is an important feature of the pathophysiological response to the ischemic injury. Therefore, anti-inflammatory strategies may be a useful therapy for treatment of acute stroke, one of the leading causes of death and disability worldwide but whose acute therapeutic management is limited to thrombolysis.

The peroxisome proliferator-activated receptors (PPAR) are ligand-dependent nuclear transcription factors, belonging to the nuclear hormone receptor superfamily of nuclear receptors, that have been implicated in diverse biologic processes such as early development, cell proliferation, differentiation, apoptosis, lipid and glucose metabolism, and cancer (1–5). Several members of this family have been described including the central nervous system (reviewed in [8–10]). Compounds belonging to the thiazolidinedione (TZD) family such as pioglitazone, troglitazone, and rosiglitazone, known to activate PPARγ, have been shown to possess neuroprotective properties in animal models of stroke. Such effects seem to be mediated through antiinflammatory as well as antioxidant mechanisms (11–13). Another PPARγ agonist, the endogenous cyclopentenone prostaglandin 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), is also neuroprotective after experimental stroke (11); in addition, increased plasma 15d-PGJ2 concentration has been associated with good neurologic outcome and smaller infarct volume after atherothrombotic ischemic stroke in humans, suggesting a neuroprotective effect of 15d-PGJ2 in this setting (14).

Because antiinflammatory actions of the TZD-unrelated PPARγ synthetic agonist L-796,449 (3-chloro-4-(3-(3-phenyl-7-propylbenzofuran-6-oxoxy) propylthio) phenyl acetic acid) have been demonstrated in macrophages (15), we have decided to investigate its effects on stroke outcome in a rodent model of cerebral ischemia by permanent occlusion of the middle cerebral artery (MCAO).

MATERIALS AND METHODS

Materials

L-796,449 was donated by Merck Sharp Dohme (Rahway, NJ). The other reagents were obtained from Sigma (Madrid, Spain) or as indicated in the text.

Animals

Adult male Fischer rats weighing approximately 250 g were used. Rats were housed individually under standard conditions of temperature and humidity and a 12-hour light/dark cycle.
cycle (lights on at 8:00 AM) with free access to food and water. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (following DC 86/609/EU and 2003/65/CE).

**Middle Cerebral Artery Occlusion**

Animals were anesthetized with 1.5% halothane in a mixture of 70% nitrogen/30% oxygen, and body temperature was maintained at physiological levels using a heating pad throughout the surgery procedure and during postsurgery recovery. Permanent focal cerebral ischemia was induced by occlusion of the ipsilateral middle cerebral artery (MCA) as described (16). Rats in which the MCA was exposed but not occluded served as sham-operated controls (SHAM). After surgery, subjects were returned to their cages and allowed free access to water and food.

**Experimental Groups**

Several groups were used for determinations of infarct size, neurologic assessment, and determination of biochemical and molecular parameters: MCAO 10 minutes before an intraperitoneal (IP) injection of saline (MCAO; n = 10) or DMSO (vehicle; 10% in saline; n = 10) and MCAO 10 minutes before IP injection of the thiazolidinedione-unrelated PPARγ agonist L-796,449 1mg/kg (MCAO1L-796,449; n = 10). An additional group consisted of SHAM-operated animals 30 minutes before IP injection of saline (SHAM; n = 6). An injection volume was ≤ 400 μL/250 g body weight. Animals were killed from 1 hour to 7 days after surgery and samples were collected.

**Infarct Size**

Infarct outcome was assessed 2 and 7 days after the occlusion with an overdose of sodium pentobarbital. Animals were killed and brain was removed and cut into 6 2-mm-thick coronal slices, which were stained with 1% TTC (2,3,5-triphenyl-tetrazolium chloride) in 0.1 M phosphate buffer. Infarct volumes were measured by sampling each side of the sphere. Infarct areas over the extent of the infarct calculated as an orthogonal projection.

**Protein Expression in Brain Homogenates and in Cytosolic and Nuclear Extracts**

Rats were killed by an overdose of sodium pentobarbitone and ipsilateral cortex was dissected from the MCA territory corresponding to the infarct and surrounding area. For determination of iNOS, COX-2, MMP-9, and HO-1 protein expression levels, rats were killed 18 hours after MCAO. Brain samples were homogenized by sonication for 10 seconds at 4°C in 4 volumes of homogenization buffer containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 50 mM Tris brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 4°C at 12,000 g for 20 minutes and the pellet was discarded.

