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Activation of p38 Mitogen-Activated Protein Kinase and Activator Protein-1 during the Promotion of Neurite Extension of PC-12 Cells by 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2

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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 in such cells as adipose cells and macrophages. Herein, we 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, a marker of cell differentiation. However, a 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 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(pyridyl)1H-imidazole (SB203580), 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 because 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 neuroblastoma 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 (PGE_2 and PGD_2 receptors) 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 by GPCR either. 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 PC-12 cells.

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 classic prostaglandins, which act after binding to cell surface G-protein coupled receptors (GPCRs), 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. In 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).

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ABBREVIATIONS: 15-deoxy-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PG, prostaglandin; PPAR-γ, peroxisome proliferator activated receptor-γ; MAP, mitogen-activated protein; JNK, Jun N-terminal kinase; ERK, extracellular signal regulated kinase; AP-1, activator protein 1; NF-κB, nuclear factor κB; SP-1, simian virus 40 promoter factor 1; NGF, nerve growth factor; DP, prostaglandin D_2 receptor; EP, prostaglandin E_2 receptor; GPCR, G protein-coupled receptor; BW A868C, 3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2- hydroxyethylamino)hydantoin; AH6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; BW755C, 3-amino-1m-(trifluoromethyl)-phenyl-2-pyrazoline; COX, cyclooxygenase; EGF, epidermal growth factor; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(pyridyl)1H-imidazole.

However, the signaling pathway(s) involved in the promoting ability of 15-deoxy-PGJ_2 on the NGF-induced neurite extension remains unclear.

It has been demonstrated that signals from mitogen-activated protein (MAP) kinase classes [known as Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAP kinase] are generally involved in NGF-induced neuronal differentiation of PC-12 cells (Leppa et al., 1998; Morooka and Nishida, 1998; Iwasaki et al., 1999). Even though these protein kinase signaling systems are evolutionarily related, they convey distinct signals. That is, depending on the type of stimuli and nature of cells, different classed of MAP kinase have been up-regulated. Bone morphogenic protein 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 at 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 PC-12 cell differentiation (Furukawa et al., 1998).

In this study, we first demonstrated that 15-deoxy-PGJ_2 promoted the NGF-induced neurite extension in a PPAR-γ independent manner; thereafter, we focused our investigation on 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 15-deoxy-PGJ_2 with that by other structure-related compounds (PGD_2 and PGE_2) on neurite extension and further examined whether 15-deoxy-PGJ_2 promotes neurite extension via a GPCR. In the present study, we show that 15-deoxy-PGJ_2 promotes the differentiation of PC-12 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. PC-12 cells, a rat cell line derived from a pheochromocytoma, and neuroblastoma SK-N-MC cells, a human neuron cancer cell line, that have the ability of differentiation were maintained on tissue culture plastic in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C under an atmosphere of 5% CO_2/95% air. The culture media was changed three times per week, and cultures were passaged at ~70 to 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. Various doses of PGD_2, PGE_2 and 15-deoxy-PGJ_2 (obtained from Cayman Chemicals, Ann Arbor, MI) with or without NGF (50 ng/ml) and antagonists of PGD_2 receptor (DP) (BW A868C) and PGE_2 receptor (EP) (AH6809) were added into medium and the cells were cultured either for 72 h to assay neurite extension, neurofilament, and PPAR-γ expressions or for ~0.5 to 3 h to assay the transcription factor activation. Expression of MAP kinase classes was determined after 24-h 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 brief, embryonic midbrain tissues were dissociated into individual cells by successive digestion with Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (Invitrogen) containing 1% trypsin. Cells were then resuspended in Dulbecco's modified Eagle's medium nutrient and Ham's F-12 nutrient mixture (1:1 mixture; Sigma Chemical Co., St. Louis, MO) with 10% NuSerum (25% newborn calf serum; BD Biosciences, San Jose, CA), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. The cells were then adjusted to give 5×10^6 cells/ml. Ten microliters of cell suspension was added to each well, and the cells were incubated for 2 h at 37°C. Two hundred microliters of the culture medium was then added to each well, and the cells were cultured for 48 h. 15-deoxy-PGJ_2 (0.5 or 1 μM) was then added into medium, and the cells were cultured for 48 h 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 neurites per unit area of the culture (number per square millimeter) using photo images of cells produced by 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 method described previously (Hong and Glauert, 1998). Briefly, the cultured cells were washed three times with ice-cold phosphate-buffered saline, pH 7.6, and pelleted. The pellets were resuspended in 400 μl of nonradioactive buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, and then centrifuged at 11,000*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 dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. After centrifugation at 11,000*g* for 6 min, the supernatant contained the nuclear proteins. The protein level was determined by a microplate modification of the Bradford (1976) 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, Madison, WI). In brief, 10 μg of nuclear protein was incubated in a total volume of 25 μl 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 of [γ-^{32}P]ATP-labeled oligonucleotide containing AP-1, SP-1, or NF-κB binding sites at room temperature. For the competition assay, 50×, 100×, or 200× excesses of unlabeled double-stranded oligonucleotide of the AP-1, SP-1, or NF-κB binding site were used as specific competitors. Excess (200×) of labeled double-stranded oligonucleotide of the SP-1 (or AP-1 for SP-1) binding site was used as a nonspecific competitor. Ten micrograms 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% nondenatured polyacrylamide gel at 150 V for 2 h. 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 phenylmethylsulfonyl fluoride, 10 μl/ml aprotinin, 1% Igepal CA 630 (Sigma Chemical), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA,

