15-Deoxy-Δ12,14-Prostaglandin J2 Protects PC12 cells from LPS-Induced Cell Death Through Nrf2 pathway in PPAR-γ Dependent Manner

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**A B S T R A C T**

**Introduction:** The inflammatory response requires a coordinated integration of various signaling pathway including cyclooxygenase (COX). COX catalyzes the formation of prostaglandins from arachidonic acid. Among prostaglandins, 15-Deoxy-D12, 14-prostaglandin J2 (15d-PGJ2), an endogenous ligand of Peroxisome proliferator-activated receptor-gamma (PPAR-γ), has been demonstrated to have anti-inflammatory actions. In this study, we investigated whether 15d-PGJ2 as a PPAR-γ ligand could exert neuroprotective effects in rat pheochromocytoma (PC12) cells in PPAR-γ dependent manner.

**Methods:** In our experiment, using PC12 cells, the levels of NF-κB, Nrf2, γ-glutamylcysteine synthetase (γ-GCS), hemeoxygenase (HO-1) and apoptosis factors were determined using Western blot in different groups. Also cell viability was determined by the conventional MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) reduction assay and two staining involved Hoechst staining and Acridine Ordange/Ethidium Bromide staining respectively.

**Results:** Our results show that NS-398, a selective COX-2 inhibitor and 15d-PGJ2, a natural potent ligand of PPAR-γ, were neuroprotective through modulation of at least three different, but related pathways and molecules, including NF-κB and Nrf2 signaling pathway. Our data showed that 15d-PGJ2 and NS-398 induced Nrf2 signaling pathway and its downstream factors such as HO-1 and γ-GCS, while 15d-PGJ2 and NS-398 decreased NF-κB level. Interestingly, the observed protective effects were mediated through PPAR-γ-dependent mechanisms, as they reversed by GW9662, an irreversible antagonist of PPAR-γ receptor.

**Discussion:** Thus we conclude that 15d-PGJ2 as well as NS-398 exert anti cell death effect in a PPAR-γ dependent mechanisms.

**1. Introduction**

Oxidative stress occurs when the level of pro-oxidants (reactive oxygen species (ROS) and other free radicals) exceed the ability of the cell to respond through antioxidant defense. Hydrogen peroxide (H2O2), one of the main ROS, is produced during the redox process and is recently considered as a messenger in intracellular signaling cascades. Stress, inflammation, and nitric oxide (NO) have been shown to be involved in neurodegenerative diseases such as Alzheimer disease and Parkinson's disease.

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**Key Words:** Cyclooxygenase, PPAR-γ, PC12, Nrf2, 15d-PGJ2, Neuroprotective.

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son’s disease (McGeer et al., 2004; Eftekharzadeh et al., 2010).

The inflammatory response requires a coordinated integration of various signaling pathways including cyclooxygenase (COX), NO, and cytokines. Two distinct COX isoenzymes exist, COX-1 and COX-2, which are regulated differentially (Appleby et al., 1994). Of the COX enzymes, COX-2 is found mainly in inflammatory cells and tissues and was found to be up-regulated during acute inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX which is the key enzyme in the conversion of arachidonic acid to prostaglandins (PGs), and is employed clinically and experimentally to prevent neuronal damage (Hewett et al., 2000). NS-398, a selective COX-2 inhibitor, belongs to the class of NSAIDs, thus NS-398 protects neuronal cultures from oxidative damages. Some recent studies have demonstrated that NS-398 mediated protective effects of neuronal cells against neurotoxicity induced by LPS (Hewett et al., 2000; Araki et al., 2001).

PGs are a family of oxygenated metabolites of arachidonic acid (AA), and have a diverse range of actions depending on the PG type and cell target (Yen-Chou et al., 2005; Vamecq et al., 1999). Among these compounds, 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) took considerable attention because of its ability to perform various action such as anti-inflammatory, anticarcinogenic and antioxidant properties (Vamecq et al., 1999; Lemberger et al., 1996). 15d-PGJ2 is known to be an endogenous ligand for the PPAR-γ (Lehmann et al., 1997). It has been reported that 15d-PGJ2 may also exert some of its effects via mechanisms dependent or independent of PPAR-γ (Lehmann et al., 1996; Jiang et al., 1998). It has been reported that 15d-PGJ2 can activate HO-1 in vascular smooth muscle cells via independent PPAR-γ (Lim et al., 2007). On the other hand, it has been reported to protect PC12 cells from oxidative stress (Ji-Woo et al., 2008), induce apoptosis in osteoblastic cells and inhibit proliferation of cancer cells (Na et al., 2003).