Cytosolic and nuclear extracts were prepared as described (18). Determination of p65 was performed in nuclei obtained from brains of rats killed 1 hour after MCAO, whereas for PPARγ, rats were killed 2 and 18 hours after MCAO. For IkBα, cytosolic extracts were obtained from brains of rats killed 5 hours after MCAO.

**Determination of Caspase-3 Activity**

Caspase-3 activity was determined as an indicator of apoptosis in brain homogenates. Caspase-3 activity was measured in a fluorometric assay by measuring the extent of cleavage of the fluorescent peptide substrate with a commercial kit (Molecular Probes, Eugene, OR) following the manufacturer’s recommendations. The fluorescence of the rhodamine 110-labeled caspase-3 product was determined in a fluorescence microplate reader (Fluoroskan Ascent FL; Labsystems, Helsinki, Finland).

**Western Blot Analysis**

Laemmli electrophoresis sample buffer was added to samples containing 30 μg protein and denatured by heating at 94°C for 10 minutes. Proteins were size-separated in 7% SDS-polyacrylamide gel electrophoresis (120 mA), transferred to a PVDF membrane (HybondTM-P; Amersham Biosciences Europe GmbH, Freiburg, Germany), and incubated with specific primary antibodies against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), COX-2 (Santa Cruz, 1:1000), MMP-9 (Chemicon, Temecula, CA; 1:2000), HO-1 (Labo; 1:5000, Stressgen Biotechnologies Corp., Victoria, BC, Canada), p65 (Santa Cruz; 1:1000), and IkBα (Santa Cruz; 1:1000). Proteins recognized by the antibody were revealed by ECLTM-kit following the manufacturer’s instructions (Amersham Biosciences Europe GmbH). β-actin and Sp1 levels were used as loading controls for total cytosolic and nuclear protein expression, respectively.

**Gelatin Zymography**

Gelatin zymography was performed as previously described (19) using 8% SDS-PAGE with copolymerized gelatin (2 mg/mL). Sample homogenates were subjected to electrophoresis. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 hour (3 times, 20 minutes each) and incubated for 24 hours in enzyme assay buffer (25 mM Tris, pH 7.5, 5 mM CaCl₂, 0.9% NaCl, 0.05% Na₂N). The gelatinolytic activities were detected as transparent bands.
against the background of Coomassie blue-stained gelatin. MMP-9 was identified by its molecular weight when compared with standards.

**Gel Mobility Shift Assays**

A complementary 20-nucleotide primer containing the PPAR response element (PPRE) consensus sequence, corresponding to Acyl CoA oxidase promoter, was used and labeled with \(^{32}\)P (GGGGACAGGACAAAGGTCA). For gel shift analysis, probe and nuclear protein extract were incubated in a binding buffer. Protein complexes were separated by 5% nondenaturing polyacrylamide gel electrophoresis. Excess of unlabeled oligonucleotide was added as competitor in the nuclear protein-PPRE labeled probe mixture, demonstrating the binding specificity.

**NO\(_x\) (Nitrite\(_2^–\) and Nitrate\(_3^–\)) Assay**

NO release was estimated from the amounts of nitrite (NO\(_2^–\)) and nitrate (NO\(_3^–\)) in brain homogenates. NO\(_3^–\) was calculated by first reducing NO\(_3^–\) into NO\(_2^–\) in the presence of Cd and NO\(_2^–\) was determined by a colorimetric assay based on the Griess reaction as described (20).

**Statistical Analysis**

Results are expressed as mean ± standard error of mean of the indicated number of experiments; statistical analysis involved one-way analysis of variance (ANOVA; or the Kruskal-Wallis test when the data were not normally distributed) followed by individual comparisons of means (Student-Newman-Keuls or Dunn’s method when the data were not normally distributed). \(p < 0.05\) was considered statistically significant.

**RESULTS**

**Effect of L-796,449 on Infarct Outcome after Permanent Middle Cerebral Artery Occlusion**

The administration of the PPAR\(_\gamma\) ligand L-796,449 10 minutes after the occlusion decreased MCAO-induced infarct size (Fig. 1A) determined 2 and 7 days after the ischemic injury. The neuroprotective effect of L-796,449 on infarct areas 2 days after the occlusion is evident in most slices (Fig. 1B). In addition, the animals treated with this compound also showed better scores in a neurologic assessment scale after MCAO (Table).