and 0.5% sodium deoxycholate] and centrifuged at 23,000*g* for 1 h. Equal amounts of proteins (20 μg) were separated on a SDS/12% polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Piscataway, NJ). Blots were blocked for 2 h at room temperature with 5% (w/v) nonfat 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 h at room temperature with specific antibodies. Rabbit polyclonal antibodies against PPAR-γ, ERK, p38 MAP kinase, JNK, and their phosphorylated forms, goat polyclonal antibody against COX-2, and mouse monoclonal antibody against neurofilament were used in this study at dilutions specified by the manufacturer (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive proteins were detected with the enhanced chemiluminescence Western blotting detection system. The relative density of the protein bands was quantified by densitometry using the Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY).

Immunohistochemical Staining. The cells (PC-12, neuroblastoma, and embryonic midbrain cells) treated with different doses of 15-deoxy PGJ$_2$ were cultured in LabTek chamber slides (Nalge Nunc International, Naperville, IL), and then the cells were fixed with 4.5% glutaraldehyde for 30 min. Immunohistochemical staining was performed with Vectastatin avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA). 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% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M 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 cultured for 24 h, and then the cells were treated with various doses of the prostaglandins with/without NGF or PGE$_2$ and PGD$_2$ antagonists in the presence of [^{3}H]arachidonic acid (0.4 μCi/ml; PerkinElmer Life Sciences, Boston, MA) for 48 h. The generation of prostaglandins was determined in the medium and cells as described by Akiba et al. (2001). In brief, prostaglandins were extracted and separated by thin-layer chromatography on a Silica Gel G plate using development solution [ethyl acetate/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 Life Sciences). 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 lipoxygenase 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$_2$ Enhanced NGF-Induced Neurite Extension and Expression 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, 1% serum did not increased neurite extension and expression of neuronal protein neurofilament; we therefore treated 15-deoxy-PGJ$_2$ with or without NGF to the cells cultured in 1% serum-containing medium for up to 72 h. As in another study by Satoh et al. (1999), the neurite extension was slightly increased by 15-deoxy-PGJ$_2$ itself (about 2-fold higher than control group). A slight inhibitory effect was found at the highest dose of 15-deoxy-PGJ$_2$ (3.2 μM); viability was decreased about 70% over control. However, in the presence of NGF (50 ng/ml), 15-deoxy-PGJ$_2$ promoted significant NGF-induced neurite extension (Fig. 1A). Dose- and time-dependent effects of 15-deoxy-PGJ$_2$ with/without NGF on neurite extension are summarized in Table 1. Immunoblotting was then performed to investigate the expression of neuronal differentiation markers in the PC-12 cells treated with 15-deoxy-PGJ$_2$. Coincidentally with the enhancing effect on neurite extension, 15-deoxy-PGJ$_2$ enhanced basal and the NGF-induced expression of neurofilament (Fig. 1B). In contrast, troglitazone, a synthetic PPAR-γ agonist, did not increase the NGF-induced neurite extension (Table 1) and the expression of neurofilament (data not shown). Moreover, pretreatment of the PPAR-γ antagonist bisphenol A diglycidyl ether did not alter the promoting activity of 15-deoxy-PGJ$_2$ on the NGF-induced neurite extension (Table 1) and the expression of neurofilament (data not shown).

