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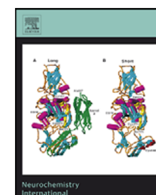

PPARγ stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)γ stimulation provides protection in several models of neurological disorders, but the mechanisms underlying these effects remain to be fully elucidated. Here we have studied whether two PPARγ agonists, pioglitazone and rosiglitazone, prevent loss of differentiated SH-SY5Y cells transiently exposed to glucose deprivation (GD). Nanomolar drug concentrations prevented GD-induced cell loss in a concentration- and time-dependent manner. These effects were abolished by malonate, a reversible mitochondrial Complex II inhibitor, while significantly potentiated by pyruvate, thus suggesting that they are related to mitochondrial function. During cell pretreatment, PPARγ agonists promoted biogenesis of functional mitochondria, as indicated by the up-regulation of PPARγ coactivator (PGC)-1α, *NRF1*, *TFAM*, cytochrome *c* oxidase subunit (CO) I and CO IV, and the increased level of mtDNA, while did not significantly change mitochondrial membrane potential. In addition, the analysis of the concentration–response and time-course curves for the protective effects and the up-regulation of mitochondrial biogenesis markers suggests that mitochondrial biogenesis and cell loss prevention are related effects. In conclusion our data indicate that a prolonged PPARγ stimulation, by repeated administration of nanomolar pioglitazone or rosiglitazone concentrations, decreases GD-induced loss of differentiated SH-SY5Y cells. In addition, they suggest that mitochondrial biogenesis may contribute to these effects.

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1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. Three PPAR isoforms (α, β/δ and γ), which are encoded by three distinct genes, have been characterized both in rodents and in humans (Berger and Moller, 2002). Among PPAR, PPARγ is a pharmacological target of particular interest. PPARγ stimulation enhances sensitivity of peripheral tissues to insulin and two synthetic PPARγ agonists, pioglitazone and rosiglitazone, are currently approved by both FDA and EMEA for the treatment of Type 2 diabetes (Yki-Järvinen, 2004).

A growing body of evidence indicates that PPARγ stimulation might exert further beneficial effects. PPARγ is expressed in several regions of the adult CNS including piriform cortex, basal ganglia and dentate gyrus, both in neuronal and non-neuronal cells (Moreno et al., 2004; de la Monte and Wands, 2006). Moreover, it has been demonstrated that PPARγ agonists reduce neuronal cell loss in *in vitro* models of neurotoxicity (Aoun et al., 2003; Zhao et al., 2006; Jung et al., 2007; Fuenzalida et al., 2007) and in *in vivo* models of cerebral ischemia-reperfusion injury (Culman et al., 2007), Parkinson's disease (PD; Breidert et al., 2002; Dehmer et al., 2004) and amyotrophic lateral sclerosis (ALS; Kiaei et al., 2005; Schütz et al., 2005). However, at cellular level, the mechanisms underlying these effects are not fully understood. Part of them have thought to be related to the ability of these drugs to regulate gene expression in neuronal cells. For example, it has been shown that pioglitazone reduces the harmful induction of cyclooxygenase-2 in response to oxidative injury in primary cortical neurons (Zhao et al., 2006), rosiglitazone induces the expression of antioxidative

Abbreviations: ALS, amyotrophic lateral sclerosis; atRA, all-*trans* retinoic acid; BSS, bicarbonate-buffered balanced salt solution; CO I, cytochrome *c* oxidase subunit I; CO IV, cytochrome *c* oxidase subunit IV; DMEM/F12, Dulbecco modified Eagle's medium/Nutrient Mixture Ham's F12; FCS, fetal calf serum; GD, glucose deprivation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide; PBS, Mg^{2+}-free phosphate-buffered saline; PD, Parkinson's disease; PGC-1α, peroxysome proliferator-activated receptor γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; SDH, succinate dehydrogenase.

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enzymes (superoxide dismutase and catalase) in SH-SY5Y human neuroblastoma cells (Jung et al., 2007) and of the anti-apoptotic protein Bcl-2 in PC12 cells (Fuenzalida et al., 2007). Nevertheless, it is reasonable that further actions could contribute to the PPARγ-mediated neuroprotective effects.

It has been demonstrated that PPARγ stimulation, through the induction of the PPARγ coactivator (PGC)-1α, promotes mitochondrial biogenesis and remodeling in several peripheral tissues such as adipose and muscle tissues (Puigserver et al., 1998; Wu et al., 1999; Puigserver and Spiegelman, 2003; Pagel-Langenickel et al., 2008). Similar effects have been recently shown in the brain of mice and in the NT-2 human neuronal-like cells after prolonged exposure to rosiglitazone or pioglitazone, respectively (Strum et al., 2007; Ghosh et al., 2007). Mitochondrial biogenesis has been proposed to contribute to the effects of PPARγ agonists in diabetic patients (Wilson-Fritch et al., 2004; Rong et al., 2007; Pagel-Langenickel et al., 2008). Though, to date, there is no evidence that it also contributes to the PPARγ-mediated neuroprotective effects.

Here we have evaluated, *in vitro*, whether pioglitazone and rosiglitazone reduce neuronal cell loss induced by transient glucose deprivation (GD) and how mitochondrial biogenesis contribute to this effect. For these purposes, we have employed differentiated SH-SY5Y human neuroblastoma cells. These cells have been already employed as cellular model to study GD-induced neuronal cell death (Russo et al., 2004; Kögel et al., 2006; Kobayashi et al., 2007). Interestingly, they express PPARγ (Valentiner et al., 2005), thus representing a valuable cellular model to study the protective effects that follow PPARγ stimulation.

2. Materials and methods

2.1. Drugs and chemicals

Dulbecco modified Eagle's medium/Nutrient Mixture Ham's F12 (1:1) (DMEM/F12), and fetal calf serum (FCS) were from Lonza (Milan, Italy). Tetramethylrhodamine ethyl ester (TMRE) was from Molecular Probe (Milan, Italy). Rosiglitazone was from Alexis (Vinci, Italy). Pioglitazone, malonic acid, L-glutamine, penicillin, pyruvate, streptomycin, all-*trans*-retinoic acid (atRA), and all other reagents were from Sigma–Aldrich (Milan, Italy). Pioglitazone, rosiglitazone and atRA were dissolved in dimethylsulfoxide, all other drugs were dissolved in the experimental buffers. Final drug concentrations were obtained by dilution of stock solutions in the experimental buffers. Final concentration of organic solvent was always less than 0.1% (vol/vol), it has no effects on cell viability and cells exposed to vehicle alone were considered as controls.

