Regulation of Inflammatory Response in Neural Cells in Vitro by Thiadiazolidinones Derivatives through Peroxisome Proliferator-activated Receptor γ Activation*

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In most neurodegenerative disorders, including multiple sclerosis, Parkinson disease, and Alzheimer disease, a massive neuronal cell death occurs as a consequence of an uncontrolled inflammatory response, where activated astrocytes and microglia and their cytotoxic agents play a crucial pathological role. Current treatments for these diseases are not effective. In the present study we investigate the effect of thiadiazolidinone derivatives, which have been recently suggested to play a role in neurodegenerative disorders. We have found that thiadiazolidinones are potent neuroprotector compounds. Thiadiazolidinones inhibited inflammatory activation of cultured brain astrocytes and microglia by diminishing lipopolysaccharide-induced interleukin 6, tumor necrosis factor α, inducible nitric-oxide synthase, and inducible cyclooxygenase type 2 expression. In addition, thiadiazolidinones inhibited tumor necrosis factor-α and nitric oxide production and, concomitantly, protected cortical neurons from cell death induced by the cell-free supernatant from activated microglia. The neuroprotective effects of thiadiazolidinones are completely inhibited by the peroxisome proliferator-activated receptor γ antagonist GW9662. In contrast the glycogen synthase kinase 3β inhibitor LiCl did not show any effect. These findings suggest that thiadiazolidinones potently attenuate lipopolysaccharide-induced neuroinflammation and reduces neuronal death by a mechanism dependent of peroxisome proliferator-activated receptor γ activation.

Inflammatory activation of neuronal and glial cells is believed to contribute to cell death and damage during neurological disease. One of the hallmarks of neurodegenerative and inflammatory pathologies is the increased number of activated astrocytes and microglia in response to the pathological stimulus (1, 2). Under normal conditions, brain microglia, the functional equivalent of macrophages in the central nervous system (3), are involved in immune surveillance and host defense against infectious agents (4). However, in response to brain injury, infection, or inflammation, microglia readily become activated in a way similar to peripheral tissue macrophages. Now, there is a growing evidence that toxic mediators, including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and nitric oxide (NO), produced by activated microglial cells might be involved in the pathogenesis of various neurodegenerative diseases such as Parkinson disease, Alzheimer disease, multiple sclerosis, and AIDS dementia (3, 5, 6). Astrocytes, which are responsible for maintaining the homeostasis of the brain tissue, also participate to a large extend in the neuroimmune responses (7, 8). Hence, it is of great interest to find a means to modulate microglial activation in central nervous system inflammatory responses for the therapeutic intervention against these neurodegenerative diseases.

Thiadiazolidinones (TDZDs) are small heterocyclic thiadiazolidinones, which were synthesized following a pathway that is based on the reactivity of N-alkyl-S-(N-(chlorocarbon)yl)amino) isothiocarbamoyl chlorides with isocyanates (9). They are small molecules with favorable ADMET-Tox-drugable properties, such as oral bioavailability and blood-brain barrier penetration (10) and they have been shown to be non-ATP competitive glycogen synthase kinase 3β (GSK-3β) inhibitors (11). TDZDs have been postulated that could be of potential therapeutic use for the treatment of Alzheimer disease and other important unmet pathologies as diabetes type II, cancer, and chronic inflammatory processes (12, 13). Preliminary in vitro studies suggest potential neuroprotective effects of TDZDs against several insults, such as 6-OHDP (14), lipopolysaccharide (LPS) and glutamate (15).

The somehow chemical structure-related derivatives to TDZDs, and recently FDA approved thiazolidinediones, such as rosiglitazone, pioglitazone, and troglitazone, are known agonists of the peroxisome proliferator-activated receptor γ (PPARγ) (16, 17). PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, which includes retinoid, steroid, and thyroid hormone recep-

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Primary Cell Cultures—Primary cortical neuronal cultures were prepared from the cerebral cortex of embryonic day 18 rats, according to published protocols (27). After removal of the meninges, the cerebral cortex was dissected and dissociated in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). After washing, cells were plated on poly-lysine (20 μg/ml; Sigma)-coated plates or coverslips, and the cultures were maintained in Neurobasal medium with B-27 supplements. In control and LPS-treated microglia was subsequently transferred to cortical neuron cell cultures, which were then incubated for an additional 24 h before cells were collected and assayed for neuronal apoptotic cell death and expression of cytokines.