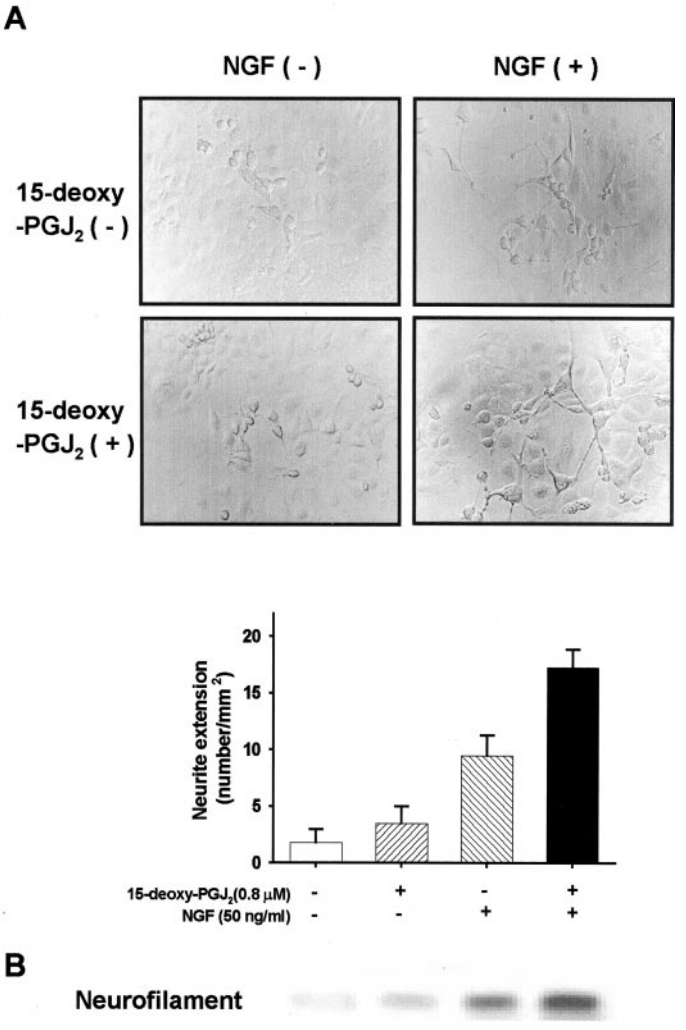

Fig. 1. Neurite extension by 15-deoxy-PGJ$_2$ with/without NGF in cultured PC-12 cells. PC-12 cells cultured with 0.8 μM 15-deoxy PGJ$_2$ in the absence or presence of NGF (50 ng/ml) for 72 h. Neurite extension was assessed by measuring the number of neurite extension per unit area of culture (number per square millimeter) as described under *Materials and Methods*. A, morphological observation (40× magnification). B, expression of neurofilament. Values are mean ± S.E. of three experiments, with triplicates of each experiment.

15-Deoxy-PGJ$_2$ Did Not Express PPAR-γ Receptor in PC-12 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 performed 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 h. Immunoreactivity against anti-PPAR-γ antibody was also analyzed by immunohistochemistry, but immunoreactivity was not found in the PC-12 cells treated with/without 15-deoxy-PGJ$_2$ (data not shown). In 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 change 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 cells and SK-N-MC neuroblastoma cells that are able to differentiate. In contrast to the expression in the PC-12 cells, PPAR-γ was expressed, and 15-deoxy-PGJ$_2$ increased its expression in these cells (Fig. 2A). Moreover, the expression of PPAR-γ correlated well with the differentiation of these cells by similar concentrations of 15-deoxy-PGJ$_2$ (0.5–1 μM) in embryonic midbrain cells (Fig. 2B) or by higher concentration (2 or 4 μM) in SK-N-MC cells (Fig. 2C).

The Promoting Effect of 15-Deoxy-PGJ$_2$ Is Not Mediated by GPCR. Considering 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 GPCR, as demonstrated by other prostaglandins such as PGD$_2$ and PGE$_2$. We therefore first explored various doses (1, 2, and 5 μM) of the structure-related PGD$_2$ and PGE$_2$, and their biological activity via GPCR, in the presence or absence of NGF (50 ng/ml) in the PC-12 cells and then assessed the neurite extension. Even the highest doses of PGD$_2$ and PGE$_2$ showed no increasing or promoting effect on the basal or NGF-induced neurite extension (Fig. 3A). In addition, the antagonists of DP (100, 200, and 500 nM BW A868C) and EP receptors (1, 5, and 10 μM 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$_2$, EGF, nor the combination of NGF and PGD$_2$ and PGE$_2$ generated 15-deoxy-PGJ$_2$ (Fig. 4C). In addition, DP receptor antagonist (500 nM BW A868C) 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 in combination with NGF did not change the levels of prostaglandins (PGD$_2$, PGE$_2$, and 15-deoxy-PGJ$_2$) (Fig. 4, A and B). NGF, prostaglandins, or the combination of NGF with prostaglandins did not change the release of arachidonic acid, and the DP receptor antagonist (500 nM BW A868C) did not significantly change the level of arachidonic acid (Fig. 4D). The inability of NGF, prostaglandins, or the combination to release arachidonic acid are in agreement with no induction of COX-2 expression in the cells treated with NGF, PGD$_2$, 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 GPCRs.