2.2. Cell cultures

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM/F12 supplemented with 10% (vol/vol) FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cell culture medium was replaced every 2 days and the cultures were maintained at 37 °C in a 95% (vol/vol) air–5% (vol/vol) CO_2 humidified incubator. Cells were used up to the 15th passage and were differentiated by treatment with atRA (5 μM) added to cell culture medium every 2 days for 5 days (Zimmermann et al., 2004). The day before the experiment, cells were plated in 24- or 6-well culture plates (0.5–0.8 x 10^6 cells/well, respectively).

2.3. Cell treatments and in vitro model of GD-induced neuronal cell loss

To study the protective effects of pioglitazone and rosiglitazone we used the experimental protocol shown in Fig. 1 of supplemental material. Cells were pretreated for 5 days with either pioglitazone or rosiglitazone (simultaneously with atRA). After a 24-h washout, cells were either transiently glucose-deprived (2 h), exposed to malonate (10 mM; 1.5 h) or glucose-deprived in the presence of malonate (1 h). The protective effects of the PPARγ agonists were measured at the end of a 24–72-h recovery period. In some experiments pyruvate (5 mM) was added during the recovery period (see Section 3 for details). GD was performed as described by Suh et al. (2007a). Briefly, the culture medium was exchanged with a bicarbonate-buffered balanced salt solution (BSS; in mM: 134, NaCl; 15.7, $NaHCO_3$; 3.1, KCl; 1.2, $CaCl_2$; 1.2, $MgSO_4$; 0.25, KH_2PO_4). The pH was adjusted to 7.2 and the solution was equilibrated with 5% (vol/vol) CO_2 at 37 °C. After a 5-min period to allow complete regression of glucose from the cells, the culture medium was completely exchanged a second time and cultures were placed at 37 °C in a 95% (vol/vol) air–5% (vol/vol) CO_2 humidified incubator. Control wells received BSS supplemented with 10 mM glucose. GD was terminated at the designated time points by replacing BSS with standard medium.

Cell loss was evaluated by measuring both cell viability and cell number at the end of the recovery period. Cell viability was determined by measuring the succinate dehydrogenase (SDH) activity by the MTT assay (Mosmann, 1983). Cell number was determined by counting the viable cells in a hemacytometer by the trypan blue exclusion test, blind by an observer.

2.4. Western blot analyses

Cell culture plates were washed with Mg^{2+}-free phosphate-buffered saline (PBS) before adding an ice-cold lysis buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (wt/vol) sodium dodecyl sulphate, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 5 mM EDTA] supplemented with 1 μl/ml of a protease inhibitors cocktail (Sigma–Aldrich), 0.1 mM $ZnCl_2$, and 1 mM phenylmethylsulphonyl fluoride. Cell lysates were centrifuged at 18,000 × g for 15 min and the supernatants were recovered. The protein concentration of cell lysates was determined using the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA) following the manufacturer's instruction. Samples containing 75 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis using an 8 or 12% (wt/vol) gel for either PGC-1α and PPARγ or cytochrome *c* oxidase subunit I (CO I) and IV (CO IV), respectively. Proteins were blotted to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) which were then incubated with SuperBlock blocking buffer (Pierce). PGC-1α was detected following incubation, with a goat polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:200 in PBS containing 0.1% (vol/vol) Tween-20 (PBS-T) overnight at 4 °C. PPARγ was detected following incubation, with a rabbit polyclonal antibody (Santa Cruz) diluted 1:200 in PBS-T for 1 h at room temperature. CO I and CO IV were detected following incubation with mouse monoclonal antibodies (Santa Cruz and Abcam plc Cambridge Science Park, Cambridge, UK, respectively) diluted 1:1000 in PBS-T overnight at 4 °C. The secondary antibodies were horseradish peroxidase-conjugated donkey anti-goat IgG (1:14,000; Santa Cruz), donkey anti-rabbit IgG (1:10,000; Santa Cruz) and sheep anti-mouse IgG (1:10,000; Santa Cruz). To confirm the homogeneity of the proteins loaded, the membranes were stripped and incubated with an anti-β-actin monoclonal antibody (1:2500, Santa Cruz). The membranes were overlaid with Western Lightning Chemiluminescence Reagent *Plus* (PerkinElmer Life Science, Cetus, Norwalk, CT, USA) and then exposed to Hyperfilm ECL film (Amersham Biosciences Corp., Piscataway, NJ, USA). Protein bands were quantified on film by densitometry, using the software Gel-Pro Analyzer 4.5, 2000 (Media Cybernetics Inc., Leiden, The Netherlands).

2.5. mtDNA extraction and quantification

mtDNA was extracted and analyzed as described by Nisoli et al. (2003) with minor modifications. Aliquots of mtDNA were loaded on ethidium bromide-stained agarose gel [1.5% (wt/vol)] and signals were analyzed by the densitometric analysis software NIH Image J 1.41 (National Institutes of Health, Bethesda, MD, USA).

2.6. RT-PCR analysis

Total RNA was extracted from cultured cells by using Omnizol (Euroclone, Milan, Italy). Resulting mRNA was reverse-transcribed by using the RevertAid HMinus Synthesis kit and oligo$(dT)_{18}$ primers (Fermentas Life Science, Milan, Italy) for 1 h at 42 °C. Reactions were terminated by heating the mixtures at 70 °C for 10 min. The resulting cDNA was used as a template for PCR amplifications. PCR amplifications were performed in 50 μl reaction mixtures containing 2.0–4.0 μl of cDNA, 5.0 μl of 10× buffer, 1.5 μl of 50 mM $MgCl_2$, 0.5 μl of 2 mM dNTPs mix, 2 U of *Taq* DNA polymerase (Fermentas Life Science), and 5.0 nM of each primer (SigmaGenosys; Table 1, Supplemental material). PCR amplicons were resolved in an ethidium bromide-stained agarose gel [1.5% (wt/vol)] by electrophoresis, and signals were quantified by the analysis software (NIH Image J 1.41). Data are expressed as the ratio of the signal obtained for each gene in one sample divided by that obtained for the reference gene (β-actin) in the same sample.