PPAR-γ, a family member of transcription factor PPARs, has been demonstrated to play an important role in the regulation of cell differentiation (Vanden et al., 1999) such as adipocytes and macrophages (Vunta et al., 2007). Recent studies have emphasized its important role in regulation of inflammatory response, cellular proliferation, and differentiation. 15d-PGJ2 and NS-398 have neuroprotective effects and are capable of inducing a pathway which is assumed to be performed by using PPAR-γ pathway. This hypothesis is assumed to be upon on neuron like-PC12 cells. Here we used PPAR-γ inhibitor (GW9662), natural ligand of PPAR-γ (15d-PGJ2), and selective inhibitor of COX-2 (NS-398) to test the hypothesis.

2. Methods

2.1. Materials

Antibodies directed against NF-κB, HO-1, γ –GCS, and β-actin were obtained from Cell Signaling Technology. Antibodies directed against Nrf2 (C-20) and Lamin B2 were purchased from Santa Cruz Biotechnology. All other reagents, unless otherwise stated, were from Sigma Aldrich (St. Louis, MO).

2.2. Cell culture and Differentiation

Rat pheochromocytoma (PC12) cells obtained from Pasteur Institute (Tehran, Iran) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, Aldrich), supplemented with 10% horse serum, 5% fetal bovine serum and 1% antibiotic mixture comprising penicillin-streptomycin, in a humidified atmosphere at 37°C with 5% CO2. Growth medium was changed three times a week. PC12 cells were differentiated by treating with nerve growth factor (NGF) (50 ng/ml) every other day for 6 days.

2.3. Drug Treatment

All compounds were dissolved in dimethyl sulfoxide (DMSO) at 10-2 M and then diluted in culture medium.

2.4. Treatment Conditions

Differentiated PC12 cells plated in 75 cm2 culture flasks. In our experiment the cells were divided into eight groups: A) control group, B) Negative control group, that received LPS (1mg/ml) for 18 h; C) NS-398 group, which received COX-2 inhibitor (NS-398) (20 μM) for 2.5 h; D) 15d-PGJ2 group, that received PPAR-γ agonist (15d-PGJ2) (5 μM) for 1 h; E) GW9662 group, that cells were incubated with PPAR-γ inhibitor (GW9662) (5μM) for 0.5 h; F) NS-398+LPS group, the cells were incubated with COX-2 inhibitor (NS-398) (20 μM) 2.5 h before LPS treatment, then the cells were exposed to LPS (1mg/ml) for 18 h; G) NS-398+15d-PGJ2+LPS group, the cells were incubated with COX-2 inhibitor (NS-398) (20 μM) 2.5 h before LPS treatment, then the cells were exposed to PPAR-γ agonist (15d-PGJ2) (5 μM) for 1 h, finally the cells were incubated with LPS (1mg/ml) for 18 h H) NS-398+15d-PGJ2+GW9662+LPS group, that PC12
cells plated in 75 cm² culture flasks, were incubated with COX-2 inhibitor (NS-398) (20 μM) 2.5 h before LPS treatment, then the cells were exposed to PPAR-γ agonist (15d-PGJ2) (5 μM) for 1 h. Finally, cells were treated with PPAR-γ inhibitor (GW9662) (5 μM) for 0.5 h before LPS-exposure. The cells were incubated with LPS (1mg/ml) for 18 h.

2.5. Measurement of Cell Viability

Cell viability was determined by the conventional MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay. The dark blue formazan crystals formed in intact cells were solubilized in DMSO and the absorbance was measured at 550 nm. Results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

2.6. Acridine Orange/Ethidium Bromide (AO/EB) Double Staining

Apoptosis was determined morphologically after staining cells with acridine orange/ethidium bromide (AO/EB) followed by fluorescence microscopy inspection. Briefly, differentiated PC12 cells were seeded in a 96-well plate and were treated with 20 μM of NS-398, 5 μM of 15d-PGJ2 and 5 μM of GW9662, followed by adding LPS (1mg/ml). After 18 h, cells were harvested and washed three times with PBS and were adjusted to a density of 1×10⁶ cells/ml of PBS. AO/EB solution (1:1 v/v) was added to the cell suspension in a final concentration of 100 μg/ml. The cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

![Figure 1.](image-url)
Figure 2. Morphological evaluation of PC12 cells in the presence of NS-398, 15d-PGJ2, and GW9662. Differentiated PC12 cells, pretreated with NS-398 (20 μM) and 15d-PGJ2 (5 μM) and GW9662 (5 μM) for 2.5 h, 1.5 h and 0.5 h respectively, in the absence and/or presence of LPS (1 mg/ml) was determined by AO/EB double staining (A). Hoechst staining (B). The morphological patterns of apoptotic cells are described in the text. All experiments were repeated three times.