Moreover, permanent MCAO induced an increase in the activity of the apoptotic parameter caspase 3 when measured 5 hours but not 18 hours after the occlusion (Fig. 2). The increase in caspase-3 activity was inhibited in those animals treated with L-796,449 (Fig. 2).

**Effect of L-796,449 on Inducible NO Synthase and Cyclooxygenase-2 (COX-2) Expression and Nitrite/Nitrate (NO\(_x^–\)) Levels After Middle Cerebral Artery Occlusion**

Occlusion of the MCA caused the expression of the inflammatory enzymes inducible NO synthase (iNOS) and COX-2 in rat brain, as shown by the levels of these proteins found 18 hours after the ischemic insult (Fig. 3A, B). The administration of L-796,449 inhibited MCAO-induced expression of iNOS but did not affect COX-2 levels at the time examined (Fig. 3A, B).

Levels of NO\(_x^–\) 18 hours after MCAO were determined as an indicator of NO synthesis. Permanent MCAO caused an increase in brain NO\(_x^–\) (379.4 ± 9.0 and 815.0 ± 8.2 pmol/mg prot in control and MCAO groups, respectively, \(n = 5\), \(p < 0.05\)) which was decreased by L-796,449 (438.2 ± 32.1 pmol/mg prot, \(n = 5\), \(p < 0.05\)).

**Effect of L-796,449 on Matrix Metalloproteinase 9 Expression after Middle Cerebral Artery Occlusion**

Occlusion of MCA caused an increase in the protein levels of matrix metalloproteinase 9 (MMP-9), a matrix
metalloproteinase that is induced by and causes damage in cerebral ischemia. L-796,449 decreased the levels of this matrix metalloproteinase after experimental stroke (Fig. 3C). Zymography studies showed that L-796,449 caused a decrease in the active form of MMP-9, but not in the proform of this enzyme (Fig. 3D).

**Effect of L-796,449 on Heme Oxygenase-1 Expression After Middle Cerebral Artery Occlusion**

Occlusion of the MCA caused the induction of heme oxygenase-1 (HO-1) when measured 18 hours after the ischemic insult. The levels of expression of this enzyme were greater in those animals treated with L-796,449 (Fig. 4).

**Effect of L-796,449 on NF-κB Nuclear Levels After Middle Cerebral Artery Occlusion**

As a sign of NF-κB activation, the nuclear levels of its subunit p65 were determined 1 hour after MCAO. Experimental ischemia caused activation of NFκB as revealed by the nuclear translocation of the NF-κB subunit p65. MCAO-induced p65 translocation was decreased in animals treated with L-796,449 (Fig. 5A).

<table>
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<th>Neurologic Score (points)</th>
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<th>MCAO + L-796,449</th>
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Data are shown as number of animals showing each neurologic score versus total number of animals per group. *, p < 0.05 versus control; #, p < 0.05 versus vehicle, n = 6.

**Effect of L-796,449 on IkBα Cytosolic Levels After Middle Cerebral Artery Occlusion**

Because IkB itself is a NF-κB-dependent gene, late IkB levels were determined as an indicator of NF-κB transcriptional activity induced after its nuclear translocation. Cytosolic IkBα levels increased 5 hours after the ischemic insult when compared with control brains (Fig. 5B). L-796,449 abolished the increase in IkBα levels induced 5 hours after MCAO.

**Expression of PPARγ in Nuclear Extracts after Middle Cerebral Artery Occlusion: Effect of L-796,449**

Western blot analysis showed the presence of PPARγ in nuclear extracts obtained from control animals (Fig. 6A). MCAO either in the absence or presence of L-796,449 did not significantly modify the expression of this receptor when measured 2 and 18 hours after the ischemic insult (Fig. 6A).

