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Neuroprotective effects of PPARγ agonists against oxidative insults in HT-22 cells

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are involved in regulating many metabolic and inflammatory processes. The present study explores the role of PPAR ligands in protecting neuronal cultures from toxic insults. For that purpose, we used WY14643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid] as a PPARα agonist, L-165041 and L-783483 as PPARβ ligands, and 15-deoxy-$\Delta^{12,14}$-PGJ2 (15d-PGJ2), troglitazone, and ciglitazone for PPARγ. Experiments were performed using HT-22, an immortalized mouse hippocampal cell line, and SK-N-SH, a human neuroblastoma cell line. Cell viability against glutamate, hydrogen peroxide (H_2O_2), and serum deprivation insults was determined using a calcein acetoxymethyl (AM) assay. Of the compounds tested, only 15d-PGJ2 and troglitazone showed a dose-dependent neuroprotection from glutamate and H_2O_2 insults in HT-22 cells. None of the PPAR agonists was protective in SK-N-SH cells. A minimum of 4–6 h preincubation with 15d-PGJ2 was required to achieve significant neuroprotection. On the other hand, troglitazone was protective even when administered simultaneously with glutamate, or for up to 8 h postglutamate insult. To investigate whether the neuroprotective effects are mediated through PPARγ, we first determined through Western blotting that HT-22 and SK-N-SH cells express PPARγ. However, the neuroprotective effects of those compounds are unlikely to be mediated through the PPARγ for two reasons: (1) various concentrations of another PPARγ agonist (ciglitazone) were not neuroprotective; (2) by itself, PPAR exhibits a low affinity for DNA, and high-affinity binding requires heterodimerization with RXR, the 9-*cis*-retinoic acid receptor; administering 9-*cis*-retinoic acid in conjunction with 15d-PGJ2 did not alter the neuroprotective effects of the latter. Our results demonstrate neuroprotective effects of 15d-PGJ2 and troglitazone that are likely independent of PPARγ.

Keywords: Neuroprotection; Peroxisome proliferator-activated receptor; 15-deoxy-prostaglandin-J2; Troglitazone; Thiazolidinedione

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily. Three different subtypes of PPAR (α, β or δ, and γ) coded by three separate genes have been identified in rodents and humans (Lemberger et al., 1996). PPARα is highly expressed in the liver and mediates the induction of enzymes of the peroxisomal fatty acid oxidation pathways (Dreyer et al., 1992). PPARβ or PPARδ is ubiquitously expressed in a broad range of mammalian tissues (Krey et al., 1997) and in the adult rat (Lemberger et al., 1996). PPARγ is highly expressed in brown and white adipose tissues and, to lesser extent, in the large intestine, retina, and some parts of the immune system (Elangbam et al., 2001). PPARγ regulates the process of adipogenesis (Chawla et al., 1994; Tontonoz et al., 1994) and is the target for the insulin-sensitizing thiazolidinedione (i.e., ciglitazone and troglitazone) class of drugs (Lehmann et al., 1995). Rosiglitazone and pioglitazone are two thiazolidinedione drugs that are clinically used for the treatment of type II diabetes. Thiazolidinediones also inhibit the proliferation, hypertrophy, and migration of vascular smooth muscle cells induced by numerous growth factors (Dubey et al., 1993; Law et al., 1996).

In addition to synthetic ligands, 15-deoxy-$\Delta^{12,14}$-PGJ2 (15d-PGJ2) appears to be a natural ligand for PPARγ with an $EC_{50} = 7.0$ μM (Kliewer et al., 1995).

We investigated the role that various PPAR ligands play in protecting neurons against glutamate, hydrogen peroxide (H_2O_2), and serum deprivation insults. In this report, we show that both 15d-PGJ2 and troglitazone protect HT-22 cells against glutamate and H_2O_2 insults.

