Expression of peroxisome proliferator activated receptor gamma in the neuronal cells and modulation of their differentiation by PPAR gamma agonists

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ABSTRACT

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2), a naturally occurring ligand activates the peroxisome proliferator-activated receptor-γ (PPAR-γ). Activation of PPAR-γ has been found to induce cell differentiation such as adipose cell and macrophage. Here it was investigated whether 15-deoxy-PGJ_2 has neuronal cell differentiation and possible underlying molecular mechanisms. Dopaminergic differentiating PC 12 cells treated with 15-deoxy-PGJ_2 (0.2 to 1.6 μM) alone showed measurable neurite extension and expression of neurofilament, markers of cell differentiation. However much greater extent of neurite extension and expression of neurofilament was observed in the presence of NGF (50 ng/ml). In parallel with its increasing effect on the neurite extension and expression of neurofilament, 15-deoxy-PGJ_2 enhanced NGF-induced p38 MAP kinase expression and its phosphorylation in addition to the activation of transcription factor AP-1 in a dose dependent manner. Moreover, pretreatment of SD 203580, a specific inhibitor of p38 MAP kinase inhibited the promoting effect of 15-deoxy-PGJ_2 (0.8 μM) on NGF-induced neurite extension. This inhibition correlated well with the ability of SB203580 to inhibit the enhancing effect of 15-deoxy-PGJ_2 on the expression of p38 MAP kinase and activation of AP-1. The promoting ability of 15-deoxy-PGJ_2 did not occur through PPAR-γ, as synthetic PPAR-γ agonist and antagonist did not change the neurite promoting effect of 15-deoxy-PGJ_2. In addition, contrast to other cells (embryonic midbrain and SK-N-MC cells), PPAR-γ was not expressed in PC-12 cells. Other structure related prostaglandins, PGD_2 and PGE_2 acting via a cell surface G-protein-coupled receptor (GPCR) did not increase basal or NGF-induced neurite extension. Moreover, GPCR (EP and DP receptor) antagonists did not alter the promoting effect of 15-deoxy-PGJ_2 on neurite extension and activation of p38 MAP kinase, suggesting that the promoting effect of 15-deoxy-PGJ_2 may not be mediated

GPCR. These data demonstrate that activation of p38 MAP kinase in conjunction with AP-1 signal pathway may be important in the promoting activity of 15-deoxy-PGJ_2 on the differentiation of PC12 cells.

Introduction

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2), is a naturally occurring downstream metabolite of PGD_2, and is produced by degradation of PGD_2 (Fukushima, 1992). In contrast to classical prostaglandins which act after binding to cell surface G-protein coupled receptors, 15-deoxy-PGJ_2 is a high-affinity ligand of the peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the nuclear hormone receptor superfamily, and has been known to induce cell differentiation of adipocytes and macrophages (Kliewer *et al.*, 1995; Tontonoz *et al.*, 1998). Activation of PPAR-γ is required for the induction of cell differentiation of these cells (Nagy *et al.*, 1998; Ricote *et al.*, 1998; Wright *et al.*, 2000). Recent study reported that 15-deoxy-PGJ_2 promoted nerve growth factor (NGF)-induced neurite extension (a marker of cell differentiation) of differentiating PC 12 cells. Contrast to the effect in adipocytes and macrophages, the activation of PPAR-γ may not be involved in the promoting effect of 15-deoxy-PGJ_2 on the neurite extension (Satoh *et al.*, 1999). However, it is unclear yet as to what signaling pathway(s) is (are) involved in the promoting ability of 15-deoxy-PGJ_2 on the NGF-induced neurite extension.

It has demonstrated that mitogen-activated protein (MAP) kinase classes, known as Jun N-terminal kinase (JNK), extra-signal response kinase (ERK) and p38 MAP kinase signals are generally involved in NGF-induced neuronal differentiation of PC-12 cells (Morooka and Nishida, 1998; Leppa *et al.*, 1998; Iwasaki *et al.*, 1999). Even though these protein kinase signaling systems are evolutionarily related, they convey distinct signals. That is, depend upon type of stimuli and nature of cells, different class of MAP kinase has been up regulated. Bone morphogenic protein (BMP) has the capacity to induce the neuronal differentiation of PC 12 cells through activation of p38 MAP kinase (Iwasaki *et al.*, 1999), whereas Ganoderma extract activated ERK (Cheung *et al.*, 2000), and staurosporine activated JNK signaling in the induction of neuronal differentiation of the PC 12 cells (Yao *et al.*, 1997).

Activation of the transcription factors such as AP-1, NF-κB and SP-1 was regulated by activation of MAP kinase signaling as downstream targets. Activation of MAP kinase and AP-1 was concomitantly decreased in the

suppression of the NGF-induced neuronal differentiation of PC 12 cells by N-acetyl-L-cysteine (Kamata *et al.*, 1996). Moreover, direct association of activation of transcription factors in the neurite extension was reported. NF-κB activation was required for SH-SY5Y neuroblastoma cell differentiation (Feng and Porter, 1999). AP-1 and SP-1 were also activated during NGF-induced PC12 cell differentiation (Frukawa *et al.*, 1998).

In this study, it was first demonstrated that 15-deoxy-PGJ_2 promoted the NGF-induced neurite extension in a PPAR-γ independent manner, we thereafter focused our investigation whether 15-deoxy-PGJ_2 exerts its ability to promote cell differentiation through enhancements of the NGF-induced activation of MAP kinases and transcription factors during differentiation of PC 12 cells after treatment of 15-deoxy-PGJ_2 with or without NGF. We next compared the promoting ability of other structure-related compounds (PGE_2 and PGD_2) on neurite extension, and further examined whether 15-deoxy-PGJ_2 promotes neurite extension via a cell surface G-protein-coupled receptor (GPCR). In the present study, we show that 15-deoxy-PGJ_2 promotes the differentiation of PC12 cells by activation of p38 MAP kinase in conjunction with AP-1 signal pathway, and its effect may be unrelated to PPAR-γ and GPCR.

Experimental Procedures

Cell culture and treatment. PC12 cells, a rat cell line derived from a pheochromocytoma and neuroblastoma SK-N-MC cells, a human neuron cancer cells which have the ability of differentiation were maintained on tissue culture plastic in Dulbecco's modified Eagle's medium and F-12 nutrient (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37℃ under an atmosphere of 5% CO_2 and 95% air. The culture media was changed 3 times per week, and cultures were passages at 70~80% confluence. To minimize the differentiation in the culture, the inactivated horse serum concentration was reduced to 1% and bovine serum was deleted from the medium. NGF (50 ng/ml) with or without various doses of 15-deoxy-PGJ_2, PGD_2 and PGE_2 (obtained from Cayman Chemicals, Ann Arbor, MI, USA), and antagonists of PGD_2 (BWA868C) and PGE_2 (AH6809) were added into medium and the cells were cultured either for 72 hr to assay neurite extension, neurofilament and PPAR-γ expressions or for 0.5 ~3 hr to

assay the transcription factor activation. Expression of MAP kinase classes was determined after 24 hr culture. After culture, the wells were washed three times with ice-cold phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.6), and viable cell numbers were determined by trypan blue dye exclusion staining under the microscope. Embryonic midbrain cells were cultured as described previously (Hong et al., 2000). In briefly, embryonic midbrain tissues were dissociated into individual cells by successive digestion with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline (Gibco, Gaithersburg, MD) containing 1% trypsin. Cells were then resuspended in Dulbecco's modified eagle's medium nutrient and Ham's F12 mixture media (1:1 mixture, Sigma Chemical Co., St. Louis, MO) with 10% NuSerum (NuSerum contains 25% newborn calf serum, Becton Dickinson, MA), 100 μg/ml of streptomycin and 100 units/ml of penicillin. The cells were then adjusted to give $5x10^6$ cells/ml. Ten μl of cell suspension was added to each well and the cells were incubated for 2 hr at 37°C. Two hundred $\mu\ell$ of the culture medium was then added to each well and the cells were cultured for 48 hr. 15-deoxy PGJ_2 (0.5 or 1 μM) was then added into medium, and the cells were cultured for 48 hr for assay of the neurite outgrowth and expression of PPAR-γ.