**Effect of L-796,449 on the Binding of Nuclear Proteins to the PPAR Response Element**

We studied by gel shift analysis whether L-796,449 increased the binding of PPAR isoforms to the PPAR response element. When studied 2 hours after the MCAO, binding of nuclear elements to the probe containing the PPRE was not significantly detected, either in absence or presence of L-796,449 (Fig. 6B). Eighteen hours after the ischemic insult, L-796,449 increased the amount of nuclear protein bound to the PPRE probe (Fig. 6B). The binding was specific as addition of excess amount of cold oligonucleotide blocked the binding (Fig. 6B).

**DISCUSSION**

We have found that a synthetic nonthiazolidinedione PPARγ agonist, L-796,449, significantly improves infarct outcome after MCAO in rats, as shown by a decrease in infarct size induced by MCAO and also by an improvement in the neurologic score of these animals. Our results also demonstrate that L-796,449 inhibits MCAO-induced expression of inflammatory mediators such as iNOS and MMP-9 and upregulates HO-1 expression, and that these effects are likely to be mediated through both PPARγ-dependent and -independent mechanisms.

The PPARγ agonist used in this study, L-796,449, is a TZD-unrelated potent PPARγ synthetic agonist with an apparent Kᵢ for this receptor of 2 nM (21). Additionally, L-796,449 has been shown to possess important actions independent of PPARγ binding (15, 21).

L-796,449 caused a significant improvement in the outcome of experimental stroke in these animals, as shown by a reduction of MCAO-induced infarct volume and by a recovery in the neurologic deficit induced by MCAO both 2 and 7 days after the occlusion. In addition, L-796,449 showed an antiapoptotic effect when determined 5 hours after the ischemic insult. Given that this compound was administered after the onset of the ischemic damage, our findings provide clues for the therapeutic management of acute ischemic stroke. Although it might be argued that access to the central nervous system is limited in a healthy brain, this is overcome in stroke
as a result of the disruption of the blood–brain barrier that occurs in cerebral ischemia (reviewed in [22]).

In the search for mechanisms involved in this neuroprotective effect, we found that L-796,449 inhibits both MCAO-induced iNOS expression and increase in the levels of NO₂⁻ and NO₃⁻, the stable metabolites of NO, in rat brain. Because iNOS mediates cytotoxicity in many cell systems, including the ischemic brain (reviewed in [23]), its inhibition may explain at least part of the neuroprotective effect of this compound. Inhibition of iNOS expression with PPARγ ligands has been similarly described in central nervous system cells after an inflammatory challenge with lipopolysaccharide (24–27) and after experimental stroke (11–13).

The inducible cyclo-oxygenase COX-2 also participates in the ischemic inflammatory cascade (28–30). Our results confirm that MCAO induces COX-2 expression in the rat brain, although L-796,449 did not affect its levels at the time studied. In this context, there are controversial results in the literature; whereas inhibition of COX-2 expression by PPARγ ligands has been shown (31, 32), other reports also demonstrate that PPARγ activation does not affect (33) or possibly increases the expression of COX-2 (34, 35).

The matrix metalloproteinase 9 (MMP-9; gelatinase B) is another inflammatory mediator that contributes to ischemic cerebral damage (36) because it participates in extracellular matrix degradation (37); we and others have shown that MMP-9 participates in the hemorrhagic transformation in acute ischemic stroke in humans (38, 39). Our data confirm that experimental stroke increases the expression of this metalloproteinase. More importantly, MCAO-induced MMP-9 expression was inhibited by L-796,449. When studied by zymography, the effect of L-796,449 was more evident on the active form of this metalloprotease. It has been reported that increased NO production is necessary for MMP-9 activation (40–42), which might constitute a potential extracellular proteolysis pathway to neuronal cell death in cerebral ischemia. Given the relevance of both interrelated signaling for cell damage, the dual inhibition of iNOS and MMP-9 expression after MCAO is likely to be one of the main mechanisms responsible for the neuroprotective effect of these compounds.