15-Deoxy-PGJ$_2$ Enhanced NGF-Induced p38 MAP Kinase Signaling. MAP kinase classes have been known to be 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 caused by 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, but not basal JNK

TABLE 1

Neurite extension by 15-deoxy PGJ$_2$ in the absence or presence of NGF

PC-12 cells were cultured with various doses of 15-deoxy PGJ$_2$ in the absence or presence of NGF (50 ng/ml), or troglitazine alone for 72 h. Bisphenol A diglycidyl ether (BADGE) was pretreated 2 h 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 as described under *Materials and Methods*. Values are mean ± S.E. of three experiments performed in triplicate.

Treatment	NeuriteExtension		
	24 h	48 h	72 h
		number/cm^2	
Control	1.4 ± 0.4	1.6 ± 0.8	1.9 ± 0.9
15-deoxy PGJ$_2$			
0.2 μM	1.9 ± 0.7	2.3 ± 0.5	2.5 ± 1.3
0.4 μM	2.4 ± 1.1	2.5 ± 1.4	3.7 ± 1.3
0.8 μM	2.3 ± 1.4	2.9 ± 0.7	3.7 ± 0.8
1.6 μM	2.6 ± 1.6	2.8 ± 0.6	3.2 ± 1.4
3.2 μM	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-deoxy PGJ$_2$			
0.2 μM	10.3 ± 1.6	11.5 ± 1.3	13.1 ± 1.5
0.4 μM	11.3 ± 0.9	14.1 ± 1.6	16.3 ± 1.3
0.8 μM	14.2 ± 2.9	16.8 ± 1.6	18.8 ± 2.6
1.6 μM	15.3 ± 2.7	16.3 ± 3.7	15.8 ± 3.7
3.2 μM	13.5 ± 2.3	14.3 ± 2.1	13.1 ± 1.5
Troglitazone			
1 μM	2.1 ± 0.3	2.5 ± 1.2	2.5 ± 1.5
5 μM	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			
20 μM	13.2 ± 2.6	16.3 ± 2.6	17.8 ± 1.9
50 μM	15.3 ± 2.7	15.3 ± 3.9	16.2 ± 2.1

and ERK and their phosphorylated forms, in a dose-dependent manner (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 cotreated. However, 15-deoxy-PGJ$_2$ did not affect the NGF-induced expression of ERK and JNK and their phosphorylated forms either (Fig. 5B). We further examined the possibility that the GPCR response to 15-deoxy-PGJ$_2$ in turn evokes signal pathway cascades that involve the p38 MAP kinase. We employed the specific antagonists of GPCRs (DP and EP receptors) in the cells treated with NGF or NGF with 15-deoxy-PGJ$_2$. Neither of the antagonists inhibited NGF or NGF with 15-deoxy-PGJ$_2$–induced activation of p38 MAP kinase (i.e., increase of the expression of phosphorylated form of p38 MAP kinase). In addition, neither PGD$_2$ nor PGE$_2$, acting in their biological capacity via GPCR, induced the activation of p38 MAP kinase (Fig. 5, C 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-κB. 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 for 1 h (Fig. 6A) with 0.8 μM of 15-deoxy-PGJ$_2$ (Fig. 6B), whereas the highest activation of SP-1 was seen in the cells treated for 30 min (Fig. 6A) with 0.8 μM 15-deoxy-PGJ$_2$ treatments (Fig. 6B). The 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 cotreated for 1 h, whereas SP-1 activity was not further increased (Fig. 7). Interestingly, neither NGF (50 μg/ml), 15-deoxy-PGJ$_2$, nor combination of the two activated NF-κB (Fig. 7).