Figure 3. Western blot analysis to measure the effects of NS-398, 15d-PGJ2 and GW9662 on the nuclear level of NF-κB in PC12 cells. A) NF-κB response to NS-398, 15d-PGJ2 and GW9662 (20, 5 and 5 μM) on PC12 cells pretreated for 2.5 h, 1.5 h and 0.5h and then exposed to LPS (1 mg/ml) for 18 h. Twenty μg proteins were separated on SDS-PAGE, western blotted, probed with anti-NF-κB antibody and reprobed with anti-Lamin B2 antibody. (One representative western blot was shown; n=3). B) The density of NF-κB bands was measured and the ratio was calculated. The mean of three independent experiments is shown. *significantly different from control cells.*significantly different from LPS-treated cells.
Figure 4. Western blot analysis to measure the effects of NS-398, 15d-PGJ2 and GW9662 on the Nrf2 in PC12 cells. A) Nrf2 response to NS-398, 15d-PGJ2 and GW9662 (20, 5 and 5 μM) on PC12 cells pretreated for (2.5 h, 1.5 h and 0.5h) and then exposed to LPS (1 mg/ml) for 18 h. Twenty μg proteins were separated on SDS-PAGE, western blotted, probed with anti-Nrf2 antibody and reprobed with anti-Lamin B2 antibody (One representative western blot was shown; n=3). B) The density of Nrf2 bands was measured and the ratio was calculated. The mean of three independent experiments is shown. * significantly different from control cells. ** significantly different from LPS-treated cells.

Figure 5. The expression of HO-1 in PC12 cells pretreated with NS-398, 15d-PGJ2 and GW9662. A) HO-1 response to NS-398, 15d-PGJ2 and GW9662 (20, 5 and 5 μM, respectively) in PC12 cells pretreated for 2.5 h, 1.5 h and 0.5h, respectively and then exposed to LPS (1 mg/ml) for 18 h. Twenty μg proteins were separated on SDS-PAGE, western blotted, probed with anti-HO-1 antibody and reprobed with anti-β-actin antibody (One representative western blot was shown; n=3). B) The density of HO-1 bands was measured and the ratio was calculated. The mean of three independent experiments is shown. * significantly different from control cells. ** significantly different from LPS-treated cells.
2.7. Hoechst Staining

In order to further confirm apoptotic morphologically, cells were stained using Hoechst 33342 (Invitrogen, H3570) followed by fluorescence microscopy inspection. Briefly, differentiated PC12 cells were seeded in a 96-well plate and were treated with NS-398 (20 µM), then they were exposed to 15d-PGJ2 (5 µM) and GW9662 (5 µM) followed by adding LPS (1µg/ml). After 18 h, the cells were harvested and washed with PBS and were adjusted to a density of 1×106 cells/ml. Then, cells were centrifuged in 1500 rpm for 5 min, pellets were washed with PBS, and incubated with Hoechst solution (0.05mg/ml) for 5 minutes at room temperature. The cellular morphology was evaluated by Olympus microscope.

2.8. Western Blot Analysis

For Western blot analysis, the cells were lysed in buffer containing complete protease inhibitor cocktail. Subcellular fractionation of nuclear and cytoplasmic proteins were performed according to the appropriate protocols (Kutuk and Basaga, 2003). The proteins were electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ElectroChemiluminescence (ECL) reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by ImageJ, measuring integrated density of bands after background subtraction. Protein concentrations were determined according to Bradford’s method (Bradford, 1976). A standard plot was generated using bovine serum albumin.

2.9. Data analysis

All data are represented as the mean ± S.E.M. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by an appropriate post-hoc test to analyze the differences. The statistical significances were achieved with P< 0.05. (* or # P< 0.05, ** or ## P<0.01 and ### P<0.001).
3. Results

3.1. NS-398 and 15d-PGJ2, Singly and/or in Combination, Protected the PC12 Cells from LPS-Induced Cytotoxicity, via a PPAR-γ-Dependent Mechanism.