Heme oxygenase-1 is a stress-related protein of 32 kDa that catalyzes the rate limiting step in heme degradation, yielding equimolar quantities of biliverdin, CO, and iron (43). HO-1 is induced by a wide variety of stimuli that cause oxidative stress (44, 45), including cerebral ischemia (46, 47), a setting in which the main actions of this enzyme are neuroprotective (48, 49), as also demonstrated in other central nervous system pathophysiological conditions (50). Our results confirm the expression of HO-1 after experimental stroke; in addition, we also show that L-796,449 enhances MCAO-induced HO-1 expression. In this context, other PPARγ agonists such as 15-deoxy-Δ12,14-prostaglandin J₂ (15-dPGJ₂)
have been shown to reduce myocardial infarct size concomitantly to the induction of HO-1 in human cardiac myoblasts (51) and to exert antiinflammatory actions through a mechanism that involves the action of HO-1 and its product CO (52). Similar antiinflammatory mechanisms may take place after administration of L-796,449 in our model. Interestingly, several data on the literature strongly support the notion that such actions of 15d-PGJ_2 on HO-1 are not shared by TZDs, are independent of PPARγ-activation, and are very likely mediated by oxidative stress caused by the production of reactive oxygen species (51, 53-55). Because we have previously shown that L-796,449 increases the synthesis of reactive oxygen species after inflammatory stimuli (15), this effect could account for the increased expression of HO-1 as a result of an enhanced oxidative environment and suggests that L-796,449-induced upregulation of HO-1 is PPARγ-independent.

As suggested previously, there is a great deal of controversy on the role of PPARγ to explain the anti-inflammatory actions of its agonists. Our results show the presence of PPARγ in brain nuclear extracts in agreement with early work describing its mRNA and protein patterns in brain (56, 57). More importantly, gel shift analysis aimed to determine whether PPARγ is activated by L-796,449 in our setting showed that this compound increases the amount of protein bound to a PPRE only at the latest time studied, strongly suggesting that at those times, PPARγ is activated by L-796,449 and may mediate at least some of the antiinflammatory effects of this drug.

It has been proposed that transrepressional inhibition of the transcriptional activity of NF-κB, a transcription factor that plays a key role in the activation of inflammatory response genes such as iNOS and COX-2 (58), mediates the
with a32PPRE-labeled probe on different pools of nuclear proteins. Electrophoretic mobility gel shift assay was performed on nuclear proteins binding to a PPRE-containing consensus PPRE before loading. Group was mixed with a 100-fold molar excess of unlabeled labeled “cold probe,” the nuclear extract from the control extracts obtained 2 and 18 hours after MCAO. For the lane error of mean, n = 5, *, p < 0.05 versus control.

Anti-inflammatory actions of PPARγ agonists (6, 7, 59). In resting cells, NF-κB is sequestered in the cytoplasm by association with an inhibitory protein IκB. In response to signaling by certain stimuli, IκB kinase (IKK) is activated and phosphorylates IκB on 2 serine residues. IκB is then ubiquinated and degraded by the proteasome, freeing NF-κB to migrate into the nucleus and activate gene expression (58). At this step, PPARγ activation is able to antagonize NF-κB-mediated transcriptional activity by an effect that does not appear to involve direct DNA binding, but through the recruitment of co-activators required for NF-κB signaling (6, 7, 59). Alternatively, as commented previously in this article, it has been shown that antiinflammatory actions of at least some PPARγ agonists occur through PPARγ-independent mechanisms (reviewed in [60]). In this context, we and others have demonstrated that L-796,449 is able to inhibit NF-κB activation in macrophages in a PPARγ-independent manner (15) by inhibiting IκB kinase activity and therefore obstructing its nuclear translocation. Our present results show that MCAO induces NF-κB activation, as shown by the translocation of p65 to the nucleus at early times, and that this effect is reduced by L-796,449, thus indicating a direct inhibitory action of L-796,449 on NFκB translocation independent of PPARγ. In addition, we studied the levels of the inhibitory protein IκBα, which is rapidly induced after NF-κB activation (61, 62), thus constituting an indicator of NF-κB transcriptional activity. Our results show that IκBα is remarkably increased in the brain of MCAO-exposed animals 5 hours after the occlusion, indicating prior NF-κB activation, and that this effect is completely blocked by L-796,449 demonstrating that this compound efficiently inhibits NF-κB signaling. Taking together, the data obtained from both gel shift assays and NF-κB activity we may suggest that, at early times, L-796,449 exerts PPARγ-independent effects by direct inhibition of NF-κB activation, whereas at later times, PPARγ activation is likely to be implicated.

In summary, our results show that synthetic, TZD-unrelated PPARγ agonists such as L-796,449, through inhibition of the inflammatory iNOS and MMP-9 and upregulation of the cytoprotective HO-1, may be effective in management of acute stroke.

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