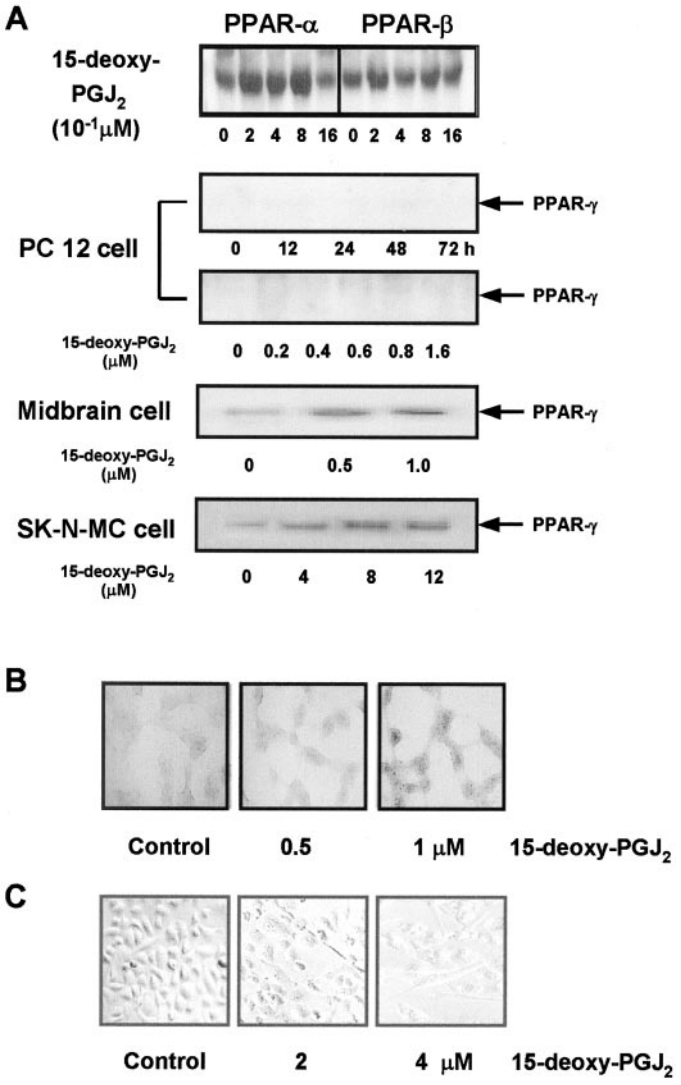

Fig. 2. Effect of 15-deoxy-PGJ$_2$ on the expression of PPAR-γ (A), and on the neurite extension of neuroblastoma (B) and embryonic midbrain cells (C). PC-12 cells were cultured with various doses of 15-deoxy-PGJ$_2$ for 72 h. Neuroblastoma (SK-N-MC) cells were precultured for 48 h, and then exposed with 15-deoxy PGJ$_2$ for 24 h. Midbrain embryonic cells were isolated from rat embryos on gestation day 12 and cultured for 48 h, and then the cells were treated with 15-deoxy PGJ$_2$ for 24 h. Expression of PPAR-γ (A) and neurite extension (B and C) were determined as described under *Materials and Methods*. Similar results were obtained from three experiments.