Antibodies—Mouse monoclonal antibodies to β-tubulin, MAP2, and anti-glial fibrillary acidic protein were from Sigma. Mouse anti-α2-4 monoclonal antibody was purchased from Serotec (Duseldorf, Germany). Monoclonal anti-CNPase was from Sternberger (Baltimore, MD), and polyclonal anti-IL-6, anti-TNF-α, anti-COX-2, and anti-iNOS antibodies were from Santa Cruz (Santa Cruz Biotechnologies, CA).

Immunocytochemistry—Neurons were stained with an antibody against β-tubulin, a marker for microtubules. Microglia were visualized by staining for the CR3 complement receptor with polyclonal antibody Ox42, and astrocytes were stained with an antibody against anti-glial fibrillary acidic protein, an intermediate filament protein whose synthesis is restricted to astrocytes. At the end of the treatment period, the cultures, grown on glass coverslips in 24-well cell culture plates, were washed with phosphate-buffered saline, fixed for 30 min with 4% paraformaldehyde at 25 °C, and permeabilized with 0.1% Triton X-100. After 5–10 min, the cells were incubated with 0.1% Tween 20 for 10 min, and the remaining microglia cells and then trypsinized and expanded at a 1:5 ratio in complete medium. In these conditions, the purity of the neuronal population was >98%, as determined by immunofluorescence analysis using anti-β-tubulin and anti-microtubule-associated protein 2 antibodies. All experiments were carried out with 7-day-old cultures.

Astrocytes were prepared from neonatal (P2) rat cerebral cortex. Briefly, after removal of the meninges the cerebral cortex was dissected, dissociated, and incubated with 0.25% trypsin, EDTA at 37 °C for 1 h. After centrifugation, the pellet was washed three times with Hanks’ balanced salt solution (Invitrogen), and the cells were plated on non-coated flasks and maintained in Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1) containing 10% fetal bovine serum. After 15 days the flasks were agitated on an orbital shaker for 12 h at 250 rpm at 37 °C. The supernatant was collected, centrifuged at 1500 × g for 10 min, and the cellular pellet containing the microglial cells resuspended in HAMS/Dulbecco’s modified Eagle’s medium (1:1) containing 10% bovine serum and seeded at a density of 2–4 × 10^5 cells/cm^2 on uncoated plates. Cells were allowed to adhere for 7 days, after which the medium was removed to eliminate non-adherent oligodendrocytes, and new fresh medium containing 10 ng/ml granulocyte-macrophage colony-stimulating factor was added. The purity of microglia obtained by this procedure was >98% as determined by immunofluorescence with the Ox-42 antibody.

Measurement of Apoptosis—To calculate the extent of apoptotic cell death, cortical neuronal cultures were treated or not with NP00111 or NP01138, incubated with glutamate (100 μM), staurosporine (50 nM), or cell-free supernatant from LPS-stimulated microglia, and phosphatidylyserine exposure on the surface of apoptotic cells was detected by confocal microscopy after staining with Annexin V-fluorescein isothiocyanate (Bender MedSystems, Vienna, Austria).

TNF-α Assay—TNF-α secreted in astrocytes and microglial cultures was measured by specific enzyme-linked immunosorbent assay using rat monoclonal anti-rat TNF-α antibody as the capture antibody and horseradish peroxidase polyclonal anti-rat TNF-α as the detection antibody (R&D Systems).