Measurement of neurite extension. The differentiation of the PC 12 cells was assessed by measurement of the number of extended neurite (>2 mm). Neurite extension was quantified by measuring the number of neurite per unit area of the culture (number/ mm^2) using photo-image of cells by an Image Gauge (version 3.12, Fuji Photo Co., Tokyo, Japan).

Nuclear extract and gel mobility shift assay. Gel mobility shift assay was done using a slight modification of a previously described method (Hong and Glauert, 1999). Briefly, the cultured cells were washed three times with ice-cold phosphate buffered saline (PBS, pH, 7.6) and pelleted. The pellets were resuspended in 400 μl of cold buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged at 11,000 x g for 4 min to remove everything except the nuclei. The pellets were resuspended in a second buffer containing 20 mM HEPES, 20% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF. After centrifugation at 11,000 x g for 6 min, the supernatant contained the nuclear proteins. The protein level was determined by a microplate modification of the

Bradford method (Bio-Rad Bulletin 1177, Bio-Rad Lab., Richmond, CA). The DNA binding activity of transcription factors was assayed according to the manufacturer's instructions (Promega Co., Madison, WI). In brief, 10 μg of nuclear protein was incubated in 25 μl total volum of incubation buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 4% glycerol, 0.08 mg/ml salmon sperm DNA) at 4° C for 15 min followed by another 20 min incubation with 100 μCi [γ-^{32}P] ATP-labeled oligonucleotide containing AP-1, SP-1 or NF-κB binding site at room temperature. For the competition assay, 50x, 100x or 200x excesses of unlabeled double-stranded oligonucleotide of the AP-1, SP-1 or NF-κB binding site were used as specific competitors. 200x excess of labeled double-stranded oligonucleotide of the SP-1 (or AP-1 for SP-1) binding site was used as a nonspecific competitor. Ten μg of antibodies to c-jun, c-fos, p50 and p65 were added to the binding reaction for the supershift assay of AP-1 and NF-κB. The DNA-protein binding complex was run on a 6% non-denatured polyacrylamide gel at 150 volts for 2 hr. Gels were dried and autoradiographed using Kodak MR film at -80° C overnight.

Western blotting. Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chem. Co.), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000 g for 1 hr. Equal amount of proteins (20 μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked for 2 hrs at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was then incubated for 3 hr at room temperature with specific antibodies. Rabbit polyclonal antibodies against PPAR-γ and ERK, p38 MAP kinase, JNK and their phsphoylated forms, and goat polyclonal COX-2 antibody, and mouse monoclonal antibody (Santa Cruz, CA, USA Santa Cruz Biotechnology Inc.) against neurofilament were used in this study at dilutions specified by the manufacturer. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL western blotting detection system. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Com., Rochester,

NY).

Immunohistochemical staining. The cells (PC-12, neuroblsatoma cell and embryonic midbrain cells) treated with different dose of 15-deoxy PGJ_2 were cultured in LabTek chamber slides (NUNC), and then the cells were fixed with 4.5% glutaldehyde for 30 min. Immunohistochemical staining was performed with Vectstatin avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). The primary antibody against human PPAR-γ (2.5 μg/ml) was used. The color of the cells was developed by immersion in a peroxidase substrate solution containing 0.05% of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.05M Tris (pH 7. 4) for 5 min. Positive staining was indicated as dark green or brownish black deposits.

Assay of generation of prostaglandins. The cells were labeled with ^{3}[H] arachidonic acid (0.4 μCi/ml, Perkin Elmer Life Sciences) for 24 hr, then the cell were treated with NGF in the presence of various doses of the prostaglandins with/without PGE_2 and PGD_2 antagonists for another 24 hr. The generation of prostaglandins was determined in the medium and cells as described by Akiba et al (2001). In briefly, prostaglandins were extracted and separated by thin layer chromatography on a Silica Gel G plate using development solution (ethylacetate/isooctane/acetic acid/water: 110/50/20/100, v/v). The area corresponding to each prostaglandin was scraped off, and the radioactivity was determined by the liquid scintillation counter (PerkinElmer, Wellesley, MA, USA). Similarly, the liberation of arachidonic acid was determined in the medium and cells after treatment of the cells with the materials described above in the presence of BW755C (a COX and lipooxygenase inhibitor). Arachidonic acid was extracted and separated using petroleum ether/diethyl ether/acetic acid (40:40:1. v/v) as the development system.

Statistics. Data were analyzed using one-way analysis of variance followed by Bonferroni's test as a post hoc test. Differences were considered significant at $p<0.05$.

Results

15-Deoxy-PGJ$_2$ Eenhanced NGF-Induced Neurite Extension and Eexpression of Neurofilament. We first investigated whether serum in culture could increase the extension of neurite growth, a marker of cell differentiation. The neurite extension was increased in the culture with 10% heat-inactivated horse serum and 5% fetal bovine serum (data not shown). However, one % serum did not increased neurite extension and expression of neuronal protein neurofilament, we therefore treated 15-deoxy-PGJ$_2$ with/without NGF to the cells cultured in 1% serum-containing medium for up to 72 hrs. As similar to other study (Satoh *et al.*, 1999), the neurite extension was slightly increased by 15-deoxy-PGJ$_2$ itself (about 2 fold higher than control group). However, in the presence of NGF (50 ng/ml), 15-deoxy-PGJ$_2$ promoted significantly NGF-induced neurite extension (Fig. 1A). Dose and time dependent effect of 15-deoxy PGJ$_2$ with/without NGF on neurite extension was summarized in table 1. Immunoblotting was then performed to investigate the expression of neuronal differentiation marker in the PC 12 cells treated with 15-deoxy-PGJ$_2$. Coincidently with the enhancing effect on neurite extension, 15-deoxy-PGJ$_2$ dose dependently enhanced basal and the NGF-induced expression of neurofilament (Fig. 1B). In contrast, troglitazone, a synthetic PPAR-γ agonist increased neither the NGF-induced neurite extension (Table 1) nor the expression of neurofilament (data not shown). Moreover, pretreatment of PPAR-γ antagonist, bisphenol A diglycidyl did not alter the promoting activity of 15-deoxy-PGJ$_2$ on the NGF-induced neurite extension (Table 1) and expression of neurofilament (data not shown).