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2. Materials and methods

2.1. Materials

WY14643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid], ciglitazone, troglitazone, 9-*cis*-retinoic acid, and 15d-PGJ2 were purchased from Biomol (Plymouth Meeting, PA). L-165041 and L-783483 were kindly provided by Merck Research Laboratories.

2.2. Cell lines

HT-22, an immortalized mouse hippocampal cell line, and SK-N-SH, a human neuroblastoma cell line, were used. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA). The HT-22 line was originally selected from HT-4 cells based on glutamate sensitivity. HT-4 cells were immortalized from primary hippocampal neurons using a temperature-sensitive SV-40 T antigen (Morimoto and Koshland, 1990). SK-N-SH cells were obtained from ATCC (Manassas, VA). HT-22 and SK-N-SH cells were grown to confluency in Dulbecco's modified essential medium (DMEM) and RPMI-1640 media, respectively, and supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS) and 5 mg/ml gentamicin at 37 °C under 95% air/5% CO_2. HT-22 cells were plated at a density of 50,000 cells/ml (5000 cells/well), and SK-N-SH cells were plated at a density of 120,000–150,000 cells/ml (12,000–15,000 cells/well) in 96-well plates. In most studies, wells were pretreated with PPAR ligands over a wide dose range at various times prior to being subjected to either glutamate, hydrogen peroxide, or serum deprivation insults. In some studies, the insults were applied prior to the addition of the PPAR ligand.

2.3. Cell viability

About 13–24 h postinsult time, viability of cells was determined using a calcein acetoxymethyl (AM) assay. Calcein AM, 2.5 μM in phosphate-buffered saline (PBS), was added to cells. After 25 min of incubation, live cells were distinguished by the presence of intracellular esterase activity, which cleaves the calcein AM dye, producing a bright green fluorescence. Viability was measured in relative fluorescent units (RFU), and expressed as percentage of vehicle-treated control values. The calcein assay was designed to produce results that accurately reflect viable cell numbers. At cell concentrations below 10,000 cells/well (96-well plate) for HT-22 and 15,000 cells/well for SK-N-SH, a linear relationship between RFU and cell number was achieved for HT-22 cells ($r^2 = 0.9997$) and for SK-N-SH ($r^2 = 0.9754$) (data not shown).

2.4. Immunoblotting

Harvested SK-N-SH and HT-22 cells were homogenized in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM dithioerythritol (DTE), and 10 μg/ml aprotonin. Cells were sonicated to disrupt cell membranes, and the soluble (cytoplasmic) and pellet fractions were separated by centrifugation. The homogenate was centrifuged at 100,000 × *g*, and the soluble fraction was collected. Samples were adjusted by addition or dilution to 0.05% Triton X-100. Equal amounts of proteins, as determined by the Bradford method, were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) in a BioRad (Hercules, CA) transblot electrophoresis apparatus at 100 V for 2 h using Towbin's buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol). The membranes containing immobilized proteins were blocked with 5% skim milk in Tris salt (TS) buffer (20 mM Tris, pH 7.5, and 0.5 M NaCl). A polyclonal PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA) that cross-reacts with both human and mouse PPAR-γ was prepared in TS buffer and added to the transblots for overnight incubation. After washes, the membranes were incubated with a goat antirabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody as the secondary antibody in TS buffer. Immunoreactive bands were visualized by a standard enhanced chemiluminescence (ECL) procedure.

2.5. Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $P < 0.05$ was considered significant for all experiments. The values are reported as the mean ± S.E.M.

3. Results

3.1. PPARα and PPARβ agonists

3.1.1. Effects of pretreatment with PPARα or PPARβ agonists on glutamate toxicity in HT-22 cells

WY14643 is a potent activator of PPARα (Issemann and Green, 1990; Dreyer et al., 1992) with an approximate EC_{50} of 1.5 μM (Issemann and Green, 1990). L-783483 and L-165041 activate PPARβ with EC_{50} values of approximately 5 nM and 0.5 μM, respectively (Berger et al., 1999). Pretreatment of HT-22 cells with WY14643 (1–40 μM), L-783483, or L-165041 (1 nM–50 μM) failed to prevent glutamate-induced cell death (data not shown).