2.7. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was measured with the fluorescent probe TMRE as previously described (Rao and Norenberg, 2004) with minor modification. Briefly, cells were loaded with TMRE in standard fresh medium at a final concentration of 50 nM for 30 min. Emitted fluorescence was examined on a Leica inverted microscope equipped with multivariant fluorescent filters. The fluorescence emission was recorded at 590 nm. Random fields with a similar degree of cell density in each experimental group were captured with an air cooled digital camera and fluorescence intensity from each cell (30–40 cells/group) was analyzed using NIH Image J 1.41 software.

2.8. Data analysis

Results are expressed as means ± S.E.M. of at least three experiments. Statistical significance was evaluated by one-way ANOVA followed by the post hoc Dunnet's test.

Differences were considered statistically significant when $p < 0.05$. Data were fitted as sigmoidal concentration–response or time-response curves and analyzed with a four-parameter logistic equation using the software Origin 6.0 (Microcal Software, Northampton, MA, USA). EC_{50} and ET_{50} were the molar concentration of drug that exerts 50% of the maximal effect and the time required to produce 50% of the maximal effect produced by pioglitazone (100 nM).

3. Results

3.1. PPARγ expression in SH-SY5Y neuroblastoma cells

It has been reported that several human neuroblastoma cell lines, including SH-SY5Y line, express PPARγ (Valentiner et al., 2005). To investigate whether our clone expresses this receptor and how either cell differentiation or PPARγ stimulation changes its expression level, we carried out Western blot analyses of cell extracts prepared from cells exposed to either atRA alone (5 μM, 5 days) or pioglitazone (100 nM, 1–5 days) plus atRA. A single band at 55 kDa was detected in untreated cells. As expected for a PPARγ agonist (Kim et al., 2003), pioglitazone gradually and significantly increased PPARγ expression level and on day 5 it was 1.49 ± 0.06-fold over pioglitazone-untreated cells ($p < 0.01$; $n = 4$) (Fig. 1A).

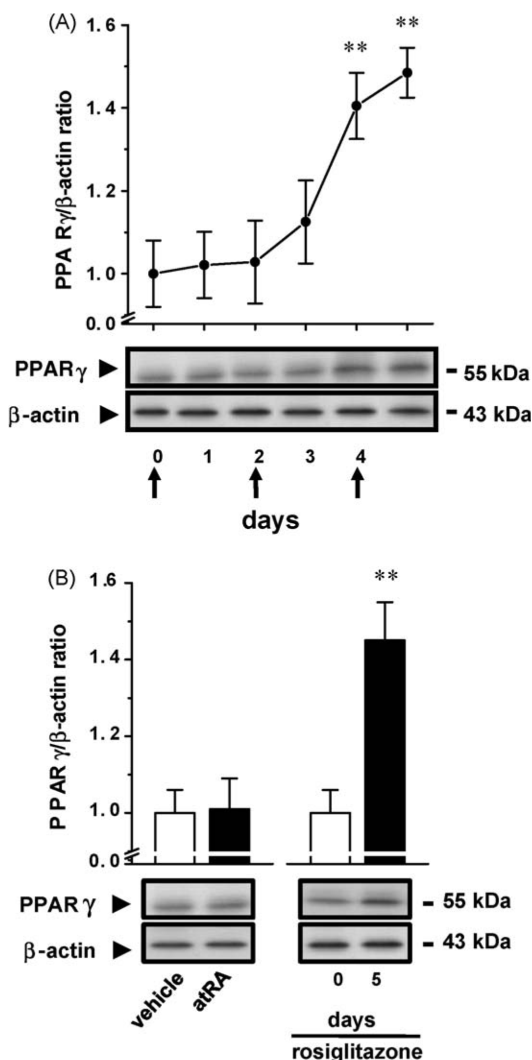

Fig. 1. Expression of PPARγ in SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were treated with either pioglitazone (100 nM; 1–5 days) plus atRA (5 μM) (A), rosiglitazone (10 nM, 5 days) plus atRA or atRA alone (5 days) (B). PPARγ expression was evaluated by Western blot analysis; β-actin was used as internal control. Pictures shown are representative of three to four independent experiments and data are means ± S.E.M. $^{**}p \leq 0.01$ *vs.* untreated cells (day 0).

Similarly, rosiglitazone (10 nM, 5 days) significantly up-regulated PPARγ expression (1.45 ± 0.10-fold over rosiglitazone-untreated cells; $p < 0.01$; $n = 3$; Fig. 1B). In contrast, neither atRA nor vehicle alone changed the PPARγ expression level (Fig. 1B). Notably, GD (see below) did not change PPARγ expression level (data not shown).

These data indicate that differentiated SH-SY5Y neuroblastoma cells can be a valuable model to study the PPARγ-mediated effects on neuronal cells.

3.2. Effects of PPARγ agonists on GD-induced neuronal cell loss

To investigate whether PPARγ stimulation could prevent neuronal cell death due to impairment of cellular energetic homeostasis, we studied the effects of cell pretreatment with either pioglitazone or rosiglitazone on GD-induced neuronal cell loss. GD (2 h) significantly decreased cell viability (−52.6 ± 4.8%, compared to control cells; $p < 0.01$; $n = 6$; Fig. 2A). Pioglitazone and rosiglitazone (10 pM to 1 μM, 5 days) significantly prevented this effect in a concentration-dependent manner with a maximum at 100 and 10 nM (78.9 ± 9.4% and 80.0 ± 9.5% over glucose-deprived cells; $p < 0.01$; $n = 6$; Fig. 2A), respectively; the EC_{50}s calculated were 5.1 ± 1.2 and 0.6 ± 0.2 nM, respectively. The protective effects exerted by the two PPARγ agonists increased in a time-dependent manner; they were significant on day 3 ($p < 0.05$; $n = 4$) and maximal on days 4–5 ($p < 0.01$; $n = 4$; Fig. 2B). Notably, cell pretreatment with either pioglitazone (100 nM, 5 days) or rosiglitazone (10 nM, 5 days) only slightly increased basal SDH activity (+13.0 ± 16.8% and +5.8 ± 9.8% compared to control cells, respectively; $n = 4$), thus indicating that the protective effects we observed does not depend either on the increased basal SDH activity or on cell proliferation. Since MTT assay provides an indirect measure of cell loss we confirmed the above results by determining the number of viable cells at the end of the experiments. GD (2 h) produced a 51.8 ± 11.1% of cell loss that was significantly prevented by either pioglitazone (100 nM; 5 days) or rosiglitazone (10 nM; 5 days): −76.8 ± 13.8% and −78.5 ± 16.3% over glucose-deprived cells, respectively ($p < 0.01$; $n = 3$; Fig. 2C).