15-Deoxy-PGJ$_2$ did not Express PPAR-γ receptor in PC12 Cells. To examine whether the promoting effect of 15-deoxy-PGJ$_2$, a natural ligand of PPAR-γ may require the activation of PPAR-γ in the processes of neurite extension, we did western blotting to determine the PPAR-γ expression by the 15-deoxy-PGJ$_2$ in the PC 12 cells. However, 15-deoxy-PGJ$_2$ did not induce the expression of PPAR-γ even after the cells were treated with 1.6 μM 15-deoxy-PGJ$_2$ for up to 72 hrs. Immuno-reactivity against anti-PPAR-γ antibody was also analyzed by immunohistochemistry, but immuno-reactivity was not found in the PC 12 cells treated with/without 15-deoxy-PGJ$_2$ (data not shown). Contrast to the PPAR-γ, the expression of PPAR-α and β was detected in PC 12 cells, but 15-deoxy- PGJ$_2$ did not increase their expression (Fig. 2A). To compare whether 15-deoxy-PGJ$_2$ could induce the PPAR-γ expression in other

differentiating neuronal cells, we then investigated the PPAR-γ expression in the embryonic midbrain cell and SK-N-MC, a neuroblastoma cell that have the function of differentiation. Contrast the PC 12 cells, PPAR-γ was expressed and the expression was increased by 15-deoxy PGJ_2 in these cells (Fig. 2A). Moreover, the expression of PPAR-γ was correlated well with the differentiation of the cells by similar concentration of 15-deoxy-PGJ_2 (0.5-1 μM in embryomic midbrain cells, Fig. 2B) or by high concentration (2 or 4 μM in SK-N-MC cells, Fig. 2C).

The promoting effect of 15-deoxy-PGJ_2 does not involve a G protein coupled receptor. Considered the absence of PPAR γ in this cell, we examined whether the 15-deoxy-PGJ_2 exerts its promoting effect on neurite extension through a G protein-coupled receptor as demonstrated other prostaglandins such as PGD_2 and PGE_2. We therefore first explored various doses (1, 2 and 5 μM) of the structure-related prostaglandin D_2 and E_2, acting their biological activity via G protein coupled receptor, in the presence or absence of NGF (50 ng/ml) in the PC 12 cells and then assessed the neurite extenson. Even the highest dose of PGD_2 and PGE_2 did not show increasing or promoting effect on the basal or NGF-induced neurite extension (Fig. 3A). In addition, in the presence of antagonist of DP receptor (100, 200 and 500 nM BWA868C) and EP receptor (1, 5 and 10 μ M AH6809) did not inhibit NGF +15-deoxy-PGJ_2-induced neurite extension (Fig. 3B). Furthermore, we also examined whether prostaglandins (PGD_2 and PGE_2) and EGF alone, or the combination of prostaglandins with EGF generates 15-deoxy PGJ_2 , a metabolite of PGD_2. Neither PGD_2, PGE_2 and EGF nor the combination of NGF and prostaglandins (PGD_2 and PGE_2) generated 15-deoxy-PGJ_2 (Fig. 4C). In addition, DP receptor antagonist (500 nM BWA868C) did not significantly change the level of 15-deoxy-PGJ_2 treated by the combination of NGF with PGD_2 and PGE_2. It was also found that prostaglandins alone or the combination with NGF did not change the level of each prostaglandins (PGD_2, PGE_2 and 15-deoxy-PGJ_2) (Fig. 4A and B). The release of arachidonic acid was not changed by NGF or prostaglandins or the combination of NGF with prostaglandins, and the DP receptor antagonist (500 nM BWA868C) did not significantly change the level of arachidonic acid (Fig. 4D). These disabilities of the NGF or prostaglandins, or the combination to release of arachidonic acid are agree with no induction of COX-2 expression in the cells treated with NGF or prostaglandins (PGD_2 and PGE_2), or the combination of NGF with

prostaglandins (Fig. 3C). These results show that the promoting effect of 15-deoxy-PGJ_2 may not be mediated by a GPCRs.

15-Deoxy-PGJ_2 Eenhanced NGF-Induced p38 MAP kinase Signaling. MAP kinase classes have been known to be a critical signal molecules in neuronal differentiation, we therefore examined whether the promoting effect of 15-deoxy-PGJ_2 on NGF-induced neurite extension could be due to the enhancement of activation of the MAP kinase signal pathway. 15-Deoxy-PGJ_2 increased basal expression of p38 MAP kinase and its phosphorylated form in a dose dependent manner, but not basal JNK and ERK and their phosphorylated forms. (Fig. 5A). Moreover, 15-deoxy PGJ_2 further enhanced the NGF-induced expression of p38 MAP kinase and its phosphorylated form when the cells were co-treated. However, 15-deoxy-PGJ_2 did not affect the NGF-induced expression of ERK and JNK, and their phosphorylation forms, either (Fig. 5B). We further examined the possibility that GPCR respond to 15-deoxy-PGJ_2, in turn evoke signal pathway cascades that involve the p38 MAP kinase. We employed the specific antagonists of G protein coupled receptors (DP and EP receptors) in the cells treated with NGF or NGF with15-deoxy-PGJ_2. Both of antagonists did not inhibit NGF or NGF with15-deoxy-PGJ_2 -induced activation of p38 MAP kinase (increase of the expression of phosphorylated form of p38 MAP kinase). In addition, PGD_2 and E_2 themselves, acting their biological activity via GPCR did not induce the activation of p38 MAP kinase (Fig. 5C and D).

15-Deoxy-PGJ_2 Activated Transcription Factor AP-1. Next, we determined whether the 15-deoxy-PGJ_2-induced neurite extensions are related to activation of transcription factors AP-1, SP-1 and NF-kB. Activation of transcription factors AP-1 and SP-1 was increased in dose and time dependent manners by 15-deoxy-PGJ_2 itself. The highest activation of AP-1 was seen in the cells treated with 0.8 μM of 15-deoxy-PGJ_2 (Fig. 6A) for 1 hr (Fig. 6B), whereas the highest activation of SP-1 was seen in the cells treated with 0.8 μM of 15-deoxy-PGJ_2 (Fig. 6A) for 30 min treatment (Fig. 6B). Enhancing effect of 15-deoxy-PGJ_2 on the NGF-induced activation of AP-1 and SP-1 was then investigated. 15-Deoxy-PGJ_2 (0.8 μM) enhanced the NGF (50 ng/ml)-induced AP-1 activation in the cells co- treated for 1 hr, whereas SP-1 activity was not further increased (Fig. 7). Interestingly, neither NGF (50 μg/ml) or 15-deoxy PGJ_2 alone nor combination of two chemicals activates NF-κB (Fig. 7).

SB203580 Inhibited Promoting Effect of 15-Deoxy-PGJ_2 on the

NGF-Induced Neurite Extension and Activation of AP-1. To examine the role of activation of p38 MAP kinase signaling pathway in the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced neuronal differentiation and activation of AP-1, PC 12 cells were pretreated for 30 min with 10 and 50 μM SB203580, a specific inhibitor of p38 MAP kinase, and then stimulated with NGF and 15-deoxy-PGJ_2 combination. SB 203580 inhibited the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced neurite extension (Fig. 8C). Consistent with the ability of SB203580 to inhibit the promoting effect of 15-deoxy-PGJ_2 on NGF-induced neurite extension of PC 12 cells, SB203580 inhibited the enhancing effect of 15-deoxy-PGJ_2 on NGF-induced p38 MAP kinase and AP-1 activation (Fig. 8A and B). In contrast, other inhibitor PD98059 inhibited neither the promoting effect of 15-deoxy-PGJ_2 on NGF-induced neurite extension nor activation of p38 MAP kinase (Fig. 8D) and AP-1 (data not shown).