Nitrite Determinations—The production of NO was monitored by measuring the content of nitrite, one of the end products of NO oxidation, by a procedure based on the diazotization of nitrite by sulfanilic acid (Griess reaction). Twenty-four hours after the incubation of astrocytes and microglia cells with LPS or neuronal cultures with conditioned medium from LPS-treated microglia, in the absence or presence of nitric-oxide synthase (iNOS), and cyclooxygenase type 2 inhibitors, including thiazolidinediones, have potent anti-inflammatory effects, such as the suppression of TNF-α and IL-1β, inducible nitric-oxide synthase (iNOS), and cyclooxygenase type 2 (COX-2) (19–25). Of relevance to central nervous system disease is that PPARγ agonists have been demonstrated to have similar anti-inflammatory effects on astrocytes and microglial cells (review in Ref. 26).

Here we demonstrate a potent anti-inflammatory effect of two TDZD compounds, NP00111 and NP01138 (see Fig. 1), in primary cultures of cortical neurons, astrocytes, and microglia. Our results indicate that both compounds act broadly to inhibit the production of proinflammatory and neurotoxic products elaborated by LPS-stimulated astrocytes and microglial cells. Concomitantly, TDZDs also exert a neuroprotective effect in primary cultures of cortical neurons treated with cell-free supernatant from LPS-stimulated microglia. Our results suggest that the effects of TDZDs are dependent on PPARγ activation, as the PPARγ antagonist GW9662 abolished them. The capacity of TDZDs to suppress the expression of inflammatory cytokines and their protective action on neurons suggests that these agents may be of value in the treatment of Alzheimer disease or other inflammatory diseases.

EXPERIMENTAL PROCEDURES

Primary Cell Cultures—Primary cortical neuronal cultures were prepared from the cerebral cortex of embryonic day 18 rats, according to published protocols (27). After removal of the meninges, the cerebral cortex was dissected and dissociated in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). After washing, cells were plated on poly-lysine (20 μg/ml; Sigma)- and gelatin (250 μg/ml; Sigma)-coated plates or coverslips, and the cultures were maintained in Neurobasal medium with B-27 supplements. In these conditions, the purity of the neuronal population was >98%, as determined by immunofluorescence analysis using anti-β-tubulin and anti-microtubule-associated protein 2 antibodies. All experiments were carried out with 7-day-old cultures.

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Nitrile Determinations—The production of NO was monitored by measuring the content of nitrite, one of the end products of NO oxidation, by a procedure based on the diazotization of nitrite by sulfanilic acid (Griess reaction). Twenty-four hours after the incubation of astrocytes and microglia cells with LPS or neuronal cultures with conditioned medium from LPS-treated microglia, in the absence or presence of nitric-oxide synthase (iNOS), and cyclooxygenase type 2.
of TDZDs, 50 µl of sample aliquots were mixed with 50 µl of Griess reagent in 96-well plates and incubated at 25 °C for 10 min. The absorbance at 550 nm was measured on a microplate reader.

**Transient Transfections—** Murine hippocampal neuronal cell line HT22 was propagated and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 40 µg/ml gentamicin, and 2 mM glutamine at 37 °C and 5% CO₂. Semiconfluent cultures were transfected with the reporter plasmid pPPRE-tk-luc, containing three PPARγ consensus binding sites upstream of a minimal promoter, together with PPARγ and RXRα expression vectors, using Transfast (Promega Corporation, Madison, WI) according to the manufacturer’s guidelines. Typically, cells received 0.2 µg of luciferase reporter plasmid, 0.2 µg of PPARγ, and 50 ng of RXRα and were harvested 24 h after treatment with rosiglitazone (30 µM), NP00111 (50 µM), NP01138 (50 µM), and/or 9-cis retinoic acid (9-cis-RA, 1 µM), for determination of luciferase and β-galactosidase (to determine transfection efficiency) activities. Each transient transfection experiment was repeated at least three times in triplicate.

**Statistical Determinations—** The data shown are the means ± S.D. of at least three independent experiments. Statistical comparisons for significance between cells with different treatments were performed using the Student’s t test, with p ≤ 0.05.