These results show that prolonged PPARγ stimulation, by repeated administration of nanomolar concentrations of either pioglitazone or rosiglitazone, decreases GD-induced loss of differentiated SH-SY5Y neuroblastoma cells.

3.3. Effects of PPARγ agonists on PGC-1α expression and function in SH-SY5Y cells

Since, it has been shown that PPARγ stimulation up-regulated PGC-1α in a variety of cell types (Wu et al., 1999; Hondares et al., 2006), including neuronal cells (Strum et al., 2007; Ghosh et al., 2007), we hypothesized that the protective effects we observed could be related to the induction of this protein. To test our hypothesis, first, we exposed SH-SY5Y cells to pioglitazone (100 nM; 1–5 days) and measured the expression of *PPARGC1A* gene. As depicted in Fig. 3A, untreated cells showed a low basal expression of this gene. Drug administration significantly modulated *PPARGC1A* expression: *PPARGC1A* peaked at 24 h (mean 1.85 ± 0.15-fold over basal level; $p < 0.01$; $n = 4$) and returned near the basal level (mean 1.23 ± 0.10-fold over basal level) 48 h after each pioglitazone administration. Western blot analysis showed that pioglitazone progressively and significantly increased PGC-1α expression with the maximum on days 4–5 (1.43 ± 0.07-fold over control cells; $p < 0.01$; $n = 4$, Fig. 3B). Moreover, pioglitazone (10 pM to 1 μM; 5 days) increased PGC-1α expression level in a concentration-dependent manner with the maximum (+1.39 ± 0.06-fold over control cells; $p < 0.01$; $n = 4$; Fig. 3C) at 100 nM; the EC_{50} calculated was 3.9 ± 3.3 nM. Similarly, rosiglitazone (10 nM) significantly up-regulated PGC-1α expression level, and on day 5 it was

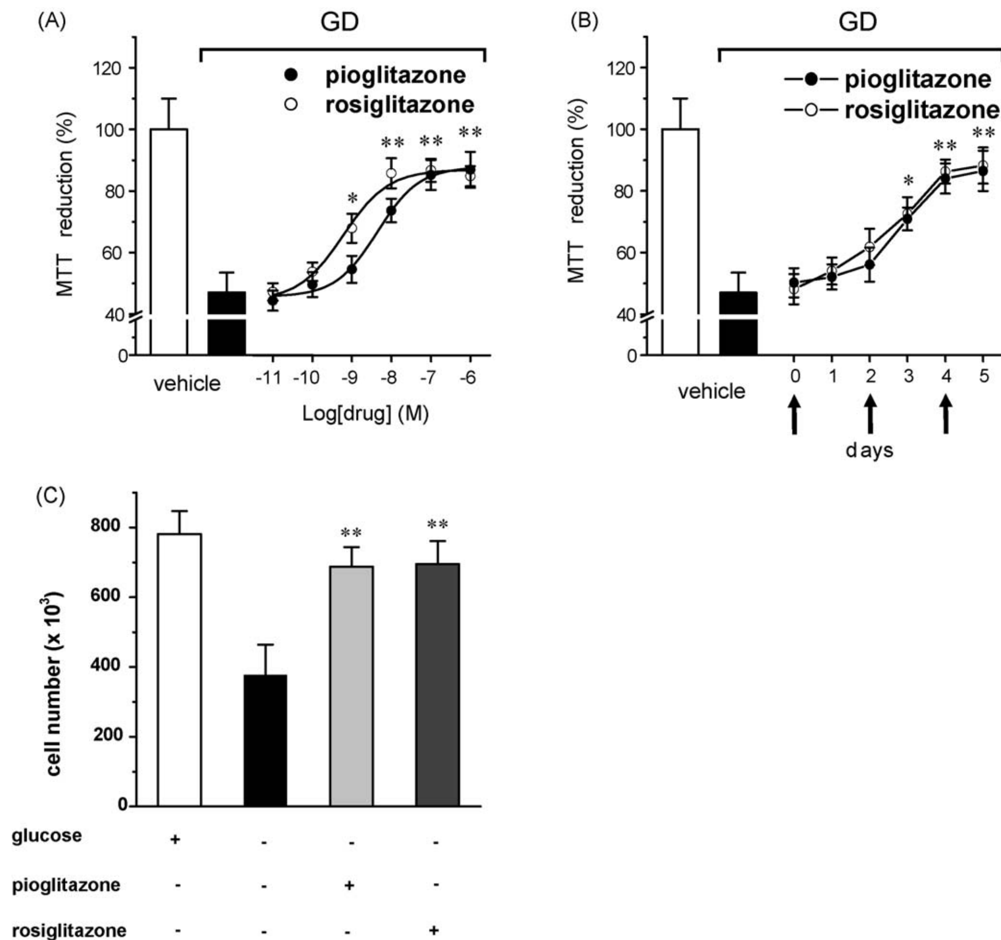

Fig. 2. Effects of PPARγ agonists on GD-induced cell loss. (A) Concentration–response curves of the effects of pioglitazone and rosiglitazone on GD-induced decrease of cell viability. SH-SY5Y cells were untreated (vehicle alone) or treated with either increasing pioglitazone or rosiglitazone concentrations (10 pM to 1 μM) for 5 days before GD. Cell viability was measured at the end of the experiment by using the MTT assay. (B) Time-course of the effects of pioglitazone and rosiglitazone on GD-induced decrease of cell viability. SH-SY5Y cells were untreated or pretreated with either pioglitazone (100 nM) or rosiglitazone (10 nM) for 1–5 days (black arrow indicates drug replacement). Cell viability was measured at the end of the experiment by using the MTT assay. (C) Effects of pioglitazone and rosiglitazone on cell number. SH-SY5Y cells were untreated or pretreated with either pioglitazone (100 nM; 5 days) or rosiglitazone (10 nM; 5 days). Cell number was determined by counting the cells in a hemacytometer under microscope and using trypan blue dye to exclude dead cells. Data are means ± S.E.M. of three to four experiments run in triplicate. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ *vs.* glucose-deprived cells.