DISCUSSION

It was reported that 15-deoxy-PGJ_2, a natural ligand of PPAR-γ promoted NGF-induced neurite extension, a marker of cell differentiation (Satho *et al.*, 1999). In this study, we demonstrated that 15-deoxy-PGJ_2 enhanced NGF-induced expression of p38 MAP kinase and its phosphorylation. In addition, activation of the transcription factor AP-1 was also increased in accordance with the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced neurite extension. It's ability of 15-deoxy-PGJ_2, however, may not be related to PPAR-γ activation in the PC 12 cells.

PPAR-γ, a member of the nuclear hormone receptor superfamily, is activated either by naturally occurring ligand 15-deoxy-PGJ_2 or by troglitazon, a synthetic agonist (Kliewer *et al.*, 1995; Tontonoz *et al.*, 1998). PPAR-γ is activated during induction of cell differentiation of adipocytes and macrophages (Kliewer *et al.*, 1995; Tontonoz *et al.*, 1998; Forman *et al.*, 1995), and the PPAR-γ was essentially required for adipocyte differentiation (Rosen et al., 1999). Moreover, PPAR-γ antagonist inhibits differentiation of adipocyte (Wright et al., 2000). These data suggest that PPAR-γ function is required for the induction of differentiation of adipocytes and macrophages. However, it is not true in the PC 12 cells since 15-deoxy-PGJ_2 did not induce PPAR-γ expression during neurite extension. Neither immuno-reactivity was detected by immmunohistochemical analysis using anti-PPAR-γ antibody (data not shown). In addition, a synthetic PPAR-γ□agonist troglitazone (even with 5 μM

concentration) did not have promoting activity in the NGF-induced neurite extension in this study and other (Satoh *et al.*, 1999). Moreover, PPAR-γ antagonist did not inhibit the promoting ability of 15-deoxy-PGJ_2 on the NGF-induced neurite extension. The expression of other subtypes of PPAR (PPAR α and β) was constantly induced in this cells. These data indicated that unlike its biological effect on the differentiation of adipocytes and macrophages, PPAR-γ function (expression or activation) is not involved in the promoting effect of 15-deoxy-PGJ_2 on the neurite extension in PC 12 cells. However, the involvement of PPAR-γ function in the neuronal cell differentiation in other cells cannot be exclusive since PPAR-γ was expressed, and this expression was increased by the 15-deoxy-PGJ_2 treatments in the neuronal differentiating cells such as embryonic midbrain and SK-N-MC cells. In fact, we also found that PPAR-γ expression in these cells correlated well with the cell differentiation. PPAR-γ independent mechanism in the cell differentiation has been reported recently. Vernochet et al (2002) reported that PPAR-γ independent formation of preadipocyte preceding a PPAR-γ dependent phase in the development of adipose cells from pluripotent stem cells. Therefore, the involvement of PPAR-γ may be dependent of type and nature of cells.

Considered the absence of PPAR γ in this cells, we next examined the possibility that 15-deoxy-PGJ_2 exerts its promoting effect on neurite extension through a GPCR such as DP receptor (PGD_2) or EP receptor (PGE_2). We therefore explored various doses of the structure-related compounds PGD_2 and PGE_2 in the presence or absence of NGF (50 ng/ml) in the PC 12 cells. However, these structure related compounds did not show increasing or promoting effect on the basal or NGF-induced neurite extension. In addition, in the presence of antagonists of GPCRs BWA868C (DP receptor antagonist) and AH6809 (EP receptor antagonist) did not inhibit NGF or NGF +15-deoxy-PGJ_2-induced neurite extension. It was reported that NGF stimulates arachidonic acid metabolism producing PGE_2 in the PC 12 cells in the exogenous source of arachidonic acid, and the inhibitors of arachidonic acid release prevent the nerve fiber growth in the dorsal root gaglion neurons (DeGeorge *et al.*, 1988). We therefore examined the possibility that NGF alone or combination with PGD_2 and PGE_2 could release 15-deoxy PGJ_2, a metabolite of PGD_2 and thereby have promoting effect as an autocrine signals. However, NGF with/without prostaglandins (PGD_2, PGE_2 and 15-deoxy-PGJ_2) treatment did not change the level of each prostaglandins (PGD_2, PGE_2 and

15-deoxy-PGJ_2) and arachidonic acid. These disabilities to generate 15-deoxy-PGJ_2 are agree with no increase the neurite extension, release of arachidonic acid and the induction of COX-2 expression in the cells treated with NGF or prostaglandins (PGD_2 and PGE_2), or the combination of NGF with prostaglandins. In addition, DP and EP receptor antagonists did not inhibit the NGF+15-deoxy-PGJ_2-induced neurite extension and the level of prostaglandins and arachidonic acid. The present data are not consistent with the data demonstrated by Degeorge et al (1988) who showed significant elevation of PGE_2 after NGF treatment. This discrepancy is not clear, but the status of cells and the time to assay may cause the discrepancy. However, similar to our observation, they found that the inhibitor of arachidonic acid release fail to block nerve fiber growth. These data suggest that the promoting effect of 15-deoxy-PGJ_2 may not be mediated by a GPCRs.

We therefore investigated the MAP kinase signaling and activation of transcription factor as possible molecular mechanisms underlying the promoting activity of 15-deoxy-PGJ_2 in the neurite extension of PC 12 cells. It has demonstrated that mitogen-activated protein (MAP) kinase classes, known as c-Jun N-terminal kinase (JNK), extra-signal response kinase (ERK) and p38 MAP kinase signals are generally involved in the NGF-induced neuronal differentiation of PC-12 cells (Morooka and Nishida, 1998). In this report, we demonstrated that 15-deoxy-PGJ_2 increased the induction of the expression of p38 MAP kinase and phosphorylation of p38 MAP kinase, but had no effect on the expression of JNK and ERK and their phosphorylations. In addition, to further demonstrate a causal link between the activation of p38 MAP kinase pathway and promoting activity of 15-deoxy-PGJ_2 on the NGF-induced neurite extension of PC 12 cells, we employed a specific inhibitor of p38 kinase SB 203580. Pretreatment of SB203580 inhibited the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced activation of p38 kinase. Importantly, this inhibition of the activation of p38 kinase pathway correlated well with the ability of SB203580 to inhibit the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced neurite extension. The specific activation of MAP kinase classes has been demonstrated during the neurite extension of PC 12 cells depend upon different stimuli. Similar to the effect of 15-deoxy-PGJ_2, morphogenetic protein-2, a member of the transforming growth factor-beta superfamily, specifically activated p38 MAP kinase during neuronal differentiation of PC 12 cells (Iwasaki *et al.*, 1999). However, MAP kinase/ERK pathway signal was