**RESULTS**

NP00111 and NP01138 Inhibit Inflammatory Activation of Cultured Glial Cells—The potential anti-inflammatory activity of TDZDs was first tested by evaluating the production of inflammatory mediators from cultured astrocytes and microglial cells. First, we performed a dose-response curve of nitrite production (an indirect quantification of NO generation) by astrocytes and microglia cultures. Astrocytes and microglia were incubated with different concentrations of TDZDs for 1 h, and then cells were cultured for another 24 h with LPS. As shown in Fig. 2, NP00111 and NP01138 dose dependently inhibited the production of nitrite (Fig. 2A) and the expression of COX-2 (Fig. 2B) induced by LPS stimulation. The effects of TDZDs were not caused by a loss of cell viability, as
the 24-h exposure of astrocytes and microglial cells to NP00111 or NP01138 did not significantly alter cell viability (data not shown). The suppressive effects of both TDZDs were stronger at higher concentrations (50 μM), and, therefore, we used this concentration henceforth.

We then analyzed more thoroughly the effects of TDZDs on LPS-stimulated primary cultures of astrocytes. When primary astrocytes were stimulated with LPS we observed once more a significant induction of nitrite production and also of TNF-α levels in the culture medium (2.0- and 4.0-fold, respectively), which was totally inhibited by NP00111 and NP01138 treatment (Fig. 3A). To further study the inhibitory effect of NP00111 and NP01138 on astrocyte activation, we next examined whether these compounds affected the LPS-induced intracellular accumulation of TNF-α and IL-6 (another proinflammatory cytokine). In addition, we also studied the accumulation of inducible COX-2 and iNOS. The enzyme iNOS is responsible for the generation of NO (28), and overexpression of COX-2 in activated microglia and astrocytes appears central to many neuroinflammatory conditions. We studied this by immunofluorescence analysis followed by confocal microscopy. As shown in Fig. 3, B and C, the protein levels of TNF-α and IL-6 were clearly increased after LPS treatment of astrocytes, and treatment of the cultures with NP00111 or NP01138 completely abrogated this effect. In addition, in basal conditions, COX-2 and iNOS protein levels were barely detectable in astrocyte cultures, and their content was significantly induced after LPS treatment. The addition of NP00111 and NP01138 drastically inhibited LPS-induced COX-2 and iNOS expression. A similar cellular density is present in all microphotographs, as assessed by 4,6-diamidino-2-phenylindole (DAPI) staining (data not shown).

Similar results were obtained with LPS-activated microglia. Microglia cultures treated with LPS showed an increase in the secreted levels of nitrite and TNF-α (3.0- and 4.2-fold respectively, compared with basal levels) (Fig. 4A), and, again, a complete inhibition in nitrite and TNF-α levels was observed in microglia cultures treated with either NP00111 or NP01138. Also, addition of TDZD compounds to the culture medium of microglial cells strongly inhibited the LPS-induced expression of the two cytokines, IL-6 and TNF-α, as well as COX-2 and iNOS intracellular levels (Fig. 4, B and C). A similar cellular density is present in all microphotographs, as assessed by DAPI staining (data not shown). Altogether these results suggest that NP00111 and NP01138 inhibit inflammatory activation of cultured glial cells.

NP00111 and NP01138 Protect from Inflammation-induced Neurodegeneration—We next investigated the effect of NP00111 and NP01138 on neuronal cell death induced by cell-free supernatant from LPS-stimulated microglia (SP). Rat embryonic cortical neurons were stimulated with supernatant from LPS-activated microglia (SP) in the absence or presence of TDZDs (50 μM) and the production of nitrite and TNF-α was evaluated by the Griess reaction or specific enzyme-linked immunosorbent assay. Values represent the means ± S.D. from three different experiments. ***, p ≤ 0.001 versus LPS-treated cells. B, rat primary astrocyte cultures were treated as in A, and the expression of TNF-α, IL-6, COX-2, and iNOS was evaluated by immunofluorescence analysis and confocal microscopy using specific antibodies, as described under “Experimental Procedures.” Scale bar, 20 μm. Representative results of three different experiments are shown. C, quantitative confocal analysis. The percentage of stained cells was analyzed using the analiSIS software, as described under “Experimental Procedures.”
significant induction in the number of Annexin V-positive cells compared with the control cultures (Fig. 5). The addition of NP00111 or NP01138 to neuronal cultures treated with conditioned medium from LPS-activated microglial cells suppressed neuronal cell death, suggesting that TDZDs could mediate their neuroprotective effects by blocking the effects of activated microglial-derived cytotoxic factors.