MTT assay was used to analyze the protective effects of NS-398 and/or 15d-PGJ2 against LPS-induced cell death. As shown in Fig.1 (A, B), LPS exposure resulted in a significant reduction of cell viability while in comparison, pretreatment of cells with 15d-PGJ2 and/or NS-398 protected against LPS-induced cell death, confirmed by high percentage of cell viability in the relevant groups. The protection observed in groups that received 15d-PGJ2 and/or NS-398 alone was the same as when they received both. Also, pretreatment of cells with 15d-PGJ2, NS-398, and GW9662 together did not show any significant changes in cell viability compared to control cells.

To clarify whether these protective effects were mediated through PPAR-γ, GW9662, an irreversible antagonist of PPAR-γ was used. GW9662 reversed the protection exerted by 15d-PGJ2 and/or NS-398, demonstrated the involvement of PPAR-γ in such a context.

3.2. Morphological Evaluation of Apoptosis

AO/EB staining discriminates live cells from dead ones on the basis of membrane integrity. AO is a cell-permeable nucleic acid selective dye which is taken up by both viable and nonviable cells and emits green fluorescence if intercalates into double stranded nucleic acid. EB intercalates and stains DNA, providing a red-orange fluorescence in the cells which are in the early or final stages of apoptosis and have much more permeable membranes compared to healthy cells. The result obtained from AO/EB double staining is represented in Fig. 2A. In this method, viable cells show uniform bright green nuclei with organized structure, while apoptotic cells have orange to red nuclei with fragmented or condensed chromatin. Pretreatment of cells with NS-398 (20 μΜ) and/or 15d-PGJ2 (5 μΜ) has significantly declined the extent of cell apoptosis compared to that observed in the cells exposed solely to LPS. The results obtained from Hoechst staining are consistent (Fig. 2B). In this method, viable cells show uniform dark blue nuclei, while apoptotic cells have bright blue nuclei with condensed or fragmented chromatin.

3.3. NS-398 and 15d-PGJ2 Inhibit the Nuclear Translocation of NF-κB in PC12 Cells.

The transcription factor NF-κB is one of the ubiquitous eukaryotic transcription factor that exerts pleiotropic effects via numerous intracellular signal transduction pathways, involved in the induction of pro-inflammatory genes including iNOS, COX-2, adhesion molecules, and cytokines. We found that LPS exposure increased NF-κB level in nuclear fraction by 4.48 fold compared to control group as determined by western blot. However in the groups that received NS-398 and 15d-PGJ2 alone or together, the level of NF-κB was decreased. At the same time in the groups that received GW9662, nuclear translocation of NF-κB was increased. The results illustrate that 15d-PGJ2 can decreased nuclear NF-κB level via PPAR-γ pathway [F (7, 16) = 985.41, P<0.001].

3.4. 15d-PGJ2- and NS-398-Treatment Result in the Induction of Nrf2 Pathway in PC12 Neurons.

Previous studies have reported that 15d-PGJ2 induces Nrf2 expression. In order to determine this, we measured Nrf2 induction, as Nrf2 induction can be considered as a consequence of Nrf2 signaling pathway activation. Results in Fig. 4B illustrate level of Nrf2 that was decreased in LPS-treated PC12 cells in comparison with controls, however the expression of Nrf2 increased in the presence of NS-398 compared to LPS-treated cells by about 1.65 fold. The level of Nrf2 protein increased when PC12 cell exposed to 15d-PGJ2 and NS-398 together by about 0.95 fold compared to LPS-treated cells. These results revere when GW9662 was used [F (7, 16) = 44.72, P<0.001].

Levels of HO-1 and γ-GCS protein factors were measured by Western blot (Fig.5, 6, respectively). HO-1 and γ-GCS act in downstream of Nrf2. The level of these factors increased in NS-398 or 15d-PGJ2 singly or together treatment-cells. [F (7, 16) = 125.6, P<0.001 and F (7, 16) = 485.99, P<0.001] respectively.

To clarify whether these protective effects were mediated through PPAR-γ, GW9662 was used. GW9662 reversed the protection exerted by 15d-PGJ2 and NS-398, demonstrating the involvement of PPAR-γ in such a context.
4. Discussion

Many experimental studies have indicated that COX-2 and PPAR-γ signaling pathways are multiply intertwined (Michael et al., 2003). In the present study, we used a selective COX-2 inhibitor, (NS-398), PPAR-γ ligand, (15d-PGJ2), and PPAR-γ antagonist (GW9662). Using them alone or together showed that NS-398 or 15d-PGJ2 had protective effects, while GW9662 induced apoptosis and showed no protective effects against cytotoxicity induced by LPS, suggesting that NS-398 or 15d-PGJ2 suppress apoptosis in a PPAR-γ dependent manner in PC12 cells.