activated by epidermal growth factor; and specific isoform of JNK was activated by staurosporine, a protein kinase inhibitor in PC 12 cells (Yao *et al.*, 1997;Morooka and Nishida, 1998). This effect also appears to be specific for 15-deoxy-PGJ_2 since a synthetic PPAR-γ agonist troglitazone (up to 5 μM) neither promotes NGF-induced neurite extension nor the neurofilament expression. Moreover, pretreatment of and PPAR-γ□antagonist (50 μM) did not inhibit the ability of 15-deoxy-PGJ_2 on the NGF-induced neurite extension. The effective concentration (0.4 to 1 μM) of 15-deoxy-PGJ_2 to activate p38 kinase is similar to that for promotion of the NGF-induced neurite extension in PC-12 cells. However, the requirement of effective concentration for causing of cell differentiation is likely depends on cell types. It was also found that similar range concentration (about 0.5 to 1 μM) of 15-deoxy-PGJ_2 is required for neuronal differentiation of embryonic midbrain cells to neuron, whereas neuroblastoma cells required about 2 to 4 μM concentration of 15-deoxy-PGJ_2 to achieve differentiating effect. The possibility that GPCR may respond to 15-deoxy-PGJ_2, in turn evoke signal pathway cascades that involve the p38 MAP kinase seems unlikely because the specific antagonists of GPCRs (DP and EP receptors) did not inhibit NGF with15-deoxy-PGJ_2-induced neurite extension and activation of p38 MAP kinase. In addition, PGD_2 and E_2 themselves, acting their biological activity via GPCRs did not induce the activation of p38 MAP kinase. The present study thus demonstrates that specific activation of p38 MAP kinase signaling is required for the promoting activity of 15-deoxy-PGJ_2 on the NGF-induced neurite extension, and the promoting activity of 15-deoxy-PGJ_2 on neurite extension is independent PPAR-γ and GPCR. PPAR-γ independent biological activities of 15-deoxy-PGJ_2 has been demonstrated. PPAR-γ independent induction of apoptosis of eosinophils and IL-8 production in the microvascular endothelial cell line treated with 15-deoxy-PGJ_2 were reported (Jozkowicz *et al.*, 2001; Harns *et al.*, 2002; Ward *et al.*, 2002). Moreover, it was also found that PPAR-γ and G protein coupled receptor independent apoptosis of human hepatic myofibroblasts and modulation of the production of reactive oxygen intermediates in neutrophils treated 15-deoxy-PGJ_2 (Vaidya *et al.*, 1999; Li *et al.*, 2001). Precise mechanisms how 15-deoxy-PGJ_2 activate p38 MAP kinase pathway in the promoting activity of 15-deoxy-PGJ_2 on the neurite extension are needed further studies. However, the signaling interaction between PPAR-γ (or the activator of PPAR-γ) and MAP kinase family in other biological events has been demonstrated. 15-

Lipoxygenase-1 metabolites down regulate PPAR-γ via the MAP kinase signaling pathway in colorectal carcinogenesis, a reverse differentiate response (His *et al.*, 2001). In addition, PPAR-γ was down regulated via MAP kinase-dependent pathway in the inhibition of adipocyte differentiation (Chan *et al.*, 2001). Moreover, very recent study showed that MAP kinase cascades are activated in astrocytes and preadipocytes by 15-deoxy-PGJ_2 and the thiazolidinedione ciglitazone through peroxisome proliferator activator receptor gamma-independent mechanisms (Lennon *et al.*, 2002).

Next, the effect of 15-deoxy PGJ_2 on the activation of transcription factors AP-1, SP-1 and NF-κB was investigated to examine whether activation of these transcription factors could correlate with the neurite extension and whether these transcription factors relay the MAP kinase signals in PC 12 cells after treatment of 15-deoxy-PGJ_2 with or without NGF. 15-Deoxy-PGJ_2 increased the AP-1 and SP-1 but not NF-κB activation in dose and time dependent manner. In addition, 15-deoxy-PGJ_2 further increased the NGF-induced activation of AP-1 but not SP-1. Furthermore, similar to the effect on the expression of p38 MAP kinase, pretreatment of SB203580 inhibited the promoting effect of 15-deoxy-PGJ_2 on the NGF-induced activation of AP-1. This result demonstrates that AP-1 rather than other transcription factors may be the most involved in the PC 12 cell differentiation by 15-deoxy-PGJ_2, and may act as a downstream target of p38 MAP kinase signal. The involvement of the activation of transcription factors has been demonstrated in the differentiation of PC12 cells and other cells. A significant coincided reduction of neurite extension and the DNA binding activity of transcription factor AP-1 was observed in the NGF-treated PC-12 cells carrying mutated presenilin-1 (Furukawa *et al.*, 1998). Significant roles of transcription factors SP-1 and NF-κB were demonstrated. Low dose of lead (0.025 to 0.1 μM) activated basal and the NGF (50 ng/ml)-induced SP-1 activation during PC12 cell differentiation (Crumpton *et al.*, 2001). NF-κB activation was increased during SH-SY5Y neuroblastoma cell differentiation by retinoic acid and 12-O-tetradecanoylphorbol 13-acetate (Feng and Porter, 1999). This differential activation of transcription factors can be explained that depend upon the nature and origins of cells, and stimuli, transcription factors can be specifically activated. Conclusively, the present study show that the promoting activity of 15-deoxy-PGJ_2 on the NGF-induced neurite extension of PC12 cells may not be related to the activation of PPAR-γ, but its ability may exert through

activation of the p38 MAP kinase in conjunction with AP-1 signal pathway.

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Figure legends

Fig. 1. Neurite outgrowth by 15-deoxy-PGJ_2 with/without NGF in cultured PC 12 cells. PC 12 cells cultured with 0.8 μM 15-deoxy PGJ_2 in the absence or presence of NGF (50 ng/ml) for 72 hrs. Neurite outgrowth was assessed by measuring the number of neurite extension per unit area of culture (number/mm^2) as described in materials and methods. A, morphological observation (x 40 magnification). B, expression of neurofilament. Values are mean±standard error of three experiments, with triplicate of each experiment.

Fig. 2. Effect of 15-deoxy PGJ_2 on the expression of PPAR-γ (A), and neurite extension of neuroblastoma (B) and embryonic midbrain cells (C). PC 12 cells were cultured with various doses of 15-deoxy PGJ_2 for 72 hrs. Neuroblastoma (SK-N-MC) cells were pre-cultured for 48 hrs, and then exposed with 15-deoxy PGJ_2 for 24 hrs. Midbrain embryonic cells were isolated from rat embryos on gestation day 12, and cultured for 48 hrs, and then the cells were treated with 15-deoxy PGJ_2 for 24 hrs. Expression of PPAR-γ (A) and neurite extension (B and C) were determined as described in materials and methods. Values are mean±standard error of three experiments, with triplicate of each experiment.

Fig. 3. Effect of prostaglandin D_2, E_2 and their receptor inhibitors on the neurite extension (A and B), and expression of cyclooxygenase 2 (C). PC 12 cells were cultured for 72 hrs with various doses of in the PGE_2 or PGD_2 in the absence or presence of NGF (50 ng/ml). The inhibitor of EP receptor

(BWA868C) and DP receptor (AH6809) was co-treated in the cells treated with NGF+15-deoxy-PGJ_2. Morphological observation under microscope (x 40). Similar effect was found in separated three experiments (A and B). Expression of cyclooxygenase-2. Similar experiment was found from two experiments with triplicate of each experiment (C). Level of prostaglandins and arachidonic acid from two experiments with triplicate of each experiment.