1.40 ± 0.08-fold over control cells ($p < 0.01$; $n = 3$; Fig. 3D). In all these experiments no significant effect was exerted by either atRA (5 μM, 5 days) or vehicle alone (Fig. 3D). Second, to assess the transcriptional consequences of PGC-1α induction, we measured the expression level of genes known to be induced in response to PGC-1α up-regulation. As shown in Fig. 3E, semiquantitative RT-PCR analysis showed that both pioglitazone (100 nM; 5 days) and rosiglitazone (10 nM; 5 days) significantly up-regulated the expression of both *NRF1* (+1.35 ± 0.15-fold and +1.38 ± 0.11-fold, respectively, over control cells; $p < 0.05$; $n = 4$) and *TFAM* (+1.43 ± 0.07-fold and +1.45 ± 0.15-fold, respectively, over control cells; $p < 0.05$; $n = 4$) and induced the expression of *UCP-2* (+2.74 ± 0.48-fold and +3.01 ± 0.39-fold over control cells; $p < 0.01$; $n = 4$).

These data indicate that in SH-SY5Y cells PPARγ stimulation promotes the activation of the PGC-1α-dependent genes.

3.4. Effects of pioglitazone on mitochondrial biogenesis in SH-SY5Y cells

It has been reported that PPARγ stimulation, together with PGC-1α, promotes mitochondrial biogenesis (Wu et al., 1999; Puigserver and Spiegelman, 2003). Therefore, we investigate whether PPARγ stimulation promotes similar effects in SH-SY5Y neuronal-like cells. In addition, since the similarity of the effects exerted by the two PPARγ agonists, we decided to perform the following experiments using only pioglitazone. First, we studied the effects of this drug on the expression level of CO I and CO IV (mtDNA- and nuclear DNA-encoded mitochondrial protein, respectively) recognized markers of mitochondrial cell content. As shown in Fig. 4A, pioglitazone (100 nM) significantly increased both CO I and CO IV expression in a time-dependent manner with a maximum on day 5 (+1.70 ± 0.10, +1.51 ± 0.10 over control cells, respectively; $p < 0.01$; $n = 4$). These effects were concentration-dependent in the range 10 pM to 1 μM; the maximums were at 100 nM (+1.70 ± 0.07 and +1.48 ± 0.05 over control cells, respectively; $p < 0.01$; $n = 4$) while the EC_{50}s calculated were 2.3 ± 0.9 and 4.5 ± 2.7 nM for CO I and CO IV, respectively (Fig. 4B). Second, we measured the amount of mtDNA, another marker of mitochondrial cell content (Nisoli et al., 2003). As shown in Fig. 4C, in comparison to control cells, pioglitazone (100 nM; 5 days) significantly increased the amount of mtDNA (+1.63 ± 0.08-fold over control cells; $p < 0.01$; $n = 4$; Fig. 4C). Finally, to assess whether pioglitazone affects mitochondrial function, we assessed mitochondrial membrane potential in pioglitazone-treated and -untreated cells. In comparison to vehicle, pioglitazone (100 nM, 5 days) did not significantly change mitochondrial membrane potential (Fig. 4D).

These results consistently indicate that in SH-SY5Y neuroblastoma cells PPARγ stimulation promotes biogenesis of functional mitochondria.