3.2. PPARγ agonists

3.2.1. Expression of PPARγ protein in HT-22 and SK-N-SH cells

We determined whether HT-22 and SK-N-SH cells expressed the PPARγ protein. Cytosolic extracts from both HT-

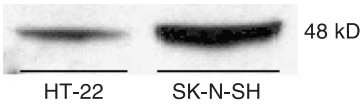

Fig. 1. Western blot analysis of PPARγ. Cytoplasmic extracts of HT-22 and SK-N-SH were subjected to Western blot analysis using an antibody to the PPARγ receptor (48 kDa).

22 and SK-N-SH cells showed a 48-kDa band that reacted with an antibody directed at PPARγ (Fig. 1).

3.2.2. HT-22 cells and glutamate toxicity

3.2.2.1. Effects of pretreatment with PPARγ agonists on glutamate toxicity in HT-22 cells. We tested 15d-PGJ2 and

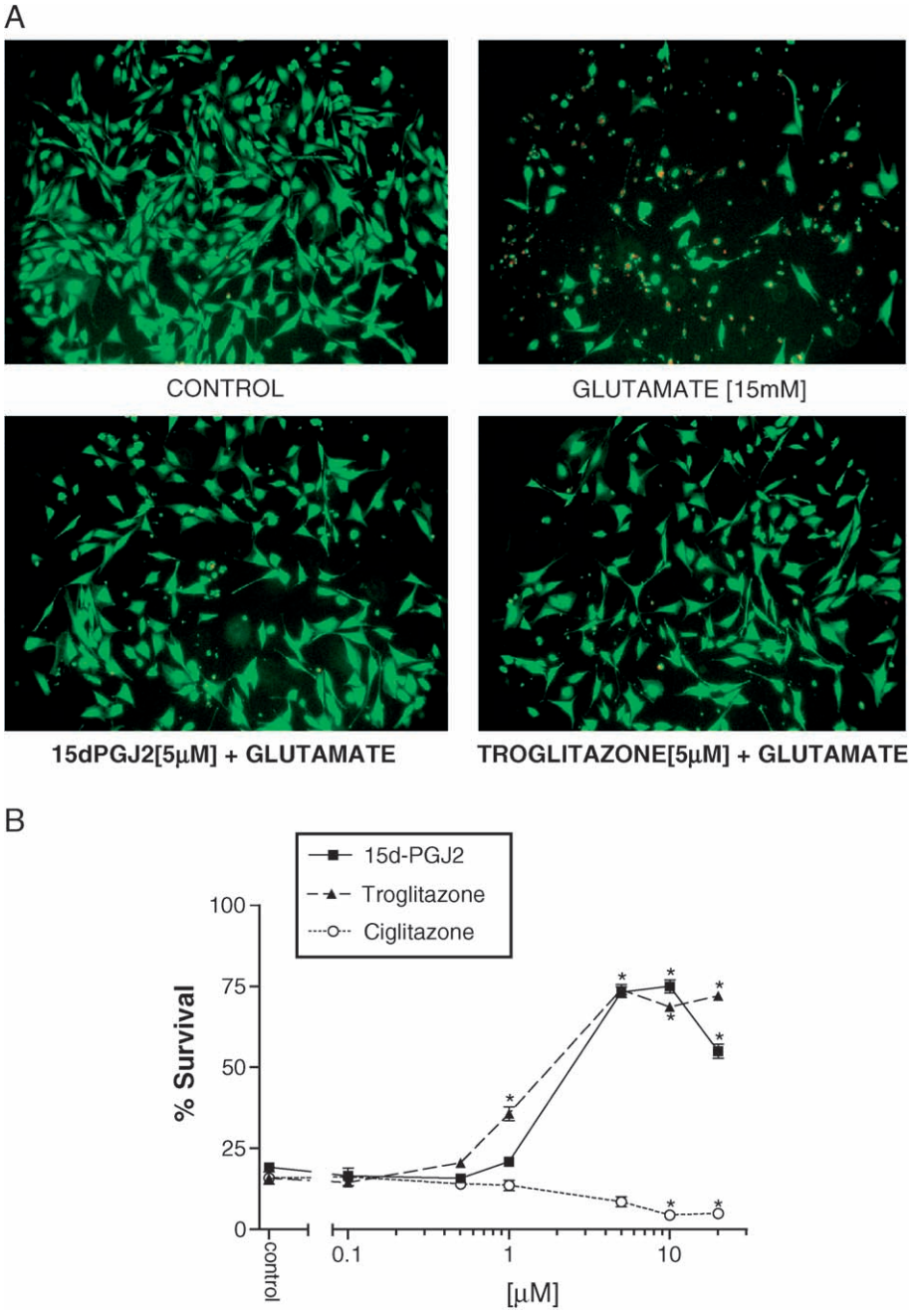

Fig. 2. Effects of PPARγ agonists on HT-22 viability during glutamate exposure. HT-22 cells were treated with 15d-PGJ2, troglitazone, or ciglitazone 24 h (h) prior to a glutamate insult (15 mM). Cell viability was determined about 16 h later. (A) Cells were stained with a propidium iodide/calcein AM solution. Shown are the neuroprotective effects of the 5-μM concentration of 15d-PGJ2 and troglitazone. (B) Data were quantified using the calcein AM assay. Results are expressed as percent survival of the control (nonglutamate-treated) cultures. Shown