Fig. 4. Levels of prostaglandins and arachidonic acid. PC 12 cells were cultured for 24 hr and then exposed with the [^{3}H]arachidonic acid, then cultured another 48 hrs with various doses of in the PGE_2 or PGD_2 in the absence or presence of NGF (50 ng/ml). The inhibitor of EP receptor (BWA868C) and DP receptor (AH6809) was co-treated in the cells treated with NGF+15-deoxy-PGJ_2. Levels of prostaglandis and arachidonic acid were determined as described in the experimental procedures. Values are mean±standard error of two experiments, with triplicate of each experiment.

Fig. 5. Effect of PGD_2, PGE_2 and 15-deoxy PGJ_2 on the expression of MAP kinase family. PC 12 cells cultured with various doses of PGD_2, PGE_2 and 15-deoxy PGJ_2. Expression of MAP kinase family in the cells treated with 15-deoxy-PGJ_2 in the absence (A) or presence of NGF (50 ng/ml, B) for 24 hrs. Expression of p38 MAP kinase in the cells treated with PGD_2, PGE_2 in the absence of presence of NGF (50 ng/ml) or inhibitor of DP , (BWA868C) or EP receptor (AH6809). Expression of MAP kinase family and their phosphorylation forms was determined by western blotting as described in materials and methods. Similar results was obtained from three experiments, with triplicate of each experiment. C1 and D1, control; C2 and D2, NGF (50 ng/ml); C3, 1 μM PGD_2; C4, 2 μM PGD_2; C5, 5 μM PGD_2; C6, 10 μM PGD_2; C7, NGF+5 μM PGD_2 ; C8, NGF+10 μM PGD_2; C9, NGF+5 μM PGD_2+5 μM AH6809; C10, NGF+5 μM PGD_2+500 nM BWA868. D3, 1 μM PGE_2; D4, 2 μM PGE_2; D5, 5 μM PGE_2; D6, NGF+1 μM PGE_2; D7, NGF+2 μM PGE_2 ; D8, NGF+5 μM PGE_2; D9, NGF+5 μM PGE_2+5 μM AH6809; D10, NGF+5 μM PGE_2+500 nM BWA868.

Fig. 6. Effect of 15-deoxy PGJ_2 on the activation of transcription factors AP-1 and SP-1. PC 12 cells cultured with 0.8 μM 15-deoxy PGJ_2 for various times (A, time course effect) or with various doses of 15-deoxy PGJ_2 for

30 min (AP-1) or 1 hr (SP-1) (B, dose effect). A DNA binding activity of AP-1 and SP-1 was determined by gel mobility shift assay as described in the materials and methods. Values are mean±standard error of three experiments, with triplicate of each experiment.

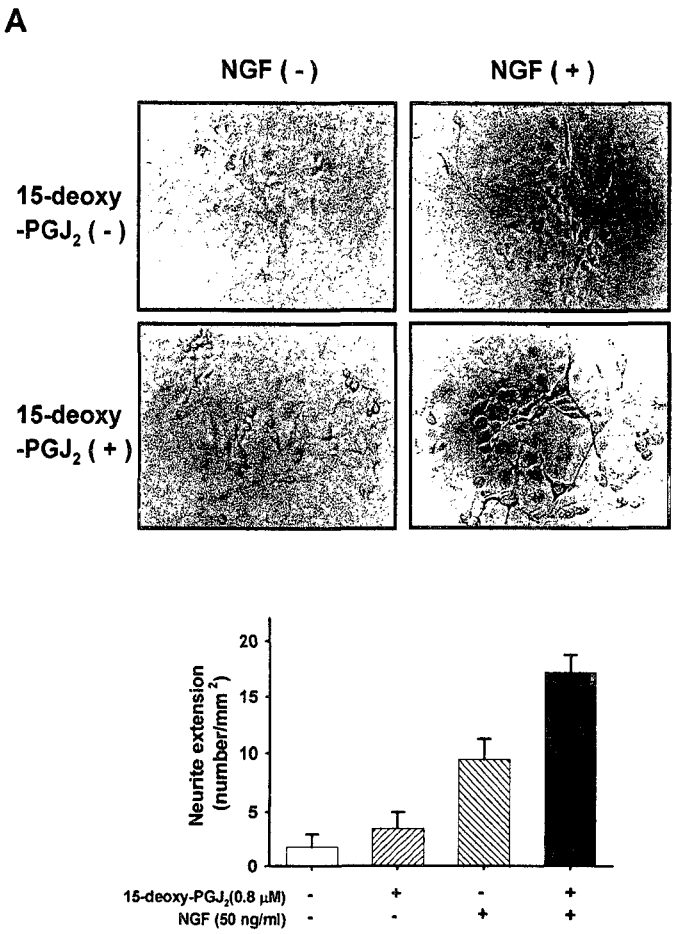
Fig. 7. Effect of 15-deoxy PGJ_2 on the NGF-induced activation of transcription factors AP-1, SP-1 and NF-κB. PC 12 cells cultured with 0.8 μM 15-deoxy PGJ_2 with NGF (50 ng/ml) for 30 min (AP-1) or 1 hr (SP-1 and NF-κB). A DNA binding activity of AP-1, SP-1 and NF-κB was determined by gel mobility shift assay as described in the materials and methods. Values are mean±standard error of three experiments, with triplicate of each experiment.