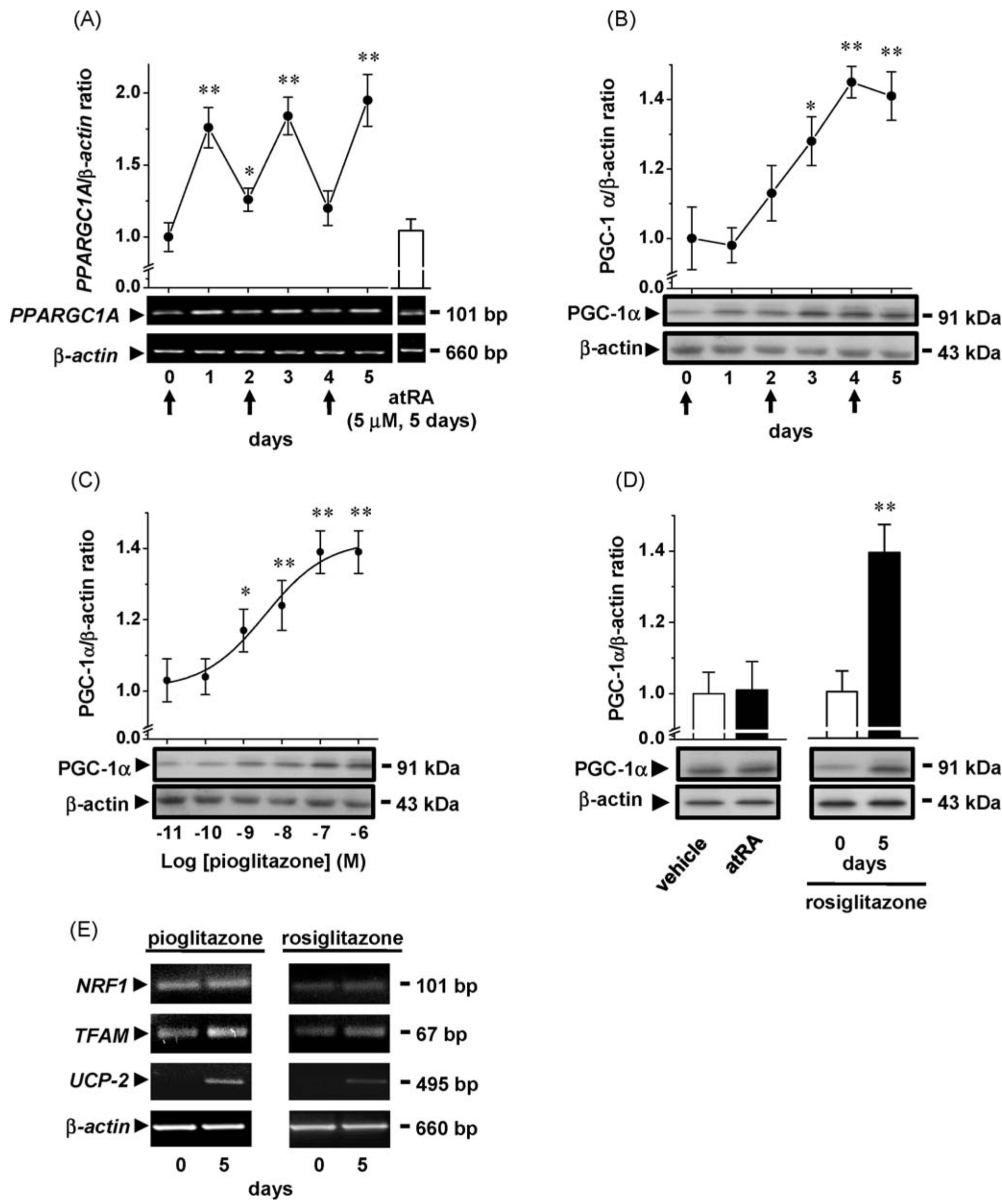

Fig. 3. Effects of PPARγ agonists on PGC-1α expression. (A) Time-course of the effect of pioglitazone on *PPARGC1A* expression. SH-SY5Y cells were untreated or treated with pioglitazone (100 nM) for 1–5 days; *PPARGC1A* expression was evaluated by semiquantitative RT-PCR analysis by using a specific primer pair and β-actin as a reference gene. (B) Time-course of the effect of pioglitazone on PGC-1α expression. SH-SY5Y cells were untreated or treated with pioglitazone (100 nM) for 1–5 days; PGC-1α expression was evaluated by Western blot analysis; β-actin was used as internal control. (C) Concentration–response curve of the effect of pioglitazone on PGC-1α expression. SH-SY5Y cells were untreated or treated with increasing pioglitazone concentrations (10 pM to 1 μM) for 5 days. PGC-1α expression was measured as above described. (D) Effects of atRA and rosiglitazone on PGC-1α expression. SH-SY5Y cells were untreated (vehicle) or treated with atRA alone (5 μM; 5 days) or rosiglitazone (10 nM; 5 days). PGC-1α expression was measured as above described. (D) Effects of pioglitazone and rosiglitazone on the expression of genes responsive to PGC-1α up-regulation. SH-SY5Y cells were untreated or treated with either pioglitazone (100 nM; 5 days) or rosiglitazone (10 nM; 5 days). *NRF1*, *TFAM* and *UCP-2* expression was evaluated by semiquantitative RT-PCR analysis by using specific primer pairs and β-actin as a reference gene. The pictures shown are representative of three to four independent experiments. Data are means ± S.E.M. of three to four independent experiments. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ *vs.* untreated cells (day 0).

3.5. Effects of pioglitazone on malonate-induced cell death

To verify whether the protective effect of pioglitazone depend on mitochondrial function, we investigated how malonate, a reversible mitochondrial Complex II inhibitor, extensively employed both *in vitro* and *in vivo* as tool to inhibit mitochondrial energy metabolism (Beal et al., 1993; Gomez-Lazaro et al., 2007), affects this effect. In particular, we used the same experimental protocol showed in Fig. 1 supplemental material and to avoid misinterpretation due to the severity of the insult, we calibrated the treatment regimen to obtain a level of cell loss similar (∼50%) to that described in Fig. 2A and B. Cell exposure to malonate (10 mM) with (1.5 h) or without glucose (1 h) decreased cell viability (−53.0 ± 7.8% and −45.3 ± 4.8%, respectively) and cell number (−53.3 ± 10.1% and −56.3 ± 11.2%, respectively). Pioglitazone (100 nM; 5 days) was unable to prevent malonate-induced cell loss either in the presence or in the absence of glucose, thus suggesting that its protective effect requires functionally active mitochondria.

3.6. Effects of pioglitazone on the pyruvate protective effect

In cells exposed to malonate in the absence of glucose, respiration is inhibited and glycolisis is restricted. In such conditions, pyruvate addition spares glycolisis and restores respiration (Kauppinen and Nicholls, 1986). Therefore, to further