are mean ± S.E.M. for $n \geq 6$. When error bars are not shown, they are smaller than the symbol used to depict the mean. *Indicates $P < 0.05$ vs. respective control.

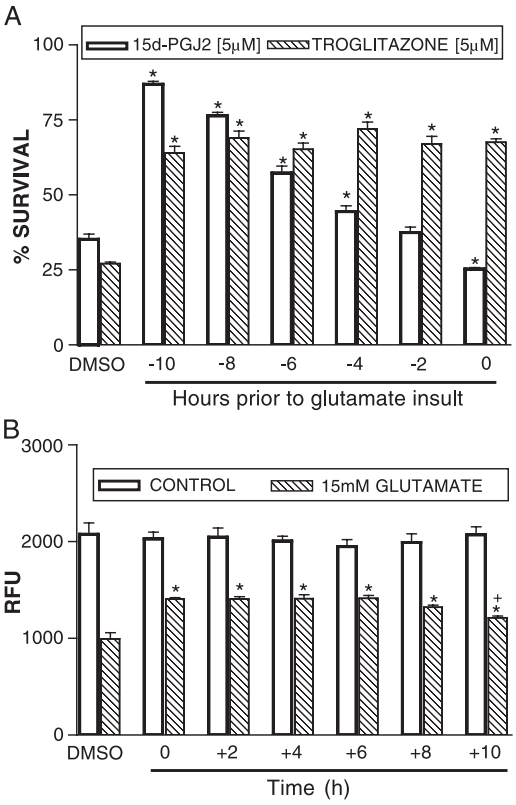

Fig. 3. (A) Effects of preincubation time on the neuroprotection by PPARγ agonists in HT-22 cells during glutamate exposure. HT-22 cells were exposed to 15d-PGJ2 (5 μM) or troglitazone (5 μM) at various times prior to glutamate insult (15 mM). DMSO controls received vehicle for either 15d-PGJ2 (clear bar) or troglitazone (striped bar). Ten hours later ($t=0$), glutamate was added. About 15 h postglutamate insult, cell viability was determined and expressed as percent survival of control (nonglutamate-treated cultures). Shown are mean ± S.E.M. of $n \geq 4$ cultures per group. *Indicates $P<0.05$ vs. respective control group. (B) Effects of posttreatment with troglitazone on HT-22 cell viability during glutamate exposure. Glutamate was first administered, then troglitazone (5 μM) was added at the times indicated. Cell viability was determined 13 h later (from $t=0$). Shown are mean ± S.E.M. for $n=6$. *Indicates $P<0.05$ vs. respective control (DMSO). $^{+}$Indicates $P<0.05$ vs. other time points.