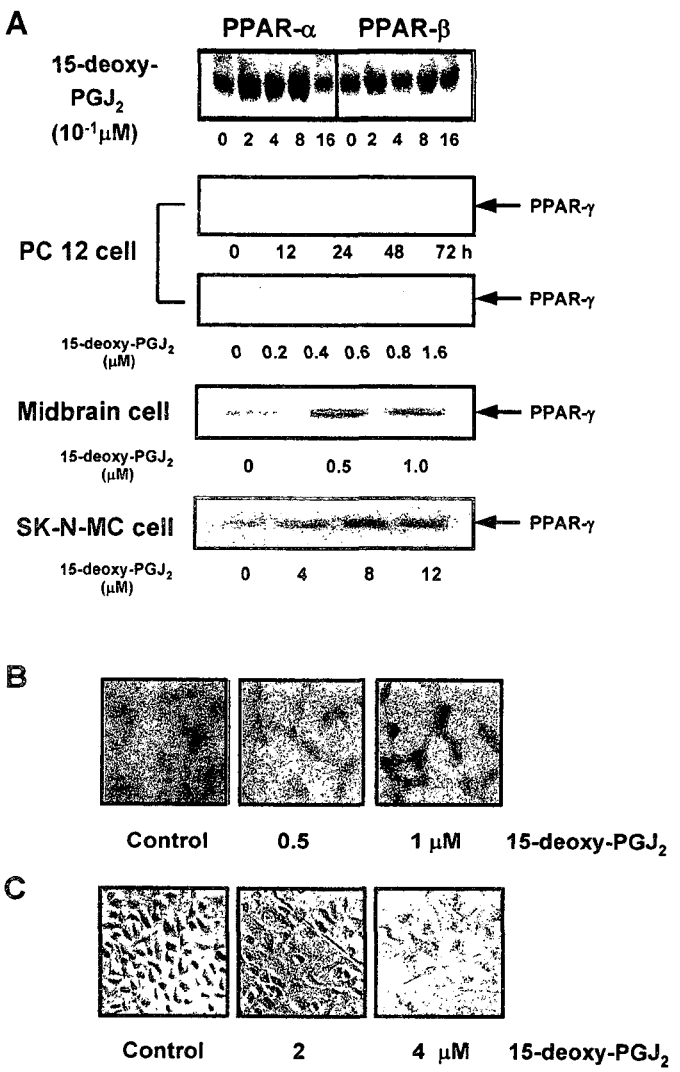
Fig. 8. Effect of SB 203580 on the enhancing effect of 15-deoxy PGJ_2 in NGF-induced activation of p38 MAP kinase (A), AP-1 neurite extension (B) and neurite extension (C), and the effect of PD98059 on the p38 MAP kinase. Cells were pre-treated with 10 or 50 μM SB203580 or 50 μM PD98059 for 30 min, and then exposed with NGF (50 ng/ml) and 15-deoxy PGJ_2 (0.8 μM) for 72 hrs to assay the neurite extension or 24 hr to assay the expression of p38 MAP kinase, or 30 min to determine the activation of AP-1. Values are mean±standard error of three experiments, with triplicate of each experiment. A1, NGF (50 ng/ml)+15-deoxy PGJ_2 (0.8 μM); A2, NGF (50 ng/ml)+15-deoxy PGJ_2 NGF (50 ng/ml)+10 μM SB 203580; A3, NGF (50 ng/ml)+15-deoxy PGJ_2 NGF (50 ng/ml)+50 μM SB 203580. D1, control, D2, NGF (50 ng/ml)+15-deoxy PGJ_2 (0.8 μM); D3, NGF+15-deoxy-PGJ_2 + PD98059 (50 μM).

Table 1. Neurite extension by 15-deoxy PGJ_2 in the absence or presence of NGF

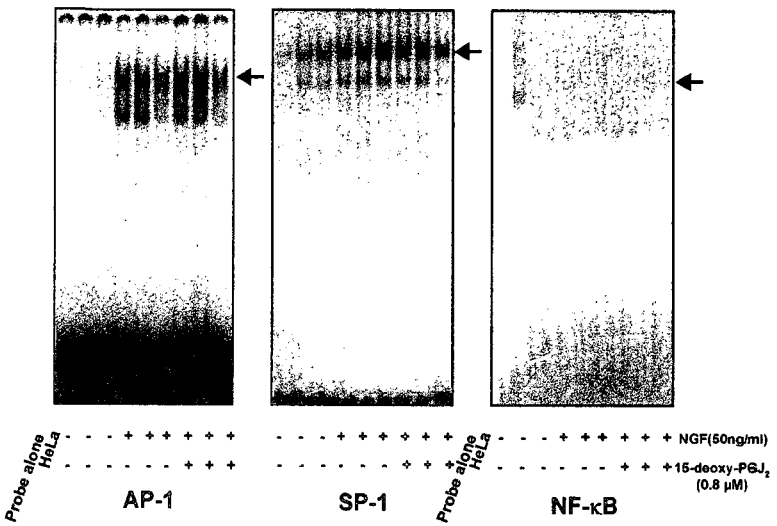
Treatment	Neurite extension (number/cm^2)		
	24 h	48h	72h
Control	1.4±0.4	1.6 ±0.8	1.9±0.9
15-deoxy PGJ_2 (μM)			
0. 2	1.9±0.7	2.3±0.5	2.5±1.3
0.4	2.4±1.1	2.5±1.4	3.7±1.3
0.8	2.3±1.4	2.9±0.7	3.7±0.8
1. 6	2.6±1.6	2.8±0.6	3.2±1.4
3.2	2.2±0.9	2.2±1.7	2.2±1.5
NGF (50 ng/ml)	6.8±3.2	7.2±1.1	9.8±1.7
NGF+15-deoxyPGJ_2 (μM)			
0.2	10.3±1.6	11.5±1.3	13.1±1.5
0.4	11.3±0.9	14.1±1.6	16.3±1.3
0.8	14.2±2.9	16.8±1.6	18.8±2.6
1.6	15.3±2.7	16.3±3.7	15.8±3.7
3.2	13.5±2.3	14.3±2.1	13.1±1.5
Triglitazone (μM)			
1	2.1±0.3	2.5±1.2	2.5±1.5
5	1.9±1.3	2.2±1.7	2.6±0.7
+NGF	7.9±1.5	7.2±2.7	8.4±1.7
BADGE (μM)			
20	13.2±2.6	16.3±2.6	17.8±1.9
50	15.3±2.7	15.3±3.9	16.2±2.1

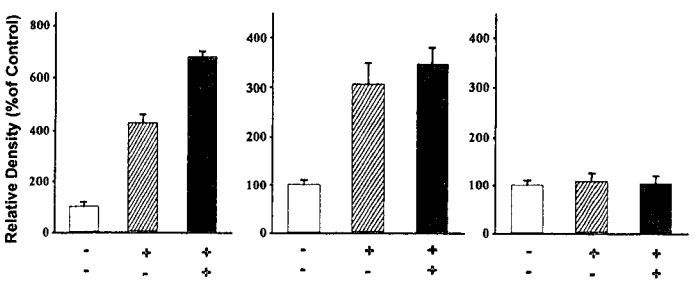
PC 12 cells were cultured with various doses of 15-deoxy PGJ_2 in the absence or presence of NGF (50 ng/ml), or triglitazone alone for 72 hrs. Bisphenol A digylycidyl ether (BADGE) was pre-treated two hour before the treatment of 15-deoxy-PGJ_2 with NGF. Neurite extension was assessed by measuring the number of neurite extension per unit area of culture (number/mm^2) as described in materials and methods. Values are mean±standard error of three experiments, with triplicate of each experiment.

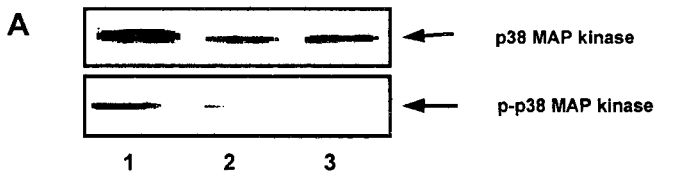

A
NGF (-)
NGF (+)
15-deoxy -PGJ2 (-)
15-deoxy -PGJ2 (+)
Neurite extension (number/mm2)
20
15
10
5
0
15-deoxy-PGJ2(0.8 μM) - + - +
NGF (50 ng/ml) - - + +
B
Neurofilament