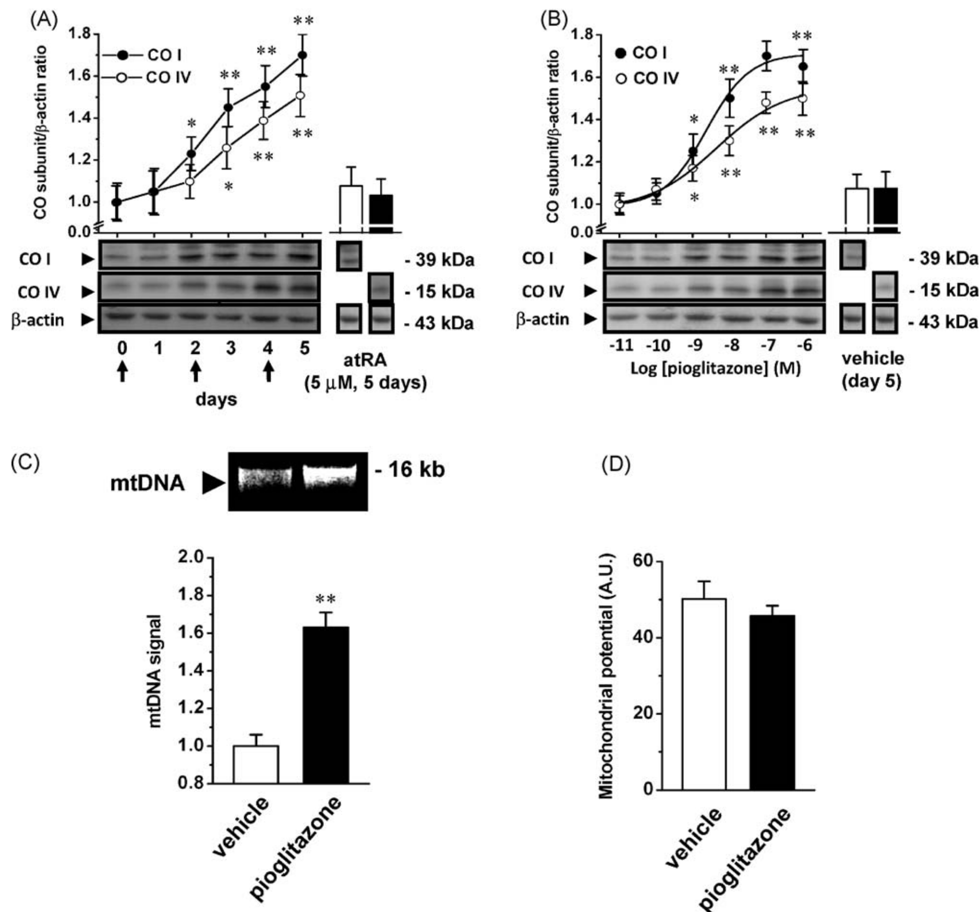

Fig. 4. Effects of pioglitazone on mitochondrial biogenesis. (A) Time-course of the effect of pioglitazone on CO I and CO IV expression. SH-SY5Y cells were untreated or treated with pioglitazone (100 nM) for 1–5 days. CO I and CO IV expression was evaluated by Western blot analysis; β-actin was used as an internal control. (B) Concentration–response curves of the effect of pioglitazone on CO I and CO IV expression. SH-SY5Y cells were untreated or treated with increasing pioglitazone concentrations (10 pM to 1 μM) for 5 days. CO I and CO IV expression were measured as described above. (C) mtDNA amount in pioglitazone (100 nM, 5 days)-treated and -untreated (vehicle) cells. (D)

investigate the contribution of mitochondria biogenesis to the protective effect of pioglitazone, we assessed the time-course of drug effect on GD plus malonate-induced cell death in the absence or presence of pyruvate (5 mM), added during the recovery period. In untreated samples, cell loss gradually increased: 54.8 ± 3.8%, 73.7 ± 6.6% and 77.6 ± 4.4% at 24, 48 and 72 h of the recovery period, respectively (Table 1). Pioglitazone decreased, albeit not significantly, cell loss (−2.8 ± 2.5%, −8.7 ± 5.0% and −9.4 ± 5.5%, at 24, 48 and 72 h, respectively, Table 1). Differently, pyruvate significantly protected cells against GD plus malonate and cell loss was reduced by 21.2 ± 2.2%, 30.6 ± 5.2% and 29.9 ± 4.0%, at 24, 48 and 72 h, respectively ($p < 0.01$ in comparison to untreated cells; $n = 4$; Table 1). Moreover, in pioglitazone pretreated cells a significantly greater decrease of cell loss was measured (37.8 ± 4.4%, 48.5 ± 3.4% and 50.2 ± 4.7%, at 24, 48 and 72 h, respectively $p < 0.01$ in comparison to cells treated with pyruvate alone; $n = 4$; Table 1), thus indicating that restoration of cell respiration by pyruvate reduced GD plus malonate-induced cell loss and this effect is significantly potentiated by pioglitazone pretreatment.

These data further confirm the hypothesis that mitochondrial biogenesis may contribute to the protective effect exerted by pioglitazone.

4. Discussion

Results here reported show that prolonged cell exposure to PPARγ agonists prevents GD-induced cell loss and suggest that mitochondrial biogenesis may contribute to this effect.

Table 1
Effects of pioglitazone on the protective effect of pyruvate.

Time after GD (h)	Cell number × 10^5 (cell loss %)			
	−Pyruvate		+Pyruvate	
	−Pioglitazone	+Pioglitazone	−Pioglitazone	+Pioglitazone
24	3.6 ± 0.3 (54.8 ± 3.8)	3.9 ± 0.2 (52.0 ± 2.5)	5.2 ± 0.2[a] (33.6 ± 2.2)	6.6 ± 0.3 (17.0 ± 4.4)[a,b]
48	2.1 ± 0.5 (73.7 ± 6.6)	2.8 ± 0.4 (65.0 ± 5.0)	4.6 ± 0.4[a] (43.1 ± 5.2)	5.9 ± 0.3 (25.2 ± 3.4)[a,b]
72	1.8 ± 0.4 (77.6 ± 4.4)	2.6 ± 0.4 (68.2 ± 5.5)	4.1 ± 0.3[a] (47.7 ± 4.0)	5.7 ± 0.4 (27.4 ± 4.7)[a,b]

SH-SY5Y cells were untreated or pretreated with pioglitazone (100 nM; 5 days) and exposed to malonate (10 mM, 1 h) in the absence of glucose (10 mM). Pyruvate (5 mM) was added during the recovery period. Cell number was determined at the end of the recovery period. Data are means ± S.E.M. of at least four experiments run in triplicate.
[a] $p < 0.01$ *vs.* pyruvate-untreated cells.
[b] $p < 0.01$ *vs.* pioglitazone- and pyruvate-untreated cells.

Previous data have shown that PPARγ agonists exert protective effects in several *in vitro* models of neurotoxicity. Our results are in agreement and extend these findings showing that cell pretreatment with either pioglitazone or rosiglitazone prevents loss of differentiated SH-SY5Y cells induced by GD. Both drugs were equally effective in preventing cell loss, while, as expected, rosiglitazone resulted more potent than pioglitazone (Willson et al., 1996). These results indicate that PPARγ stimulation underlies the protective effects. It has also been reported that pioglitazone may exert PPARγ-independent actions. Indeed, this drug activates not only PPARγ but also PPARα (albeit at higher concentrations; Sakamoto et al., 2000), binds mitoNEET (Colca et al., 2004) a 2Fe–2S mitochondrial protein and stabilizes this protein against iron–sulfur cluster release (Paddock et al., 2007). However, we did not detect PPARα in our cell model (data not shown), thus the activation of this receptor does not seem to be necessary to explain the pioglitazone protective effects we observed. On the other hand, to date, the role of mitoNEET as pioglitazone receptor, although cannot be excluded (even in our model), remain largely elusive and need further investigation.