Neurodegeneration induced by conditioned medium from
LPS-activated microglia was associated with an increase in neuronal expression of TNF-α, IL-6, iNOS, and COX-2 (Fig. 6, A and B) in a manner similar to the one observed in glial cells treated with LPS. In addition, as observed in astrocytes and microglia, the TDZD compounds NP00111 and NP01138 completely blocked the induction of these genes in neuronal cultures. A similar cellular density is present in all microphotographs, as assessed by DAPI staining (data not shown). In line with these results, and as shown for glial cells, the release of nitrite to the culture medium was significantly increased after incubation of the neurons with supernatant from LPS-activated microglia (1.9-fold compared with basal values), and this increase was completely abolished in cultures treated with both TDZDs (Fig. 6C).

NP00111 and NP01138 Protect Neurons from Apoptotic and Excitotoxic Stress—We next analyzed whether the neurodegeneration induced by other insults could also be prevented by TDZD compounds. To this end, we treated cortical neurons with 50 nM staurosporine (apoptotic stimulus) or 100 μM glutamate (excitotoxic insult) and determined apoptosis by Annexin V staining. As shown in Fig. 7, both stimuli elicited an induction in the number of apoptotic cells, similar to those found when an inflammatory stimulus was used. The addition of NP00111 or NP01138 protected neuronal cultures from both staurosporine-induced apoptosis and glutamate-induced excitotoxicity. These observations indicate that the protective role of the TDZDs is not restricted to a specific insult.

Dependence of TDZDs Effects on PPARγ Activation—The structural similarities between NP00111 and NP01138 and the antidiabetic thiazolidinediones, such as rosiglitazone (Fig. 1) prompted us to analyze a possible involvement of the nuclear receptor PPARγ in the actions of these compounds. Thiazolidinediones are known ligands of PPARγ (16), whose activation has been shown to play a key role in cellular proliferation, differentiation, and inflammation (29, 30). This fact suggests the possibility that PPARγ activation could be involved in the mediation of the effects of these TDZDs in neural cells. We first tested this hypothesis in transient transfection experiments by using a reporter construct containing three consensus PPARγ response elements (pPPRE-tk-luc). As a positive control we used rosiglitazone, a well known high affinity ligand of PPARγ. In HT22 cells transfected with pPPRE-tk-luc and expression vectors for PPARγ and its partner RXRα, a significant stimulation of luciferase activity was observed after treatment with NP01138 (Fig. 8). This increase was similar or even higher (in the case of NP01138) to the one observed for rosiglitazone. These results demonstrate that these compounds are able to activate a reporter construct containing PPARγ consensus response elements. It has been shown that the PPARγ receptor activates transcription acting as a permissive PPARγ/RXR heterodimer (review in Ref. 31). This kind of nuclear receptor heterodimers can be activated by ligands of either RXR or its partner receptor and are synergistically activated in the presence of both ligands. Therefore, we next...
analyzed the response of the PPRE construct to the RXR ligand 9-cis-RA alone or in combination with rosiglitazone, NP00111, or NP01138. Fig. 8 shows that treatment of HT22 cells with 9-cis-RA resulted in a significant increase (1.5-fold) in promoter activity. Moreover, when the cells were incubated with 9-cis-RA together with rosiglitazone, NP00111, or NP01138, a larger activation of the PPRE reporter construct was observed. These data further suggest that the effects of the TDZDs could be mediated by activation of PPAR\textsubscript{a}.