two thiazolidinediones, troglitazone and ciglitazone, for their ability to protect HT-22 cells against glutamate toxicity. HT-22 cultures were incubated with different concentrations of the three compounds for 24 h prior to the insult. Two of these compounds, 15d-PGJ2 and troglitazone, showed a dose-dependent protection against glutamate insult (Fig. 2A and B). Glutamate-treated HT-22 cells in the presence or absence of either 15d-PGJ2 or troglitazone were photomicrographed and compared to control (Fig. 2A). Peak neuroprotective concentrations for 15d-PGJ2 ranged from 1 to 10 μM, and troglitazone exhibited a dose-dependent neuroprotection over concentrations ranging from 1 to 20 μM (Fig. 2B). For 15d-PGJ2 and troglitazone, toxicity was observed at concentrations at or above 10 and 20 μM, respectively.

3.2.2.2. Effects of preincubation time on PPARγ agonists protection of HT-22 cells from glutamate toxicity. We determined the minimum pretreatment time with PPARγ agonists needed to achieve neuroprotection. HT-22 cells require exposure to 15d-PGJ2 for 4–6 h prior to the administration of glutamate to achieve significant neuroprotection. In contrast, troglitazone was equally protective when administered 10 h prior to, or simultaneously with, the glutamate insult (Fig. 3A).

3.2.2.3. Effects of posttreatment time on 15d-PGJ2 and troglitazone protection of HT-22 cells from glutamate toxicity. When added at the same time as glutamate, or up to 10 h thereafter, 15d-PGJ2 failed to display any neuroprotective effects (data not shown). Since troglitazone protected HT-22 cells when administered simultaneously with the glutamate insult, we attempted to determine the length of the delay between glutamate exposure and troglitazone treatment that would still afford neuroprotection. Glutamate was administered to HT-22 cells, and troglitazone was then added 0–10 h thereafter. The neuroprotective effects of troglitazone remained unaltered up to 6 h postglutamate insult (Fig. 3B). At 8 and 10 h postglutamate insult, troglitazone still significantly protected HT-22 cells, but the extent of protection decreased (Fig. 3B).

3.2.3. HT-22 cells and H_2O_2 toxicity

3.2.3.1. Effects of pretreatment with PPARγ agonists on H_2O_2 toxicity in HT-22 cells. We determined the effects of 15d-PGJ2 and troglitazone on HT-22 cell protection against another type of pro-oxidant insult, hydrogen peroxide. Both compounds showed a dose-dependent protection of HT-22 cells against H_2O_2 (Fig. 4). Higher concentrations (>10 and >20 μM) of 15d-PGJ2 and troglitazone, respectively, were toxic to HT-22 cells (data not shown).

3.2.3.2. Effects of preincubation time on PPARγ agonists protection of HT-22 cells from H_2O_2 toxicity. Fig. 5 shows

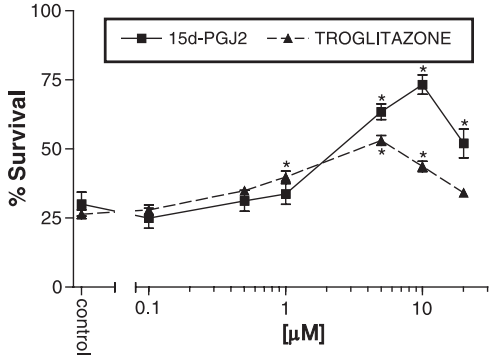

Fig. 4. Effects of PPARγ agonists on HT-22 cell viability during hydrogen peroxide exposure. HT-22 cells were pretreated with either 15d-PGJ2 or troglitazone for 24 h. H_2O_2 (30 μM) was administered and, 24 h later, cell viability was determined and expressed as percent survival of the control (non-H_2O_2-treated) cultures. Shown are mean ± S.E.M. for $n=6$ cultures/group. When error bars are not shown, they are smaller than the symbol used to depict the mean. *Indicates $P<0.05$ vs. respective control.

that 4 h of preincubation with either 15d-PGJ2 or troglitazone was required to achieve significant neuroprotection from H_2O_2 toxicity in HT-22 cells.