Interestingly, in our experiments pioglitazone and rosiglitazone exerted protective effects at nanomolar concentrations. Pharmacokinetic studies have shown that in diabetic patients the mean serum concentrations of pioglitazone and rosiglitazone were in the low micromolar range (Budde et al., 2003; Niemi et al., 2004). In addition, studies in animals have documented that these drugs passes (albeit poorly) the blood–brain barrier (Maeshiba et al., 1997). Thus, these data suggest that the concentrations effective in our *in vitro* model are comparable to that reached *in vivo*.

Consistent results we obtained when cell viability and cell number were measured, thus indicating that effectively pioglitazone and rosiglitazone decreased GD-induced cell loss. Details on the events implicated in GD-induced neuronal cell loss have been previously reported (Suh et al., 2007b). GD primarily impairs energetic homeostasis (i.e., ATP production from glycolisis and mitochondria) (Ballesteros et al., 2003; Russo et al., 2004) and secondarily triggers several events (i.e. production of reactive oxygen species, increase in intracellular Ca^{2+} level, activation of PARP-1) that can result in cytotoxicity (Suh et al., 2007a,b). Impairment of energetic homeostasis is a major cause of neuronal cell loss associated to hypoglycaemia and ischemia. In addition, it contributes to neuronal dysfunction and death observed in many other neurological disorders (Beal, 2005). Thus, our results, although preliminary, might be relevant to understand the cell protective effects exerted by PPARγ agonists. Previous studies have provided evidence that at cellular level PPARγ agonists exert protective effects through multiple mechanisms of action. Dello Russo et al. (2003) have previously shown that pioglitazone protects astrocytes against hypoglycaemic-induced cell death after just 24 h by enhancing glucose utilisation, through the increased glucose membrane transport. Here we propose an additional mechanism. During cell pretreatment repeated administration of PPARγ agonists increases PGC-1α expression. This finding is in agreement with previous data showing that these drugs induce PGC-1α expression both in non-neuronal, neuronal and neuronal-like cells (Puigserver et al., 1998; Wu et al., 1999; Puigserver and Spiegelman, 2003; Strum et al., 2007; Ghosh et al., 2007; Pagel-Langenickel et al., 2008) and with the recent demonstration that *PPARGC1A* is a PPARγ-target gene (Hondares et al., 2006). Acting as a "transcriptional booster", PGC-1α increases the expression of nuclear genes involved in determining mitochondrial biogenesis (i.e. *TFAM*) and remodeling (i.e. *UCP-2*) (Puigserver et al., 1998; Wu et al., 1999; Puigserver and Spiegelman, 2003; St-Pierre et al., 2006). In our model both pioglitazone and rosiglitazone significantly increased the expression of *NRF1*, *TFAM*, *UCP-2*. In addition, we show that pioglitazone significantly increased CO I, CO IV expression level and the amount of mtDNA, thus indicating that drug pretreatment promoted mitochondrial biogenesis and remodeling in SH-SY5Y cells. Interestingly, the analysis of the time-course and the concentration–response curves for the protective effects and the up-regulation of both PGC-1α, CO I and CO IV, indicates that cell protection and mitochondrial biogenesis are related effects (Table 2). Moreover, our results indicate that the pioglitazone protective effect depends on mitochondrial function: pioglitazone was unable to protect glucose-deprived cells in the presence of malonate, a reversible mitochondrial Complex II inhibitor, while significantly potentiated the protective effect of pyruvate against GD plus malonate. Notably, in our experiments pioglitazone did not significantly change both basal SDH activity and mitochondrial membrane potential, thereby suggesting that mitochondrial biogenesis does not significantly change basal mitochondrial activity, while increases cell resistance against GD-induced cell loss. Overall these data suggest that PPARγ agonists, through the induction of PGC-1α promote mitochondrial biogenesis and this action may contribute to the cytoprotective effects exerted by these drugs. This hypothesis is in agreement with recent findings showing that in human umbilical vein endothelial cells pioglitazone and ciglitazone (another PPARγ agonist) induce PGC-1α, promote mitochondrial biogenesis; effects that may contribute to the prevention of diabetic vascular complication (Fujisawa et al., 2009).

Table 2
Summary of the effects exerted by pioglitazone.

Effect	EC_{50} (nM)[a]	ET_{50} (days)[b]
Protective effect against GD	5.1 ± 1.2	2.9 ± 0.5
PGC-1α up-regulation	3.9 ± 3.3	2.5 ± 0.3
CO I up-regulation	2.3 ± 0.9	2.5 ± 0.1
CO IV up-regulation	4.5 ± 2.7	3.3 ± 1.3

[a] EC_{50} was determined after 5 days of cell pretreatment with increasing pioglitazone concentrations (10 pM to 1 μM).
[b] ET_{50} was determine on cells exposed to 100 nM pioglitazone for 1–5 days.

Although the mechanism(s) through which the increased mitochondrial cell content exerts protective effects remain to be explored, some hypothesis may be proposed. Mitochondria, as the site of oxidative phosphorylation, provide a highly efficient route to generate ATP from energy-rich molecules. Thus, it is reasonable to suppose that cells with increased mitochondrial content could better tolerate the impairment of energetic homeostasis caused by GD. Moreover, mitochondria biogenesis can increase cell resistance against noxious stimuli by mechanisms other than those related to ATP synthesis. Mitochondria possess an high capacity to buffer much of the cytosolic Ca^{2+} that enter the cells for example during excitotoxic insults (White and Reynolds, 1996; Nicholls and Budd, 2000), they maintain physiological levels of NAD^{+} during genotoxic stress (i.e. those produced by ROS and PARP-1 activation) and promote cell survival by the recently defined "mitochondrial oasis effect" (Yang et al., 2007). Therefore, an increased mitochondrial cell content could exert neuroprotective effects through multiple mechanisms.

In conclusion, our results show that prolonged exposure of SH-SY5Y neuronal-like cells to nanomolar pioglitazone or rosiglitazone concentrations increases mitochondrial cell content by up-regulating PGC-1α expression. This effect provides a plausible mechanism that can contribute to the protective effects exerted by the drug in our model of GD-induced cell death. Moreover, our data rise the possibility that mitochondrial biogenesis could be a potential protective mechanism of PPARγ agonists. Further studies could clarify the relevance of these findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2009.05.001.

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