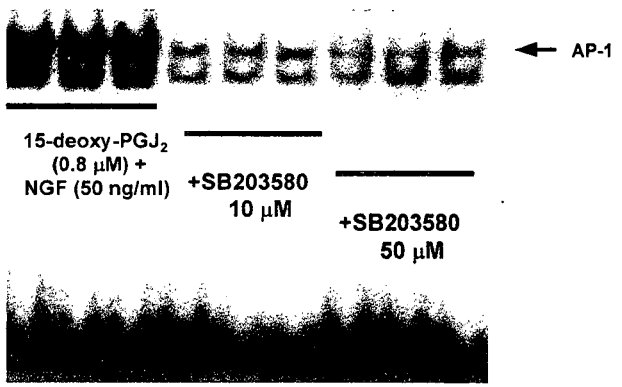

A
PPAR-α
PPAR-β
15-deoxy-PGJ2 (10-1μM)
0 2 4 8 16 0 2 4 8 16
PC 12 cell
PPAR-γ
0 12 24 48 72 h
PPAR-γ
15-deoxy-PGJ2 (μM)
0 0.2 0.4 0.6 0.8 1.6
Midbrain cell
PPAR-γ
15-deoxy-PGJ2 (μM)
0 0.5 1.0
SK-N-MC cell
PPAR-γ
15-deoxy-PGJ2 (μM)
0 4 8 12
B
Control 0.5 1 μM 15-deoxy-PGJ2
C
Control 2 4 μM 15-deoxy-PGJ2

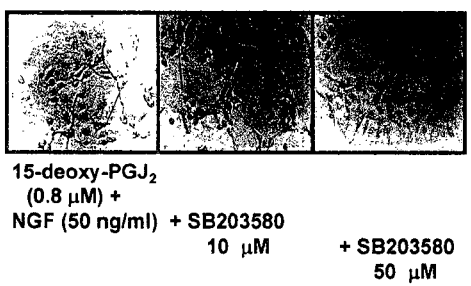
A
B
C
D
Control
NGF
PGD2
15-d-PGJ2
PGE2
+ NGF
+ BWA868C
[3H]PGE2 (cpm × 10³)
[3H]PGD2 (cpm × 10³)
[3H]15-deoxy-PGJ2 (cpm × 10³)
[3H]Arachidonic acid (cpm × 10³)
COX-2
Con
NGF (50 ng/ml)
15-d-PGJ2 (0.8 μM)
NGF+PGD2
NGF+PGE2
NGF+15-d-PGJ2

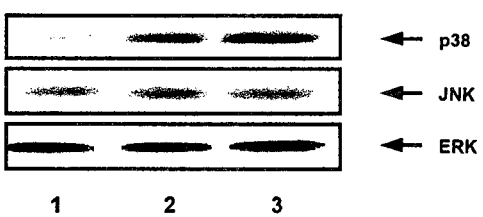

Probe alone
HeLa
NGF(50ng/ml)
15-deoxy-PGJ2 (0.8 μM)
AP-1
SP-1
NF-κB


Relative Density (%of Control)

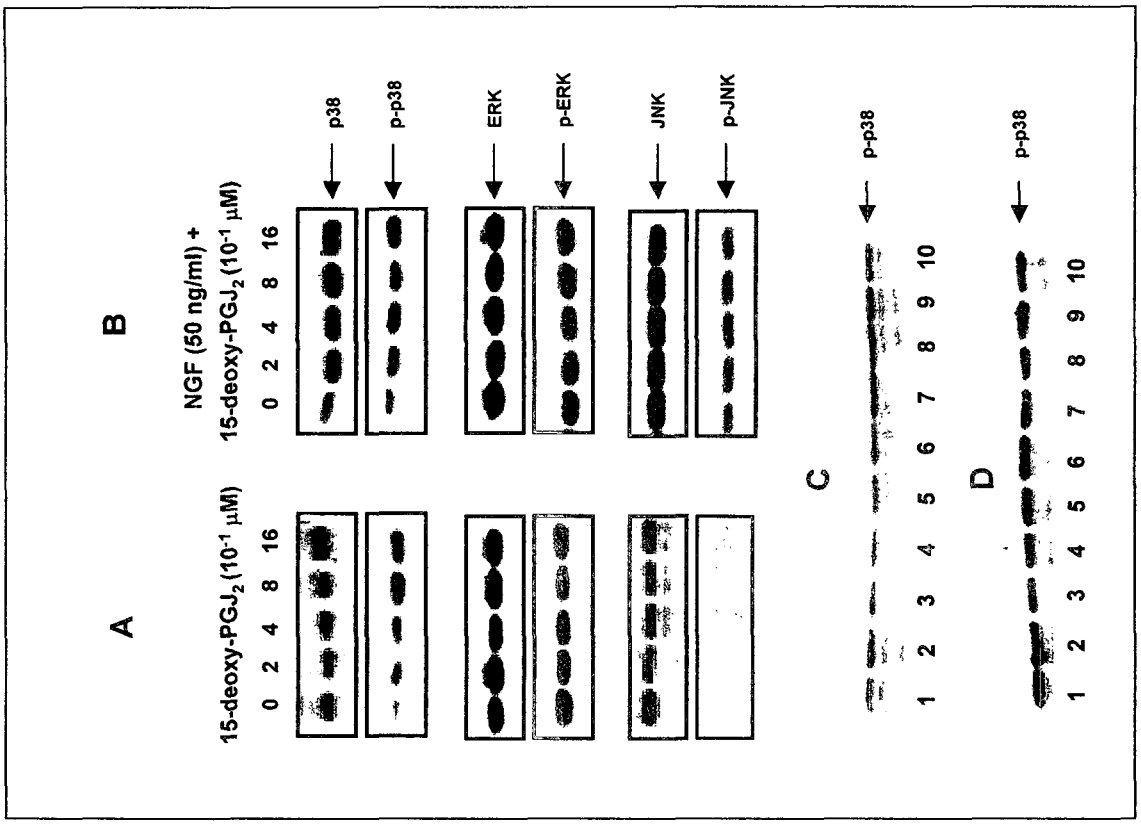

A
p38 MAP kinase
p-p38 MAP kinase
1
2
3


B
AP-1
15-deoxy-PGJ2 (0.8 μM) + NGF (50 ng/ml)
+SB203580 10 μM
+SB203580 50 μM


C
15-deoxy-PGJ2 (0.8 μM) + NGF (50 ng/ml)
+ SB203580 10 μM
+ SB203580 50 μM


D
p38
JNK
ERK
1
2
3

A
1 2 3 4 5
AP-1
Free probe
15-deoxy-PGJ2 (0.8μM)
Relative Density (% of Control)
0 100 200 300 400
0 0.5 1 3
Time (Hours)
1 2 3 4
SP-1
Free probe
15-deoxy-PGJ2 (0.8μM)
Relative Density (% of Control)
0 100 200 300 400
0 0.5 1 3
Time (Hours)
B
1 2 3 4 5 6
AP-1
Free probe
Relative Density (% of Control)
0 100 200 300 400
0 0.2 0.4 0.8 1.6 3.2
15-deoxy-PGJ2 (μM)
1 2 3 4 5
SP-1
Free probe
Relative Density (% of Control)
0 100 200 300 400
0 0.2 0.4 0.8 1.6 3.2
15-deoxy-PGJ2 (μM)


A
15-deoxy-PGJ2 (10-1 μM)
0 2 4 8 16
B
NGF (50 ng/ml) +
15-deoxy-PGJ2 (10-1 μM)
0 2 4 8 16
p38
p-p38
ERK
p-ERK
JNK
p-JNK
C
p-p38
1 2 3 4 5 6 7 8 9 10
D
p-p38
1 2 3 4 5 6 7 8 9 10