In view of the data shown above, we next analyzed whether activation of PPAR\textsubscript{a} is involved in the neuroprotective and anti-inflammatory effects of the TDZDs. Because TDZDs have also been described as GSK-3\textsubscript{b} inhibitors and it has been postulated that could be of potential therapeutic use for the treatment of Alzheimer disease, we also tested the effect of LiCl, an inhibitor of GSK-3\textsubscript{b}, on neuronal cell death induced by conditioned medium from activated microglia and proinflammatory activity. To this end, neuronal cultures were pretreated with the selective PPAR\textsubscript{a} antagonist GW9662 (32–34) before exposure to NP00111 and NP01138 and the cell-free supernatant from LPS-activated microglia. Other neuronal cultures were pretreated with LiCl prior to stimulation with the conditioned medium. As shown in Fig. 9, A and B, GW9662 suppressed both, the blocking effect of TDZDs on COX-2 induction and their neuroprotective effects in cultures of cortical neurons. Treatment of neuronal cultures with the GSK-3\textsubscript{b} inhibitor LiCl (Fig. 9, B and C) did not have any effect upon the increase in Annexin V-positive cells elicited by treatment with conditioned medium from microglia. The induction in COX-2 levels was also not altered by incubation of neuronal cultures with LiCl. These results suggest an involvement of the nuclear receptor PPAR\textsubscript{a} in the neuroprotective action of both compounds.

Finally, we analyzed whether the action of NP00111 and NP01138 on glial cells was also dependent on PPAR\textsubscript{a} activation. Astrocytes and microglial cells were then treated with...
GW9662 or LiCl, and nitrite production was measured after LPS stimulation. As can be seen in Fig. 10, the nitrite levels in the culture medium of astrocytes and microglia stimulated with LPS were very similar to those found in control cultures. On the other hand, incubation of these cultures with the PPARγ antagonist GW9662 significantly blocked the inhibitory effect of both TDZDs on nitrite production after LPS stimulation. These data strongly suggest that NP00111 and NP01138 are acting through activation of the PPARγ receptor and not through their inhibition of GSK-3β activity.

**FIG. 10. Effect of GW9662 and the GSK-3β inhibitor LiCl on nitrite production by glial cells.** Either rat primary astrocytes or microglia cultures were treated for 24 h with LPS (10 μg/ml) in the absence or presence of NP00111 or NP01138, and the expression of COX-2 and apoptosis were analyzed as described under “Experimental Procedures.” Some cultures were pre-incubated 1 h with 30 μM GW9662 prior to the addition of the TDZDs (A), or with 20 mM LiCl prior to the addition of conditioned medium from LPS-activated microglia (B). Scale bar, 20 μm. Representative results of three different experiments are shown. C, quantitative confocal analysis. The percentage of stained cells was analyzed using the analiSIS software, as described under “Experimental Procedures.”

**DISCUSSION**

In the present work, we have demonstrated that NP00111 and NP01138, two thiazolidinone compounds, suppress inflammatory activation of astrocytes and microglia in vitro and protect cortical neurons from cell death and that these effects could be mediated by a mechanism apparently dependent on PPARγ activation.

Inflammation plays a central role in the pathogenesis of several brain disorders and neural injury (35) and develops as a consequence of activation of astrocytes and microglial cells (36). Inflammatory cells generate potentially damaging nitric oxide, oxygen free radicals, proteases, arachidonic acid derivatives, and cytokines, which could be mediators of the so-called secondary damage (35). Augmented activation of microglia and increased levels of inflammatory compounds have been localized at the sites of neurodegeneration in several disorders like...
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Alzheimer disease, multiple sclerosis, AIDS-associated dementia, and posttraumatic lesions. Stimulated astrocytes are also an important cellular source of inflammatory mediators in the central nervous system, and the proliferation of astrocytes is frequently observed after brain injury (37). Here we show that NP00111 and NP01138 are strongly anti-inflammatory against LPS-induced responses in rat primary astrocytes and microglia. Our results demonstrate that NP00111 and NP01138 inhibit NO production and TNF-α release from LPS-activated astrocyte and microglia cultures. In addition, these TDZDs completely blocked intracellular accumulation of cytokines, such as IL-6 and TNF-α, and the expression of iNOS and the proinflammatory gene COX-2, in response to LPS. Hence, these results suggest that these compounds could be of therapeutic value in those brain diseases and brain damage where inflammation processes are involved.