3.2.3.3. Effects of posttreatment time on 15d-PGJ2 and troglitazone protection of HT-22 cells from H_2O_2 toxicity. Neither 15d-PGJ2 nor troglitazone protected HT-22 cells after the H_2O_2 insult was administered (data not shown).

3.2.4. HT-22 cells and serum deprivation toxicity

3.2.4.1. Effects of pretreatment with PPARγ agonists on serum deprivation toxicity in HT-22 cells. We examined whether 15d-PGJ2 or troglitazone has neuroprotective activity against serum deprivation toxicity. HT-22 cells were incubated for 24 h in a 10% fetal bovine serum-enriched medium containing either 15d-PGJ2 or troglitazone. Then, cells were exposed for about 12 h to a serum-free medium with both of the compounds present. 15d-PGJ2 as well as troglitazone failed to protect HT-22 cells against serum deprivation (data not shown). In addition, 15d-PGJ2 and troglitazone enhanced the serum deprivation-induced toxicity at or above 10 and 20 μM, respectively (data not shown).

3.2.5. SK-N-SH cells and H_2O_2 and serum deprivation toxicities

3.2.5.1. Effects of pretreatment with PPARγ agonists on H_2O_2 and serum deprivation toxicities in SK-N-SH cells. After showing that protection in HT-22 cells was insult type-specific, we investigated whether it was also cell type-specific. We used SK-N-SH, a human neuroblastoma cell line, and tested whether 15d-PGJ2 and troglitazone would protect SK-N-SH against H_2O_2 and serum deprivation insults. Neither of the compounds protected SK-N-SH against either insults (data not shown). Higher concentrations (>10 and >20 μM) of 15d-PGJ2 and troglitazone, respectively, were toxic to SK-N-SH cells (data not shown).

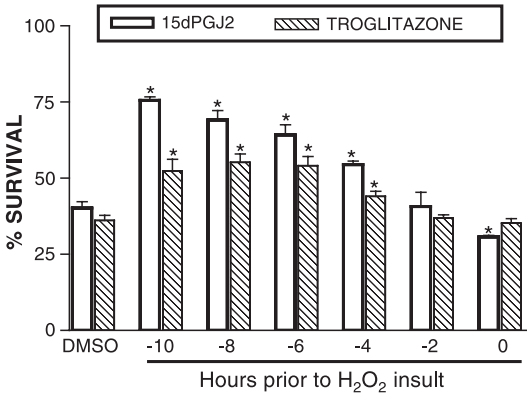

Fig. 5. Effects of preincubation time on the neuroprotection by PPARγ agonists on HT-22 cell viability during H_2O_2 exposure. HT-22 cells were exposed to 15d-PGJ2 (5 μM) or troglitazone (5 μM) at various times prior to H_2O_2 (30 μM) insult. DMSO controls received vehicle for either 15d-PGJ2 (clear bar) or troglitazone (striped bar). Ten hours later ($t=0$), H_2O_2 (30 μM) was added. About 12 h post-H_2O_2 insult, cell viability was determined and expressed as percent survival of control (non-H_2O_2-treated cultures). Shown are mean ± S.E.M. of $n=6$ cultures/group. *Indicates $P<0.05$ vs. respective control group.