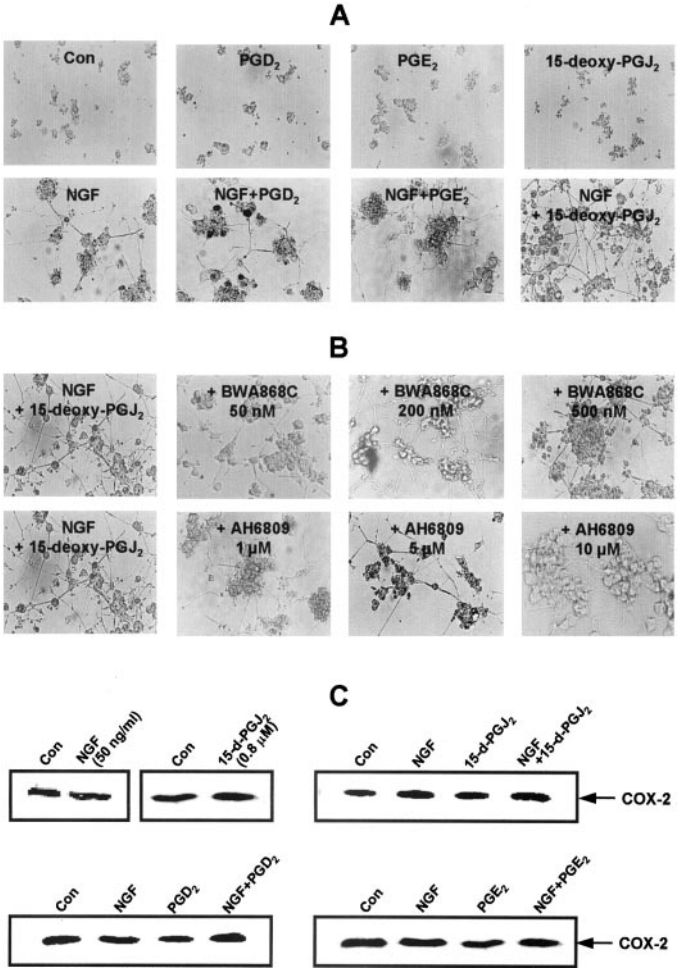

Fig. 3. Effect of PGD$_2$, E$_2$, and 15-deoxy-PGJ$_2$ on the neurite extension (A) and expression of cyclooxygenase 2 (C), and effect of DP and EP receptor inhibitors on the NGF + 15-deoxy-PGJ2-induced neurite extension (B). PC-12 cells were cultured for 72 h with various doses of PGD$_2$, PGE$_2$, and 15-deoxy-PGJ$_2$ or in the absence or presence of NGF (50 ng/ml). The inhibitor of DP (AH6809) or EP receptor (BW A868C) was cotreated in the cells treated with NGF + 15-deoxy-PGJ$_2$. Morphological observation was performed under the microscope (40×). Similar effects were found in separated three experiments (A and B). Similar results were found from two experiments performed in triplicate (C).

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 the 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 an NGF and 15-deoxy-PGJ_2 combination. SB203580 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. 8, A and B). In contrast, another 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).

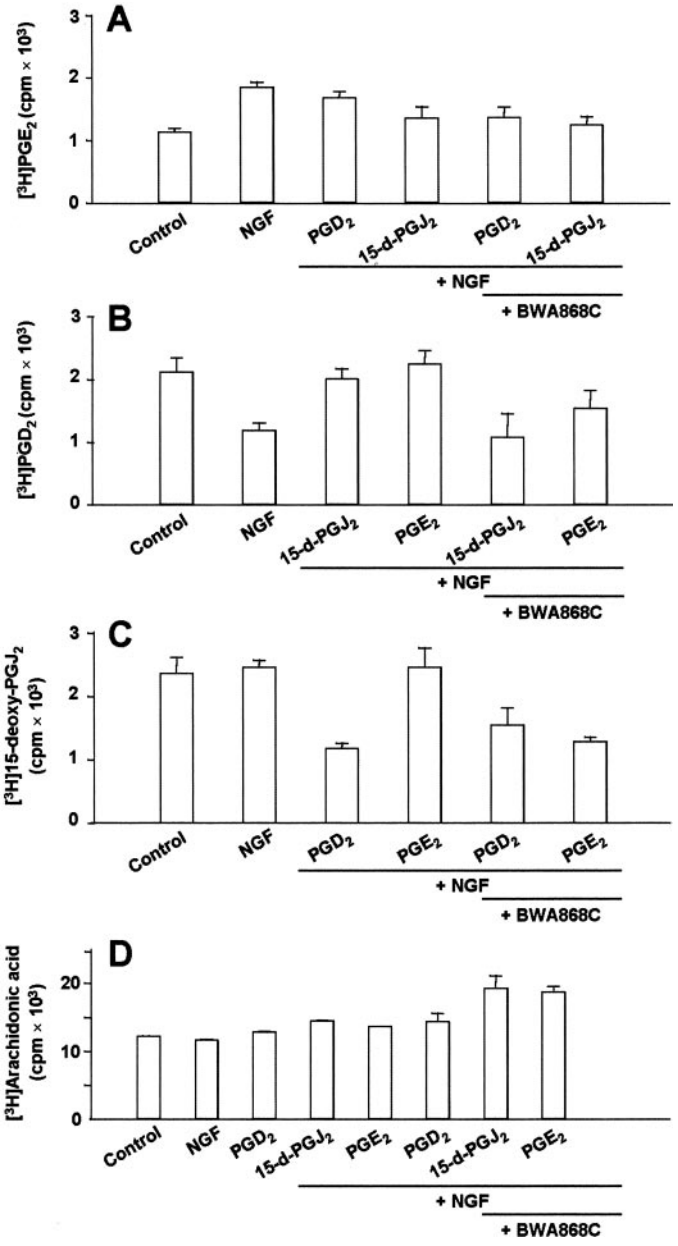

Fig. 4. Levels of prostaglandins and arachidonic acid. PC-12 cells were cultured for 24 h, and then the cells were cultured another 48 h with various doses of PGD_2, PGE_2, or 15-deoxy-PGJ_2 with/without NGF (50 ng/ml) or inhibitor of DP receptor (BW A868C) in the presence of [^{3}H]arachidonic acid for 48 h. Levels of prostaglandins and arachidonic acid were determined as described under *Materials and Methods*. Values are mean ± S.E. of two experiments with triplicate of each experiment.