Several lines of evidence suggest interrelation and cross-talk between the two molecules (Wei-Hao et al., 2009). In one hand, NSAIDs which function as non-selective COX-2 inhibitors, are known to be weak agonists of PPAR-γ (Lehmann et al., 1997). On the other hand, 15d-PGJ2 is a downstream product of COX-2 and thus inhibition of COX-2 inhibits the protective effects of PPAR-γ because of reduced 15d-PGJ2 production. Therefore, it is reasonable that addition of a PPAR-γ agonist to a COX-2 inhibitor protects PC12 cells from cytotoxicity via PPAR-γ dependent manner.

15d-PGJ2 can increase the nuclear translocation of Nrf2, leading to enhanced expression of several phase-II detoxifying enzymes as well as other antioxidant enzymes / proteins (Kim et al., 2006). Park et al. (2004) have shown that 15d-PGJ2 promotes activation of Nrf2 through its interaction with PPAR-γ.

Among enzymes up-regulated by Nrf2, we explored HO-1 and γ-GCS. Many previous studies show that HO-1 has pronounced anti inflammatory as well as antioxidative properties. HO-1, also known as heat-shock protein 32 (HSP32) (Kondo et al., 2007), is a 32-kDa protein which is transiently activated by a wide variety of noxious stimuli including oxidative stress. In agreement with previous studies have been demonstrated that HO-1 can be induced by 15d-PGJ2 in various types of cells (DH et al., 2009), in this study we show that 15d-PGJ2 can induce activation of HO-1 in PPAR-γ dependent manner. So, compounds that regulate Nrf2 pathway may be promising candidates for neuroprotection against free radical stress through induction of γ-GCS as well as Hsp32, both of which prevent accumulation of ROS.

It is believed that activation of Nrf2 signaling pathway can inhibit NF-κB nuclear translocation (Tin et al., 2008) that has been shown to be required for programmed cell death (PCD). In the context of our study, it appears that NF-κB nuclear translocation be involved in the cell death pathway, as we detected its increased nuclear level in LPS-treated cells, while it was declined in NS-398 or 15d-PGJ2-pretreated cells and increased in GW9662-pretreated PC12 cells. Previous studies show that only 15d-PGJ2 inhibits NF-κB (Rossi et al., 2000) but in our study, we found that NS-398 also inhibits NF-κB, and GW9662 increased NF-κB expression. One of the most important conclusions of this report is that 15d-PGJ2 as well as NS-398 effects exert their anti-cell death effect through PPAR-γ pathway. Previous studies reported that 15d-PGJ2 exerts its protective effect through Nrf2 pathway (Ji-Woo et al., 2008), and 15d-PGJ2 inhibits NF-κB also, but we demonstrated that both 15d-PGJ2 and NS-398 inhibited NF-κB, and activated HO-1, γ-GCS, and Nrf2 via PPAR-γ pathway. Nrf2 signaling pathway has been proved to be responsible for the induction of many cytoprotective and anti-oxidative enzymes that form a natural defense system against assaults brought by electrophils and oxidants. This pathway has a well-appreciated role in protecting cells from endogenous and exogenous stresses as well as anti-inflammatory effects. The present investigation provides strong evidences supporting the neuroprotective potential of NS-398 and 15d-PGJ2 in vitro.

In conclusion, the present study demonstrated that NS-398, a selective COX-2 inhibitor and 15d-PGJ2, a natural potant ligand of PPAR-γ, were neuroprotective through modulation of at least three different, but related pathways and molecules, including NF-κB and Nrf2 signaling pathway, as well as apoptosis. Interestingly, the observed protective effects were mediated through PPAR-γ-dependent mechanisms, as they reversed by GW9662, an irreversible antagonist of PPAR-γ receptor.

Acknowledgments

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Abbreviations

COX-2, cyclooxygenase-2; ECL, Electrochemiluminescence; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; NF-κB, Nuclear factor-κB; NGF, nerve growth factor; Nrf2, Nuclear factor-erythroid 2 p45-related factor 2; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide; ROS, reactive oxygen species.
References