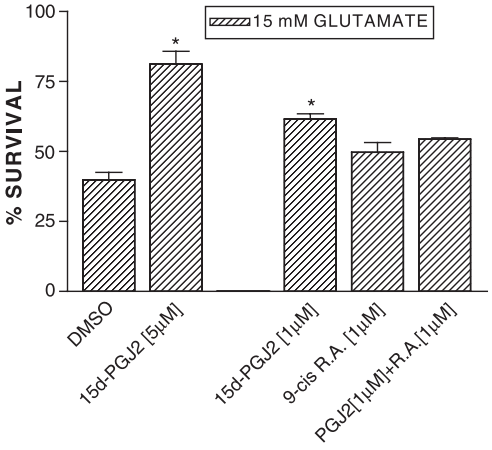

Fig. 6. Effects of 15d-PGJ2, 9-*cis*-retinoic acid, and their combination on HT-22 cell viability during glutamate exposure. The protective effect of a 5-μM concentration of 15d-PGJ2 is shown to demonstrate that the lack of additive effects of 15d-PGJ2 and 9-*cis*-retinoic acid is not due to maximizing neuroprotection at 1 μM 15d-PGJ2. Shown are mean ± S.E.M. for $n=4$ cultures/group. *Indicates $P<0.05$ vs. control (DMSO).

3.3. Interaction with 9-cis-retinoic acid

3.3.1. Effects of 9-cis-retinoic acid on the protective effects of 15d-PGJ2 against glutamate toxicity in HT-22 cells

By itself, PPAR exhibits a low affinity for DNA; high-affinity binding requires heterodimerization with RXR, the 9-*cis*-retinoic acid receptor (Dussault and Forman, 2000). We investigated whether adding 9-*cis*-retinoic acid would increase the activity of 15d-PGJ2. Fig. 6 shows that adding a 1-μM concentration of 9-*cis*-retinoic acid did not alter the neuroprotective effects of 15d-PGJ2.

4. Discussion

The present study demonstrates that 15d-PGJ2 and troglitazone protect mouse hippocampal HT-22 cells against glutamate and H_2O_2 toxicities. The neuroprotection was dose-dependent, effective against two pro-oxidant insults, glutamate and H_2O_2, and selective for 15d-PGJ2 and troglitazone, but not another PPARγ agonist, ciglitazone, nor for any PPARα or PPARβ/PPARδ agonists tested.

Despite their ability to protect HT-22 cells against glutamate and H_2O_2, both compounds failed to protect either the HT-22 or the SK-N-SH cells against serum deprivation. This absence of efficacy during serum deprivation may be due to different pathways leading to cell death during serum deprivation vs. oxidative insults. The toxicity of glutamate in HT-22 cells is mediated through oxidative stress (Murphy et al., 1989). Glutamate blocks cystine uptake by inhibiting the

glutamate/cystine antiporter (Murphy et al., 1989). Since cystine is required for glutathione (GSH) synthesis, the intracellular concentration of GSH decreases as a consequence. In as much as HT-22 cells lack ionotropic (NMDA) glutamate receptors, the glutamate-induced cell death appears to occur via a slow-onset oxidative stress (Li et al., 1997; Maher and Davis, 1996). Morphologically, glutamate-treated cells undergo a form of cell death distinct from either necrosis or apoptosis, characterized by plasma membrane blebbing and cell shrinkage, but unlike apoptosis, no DNA fragmentation is observed and the nuclei remain intact (Tan et al., 1998b). By contrast, serum deprivation appears to initiate more characteristics of apoptosis (Miller and Johnson, 1996; Tanabe et al., 1998) and is resistant to protection by either 15d-PGJ2 or troglitazone.

The neuron type specificity of the neuroprotective effects of troglitazone is supported by recent reports. Uryu et al. (2002) reported that troglitazone inhibits both postglutamate neurotoxicity and low potassium-induced apoptosis in cerebellar granule neurons. Nishijima et al. (2001) have shown that troglitazone improves the survival of rat motoneurones against brain-derived neurotrophic factor (BDNF) withdrawal, but does not promote the survival of hippocampal neurons. Additionally, neuroprotection by troglitazone has been recently demonstrated in vivo. Sundararajan et al. (2001) reported that troglitazone reduces infarct size and improves functional outcome following cerebral ischemia in rats. Heneka et al. (1999) demonstrated that PPARγ agonists protect cerebellar granule cells from cytokine-induced apoptotic cell death by inhibition of inducible nitric oxide synthase (iNOS).