Discussion

15-Deoxy-PGJ_2, a natural ligand of PPAR-γ, has been reported to promote NGF-induced neurite extension, a marker of cell differentiation (Satoh 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

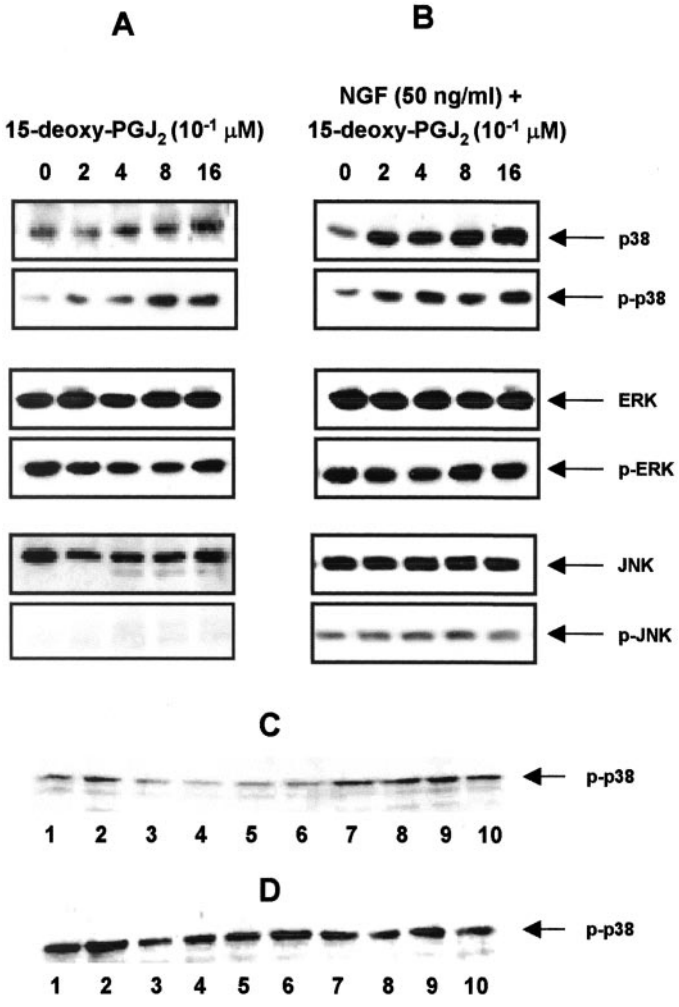

Fig. 5. Effect of PGD_2, PGE_2, and 15-deoxy-PGJ_2 on the expression of MAP kinase family. PC-12 cells were cultured with various doses of PGD_2, PGE_2, and 15-deoxy-PGJ_2 with (B)/without (A) EGF (50 ng/ml) in the absence or presence of DP (BW A868C) or EP receptor (AH6809) inhibitors (C and D). Expression of MAP kinase family and their phosphorylation forms was determined by Western blotting as described under *Materials and Methods*. Similar results were obtained from two 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 BW A868C. 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 BW A868C.

increased in accordance with the promoting effect of 15-deoxy-PGJ$_2$ on the NGF-induced neurite extension. However, the promoting ability of 15-deoxy-PGJ$_2$ on NGF-induced neurite extension may not be related to PPAR-γ or GPCR activation in the PC-12 cells.

PPAR-γ, a member of the nuclear hormone receptor superfamily, is activated either by the naturally occurring ligand 15-deoxy-PGJ$_2$ or by troglitazone, 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; Forman et al., 1995; Tontonoz et al., 1998), and the PPAR-γ was essentially required for adipocyte differentiation (Rosen et al., 1999). Moreover, PPAR-γ antagonists inhibit differentiation of adipocytes (Wright et al., 2000). These data suggest that PPAR-γ function is required for the induction of differentiation of adipocytes and macrophages. However, this is not true in the PC-12 cells because 15-deoxy-PGJ$_2$ did not induce PPAR-γ expression during neurite extension. Immunoreactivity was not detected by immmunohistochemical analysis using anti-PPAR-γ antibody (data not shown). In addition, the synthetic PPAR-γ agonist troglitazone (even at 5 μM) did not have promoting activity in the NGF-induced neurite extension in this study and in another study (Satoh et al., 1999). Moreover, the PPAR-γ antagonist bisphenol A diglycidyl ether 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 cell. These data indicate 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 because 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 mechanisms in cell differentiation have been reported recently. Vernochet et al. (2002) reported that PPAR-γ independent formation of preadipocyte preceded a PPAR-γ–dependent phase formation 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.