We have also shown that NP00111 and NP01138 not only prevent the inflammatory response by glial cells but also have a neuroprotective activity in primary cortical neurons. These compounds protect cortical neurons from apoptosis induced by cell-free medium from LPS-activated microglial cultures. In addition, TDZDs protected cortical neurons against staurosporine-induced apoptosis and glutamate-induced excitotoxicity, suggesting that these compounds are potent neuroprotective agents against a wide variety of neuronal insults. Neuroprotection by TDZDs was coincident with a decrease in cytokine, COX-2, and iNOS expression and NO production. Accumulating data indicate that, in addition to glial cells, neurons can express iNOS (38–40), whose production of NO can be neurotoxic (41, 42). Although a role for COX-2 in healthy cells is not clear, under pathological conditions, the induction of COX-2 in neurons has been well demonstrated, and this induction correlates with apoptosis. COX-2 is rapidly induced in hippocampal and cortical neurons following an electroconvulsive seizure (43). COX-2 is also induced in models of cerebral ischemia, and there is a direct correlation between the extent of COX-2 mRNA induction and the severity of subsequent tissue damage (44).

TDZDs were first described as GSK-3β inhibitors and were postulated that could be of potential therapeutic use for the treatment of Alzheimer disease and other important unmet pathologies as diabetes type II, cancer, and chronic inflammatory processes (12). So far, in vivo studies have been only performed using the tet/GSK-3β transgenic model (45) confirming the potential role of TDZDs as therapeutic drugs for Alzheimer disease. However, the results presented here indicate that the effects of two TDZDs, the NP00111 and NP01138 compounds, on neuroprotection and inhibition of glial activation by LPS stimulation are mediated by activation of the PPARγ nuclear receptor. In fact, we show that NP00111 and NP01138 can activate a reporter construct containing consensus PPARγ/RXR permisive heterodimer. Moreover, the protective effects of these TDZDs are suppressed by GW9662, a specific antagonist of PPARγ. Altogether, these results suggest that the neuroprotective and anti-inflammatory effects of NP00111 and NP01138 could be mediated through PPARγ activation. In line with these observations, there are a number of reports showing inhibitory effects of different PPARγ ligands on the production of microglia-derived proinflammatory factors (review in Ref. 26). Petrova et al. (46) were the first to report that 15-deoxy-D12,14-prostaglandin J2 (15dPG-J2), a potent ligand of PPARγ, strongly inhibited LPS-induced iNOS and subsequent NO production in the mouse microglial cell line BV-2. Subsequent studies by Bernardo et al. (22) demonstrated that 15dPG-J2 and ciclitazone attenuated TNF-α and NO production in primary cultures of microglia stimulated with LPS. It has been also revealed that a variety of PPARγ agonists, including the nonsteroidal anti-inflammatory drugs indomethacin and ibuprofen, the thiazolidinediones troglitazone and ciclitazone, and 15dPG-J2, inhibited β-amyloid stimulation of neurotoxic molecules by microglia. However, and in contrast with the results presented here, the direct application of these agonists to cortical neurons did not significantly improved neuron survival, indicating on microglia (47). Conversely, and in agreement with our results, a direct neuroprotective effect of nonsteroidal anti-inflammatory drugs and 15dPG-J2 has also been shown in cerebellar granule neurons (48). Lastly, there is in vivo evidence suggesting that 15dPG-J2 and pioglitazone can reduce the severity of experimental autoimmune encephalomyelitis (49, 50). Our results extend these studies and present new potential ligands of PPARγ as robust agents against neural injury.

Collectively, our results indicate that TDZDs can be very effective neuroprotective and anti-inflammatory compounds in neuronal cells through, at least in part, activation of the nuclear receptor PPARγ. Our study suggests possible therapeutic uses of TDZDs in certain brain disorders, such as multiple sclerosis, Parkinson, and Alzheimer diseases, where inflammatory responses play a major role.

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