In contrast to the present report of neuroprotection with 15d-PGJ2, Rohn et al. (2001) reported that incubation of cortical neurons and SH-SY-5Y human neuroblastoma with 10 μM 15d-PGJ2 induced morphological changes including neurite degeneration and nuclear condensation and fragmentation, which were consistent with neurons dying by apoptosis. At this dose of 15d-PGJ2, we observed toxicity in both HT-22 and SK-N-SH cells.

15d-PGJ2 and troglitazone displayed different preincubation time requirements for their neuroprotective effects. 15d-PGJ2 required a minimum of 4–6 h pretreatment to protect against H_2O_2 or the glutamate insults. Troglitazone required a minimum of 4 h of preincubation to protect against H_2O_2. On the other hand, it was protective even when given simultaneously with or up to 10 h after glutamate. Neither of the compounds protected cells when administered after the H_2O_2 insult. The reason for different preincubation requirements by troglitazone during the glutamate-vs.-H_2O_2 insults could be explained by the mechanism of cytotoxicity of the two insults. During glutamate exposure, reactive oxygen species production proceeds in two phases: an initial slow increase for the first 6 h, followed by a much higher rate (Tan et al., 1998a). H_2O_2, on the other hand, rapidly penetrates into cells and induces oxidation of a variety of molecules. In this situation, pretreatment for a time sufficient to allow distribution of the compound and activation of yet-to-be-determined protective mechanisms is needed.

Even though 15d-PGJ2 and troglitazone are both PPARγ agonists, we do not believe that the PPARγ receptor mediates the neuroprotective effects of these compounds for several reasons. First, both HT-22 and SK-N-SH cells express PPARγ, but SK-N-SH cells are not protected by either troglitazone or 15d-PGJ2. Second, ciglitazone, another thiazolidinedione, and a PPARγ ligand with similar affinity to 15d-PGJ2 (Lehmann et al., 1995) did not protect HT-22 cells against glutamate cytotoxicity. Third, by itself, PPAR exhibits a low affinity for DNA; high-affinity binding requires heterodimerization with RXR, the 9-*cis*-retinoic acid receptor (Benson et al., 2000; Dussault and Forman, 2000). In the present study, we saw no interaction between 9-*cis*-retinoic acid and 15d-PGJ2 on neuroprotection.

Rossi et al. (2000) reported that 15d-PGJ2 inhibits IkappaB kinase (IKK), which raises the possibility that activation of NFκB may be the mediator of the neuroprotective effects of 15d-PGJ2. In that study, the inhibitory effects of 15d-PGJ2 on IKK were evident within an hour of the treatment with 15d-PGJ2. In the present study, we observed that 4–6 h of preincubation with 15d-PGJ2 was required for neuroprotection, arguing against the inhibition of IKK as a potential mechanism of neuroprotection.

In summary, we have shown that two PPARγ ligands, 15d-PGJ2 and troglitazone, protected mouse hippocampal HT-22 cells against oxidative stress induced by glutamate or H_2O_2. Oxidative stress is implicated in a number of neurodegenerative diseases including Alzheimer's disease (Markesbery, 1997), Parkinson's disease (Jenner and Olanow, 1996), and stroke (Cherubini et al., 2000). Several lines of evidence suggest that increased oxidative stress occurs in diabetes and could have a role in the development or deterioration of peripheral insulin resistance (Rudich et al., 1999). These results may be useful in the development of novel pharmaceutical therapies for targeting neurodegenerative diseases involving an oxidative stress component. In addition, thiazolidinediones are used to treat type II diabetes mellitus, which leads to neuronal pathologies. A single agent that could target both conditions is of great benefit for diabetic patients suffering from neuropathies.

Acknowledgements

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