Considering the absence of PPAR-γ in these cells, we next examined the possibility that 15-deoxy-PGJ$_2$ exerts its promoting effect on neurite extension through a GPCR such as the DP or EP receptor. 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 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, BW A868C (DP receptor antagonist) and AH6809 (EP receptor antagonist) did not inhibit NGF or NGF + 15-deoxy-PGJ$_2$-induced neurite extension. NGF was reported to stimulate arachidonic acid metabolism, producing PGE$_2$ in the PC-12 cells when arachidonic acid was added exogenously, and the inhibitors of arachidonic acid release prevented the growth of nerve fiber in the dorsal root ganglion neurons (DeGeorge et al., 1988). We therefore examined the possibility that NGF alone or in combination with PGD$_2$ and PGE$_2$ could release 15-deoxy PGJ$_2$, a metabolite of PGD$_2$ and thereby have a promoting effect as autocrine signals. However, NGF with/without prostaglandin (PGD$_2$, PGE$_2$, and 15-deoxy-PGJ$_2$) treatment did not change the level of pros-

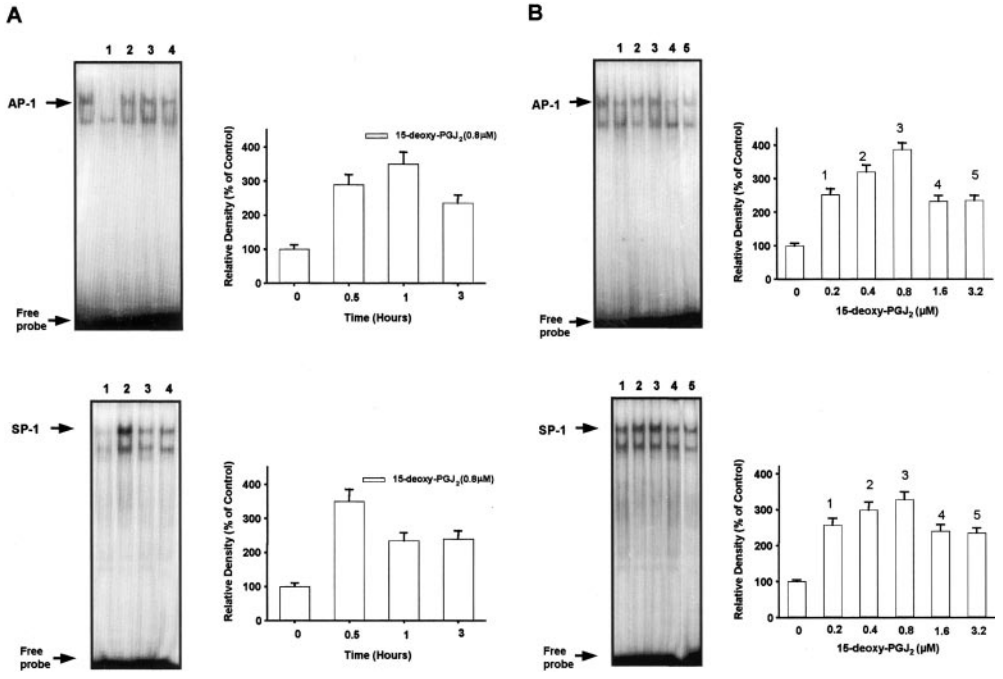

Fig. 6. Effect of 15-deoxy-PGJ$_2$ on the activation of transcription factors AP-1 and SP-1. PC-12 cells cultured with 0.8 μM 15-deoxy PGJ$_2$ for various times (A, time course effect) or with various doses of 15-deoxy PGJ$_2$ for 30 min (AP-1) or 1 h (SP-1) (B, dose effect). A DNA binding activity of AP-1 and SP-1 was determined by gel mobility shift assay as described under *Materials and Methods*. Values are mean ± S.E. of three experiments performed in triplicate.

taglandins and arachidonic acid. This inability to generate 15-deoxy-PGJ_2 is consistent with the lack of increase in 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 did not change the level of prostaglandins and arachidonic acid. The present data are not consistent with the data from DeGeorge et al. (1988) demonstrating significant elevation of PGE_2 after NGF treatment. This discrepancy is unclear, but the status of cells and the time to assay may cause the discrepancy. However, as in our observations, they found that the inhibitor of arachidonic acid release fails to block the growth of nerve fiber. These data suggest that the promoting effect of 15-deoxy-PGJ_2 may not be mediated by GPCRs.

We therefore investigated 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 signals from MAP kinase classes (JNK, ERK, and p38 MAP kinase) 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 phosphorylated forms. In addition, to further demonstrate a causal link between the activation of the p38 MAP kinase pathway and promotion of activity of 15-deoxy-PGJ_2 on the NGF-induced neurite extension of PC-12 cells, we employed the specific p38 kinase inhibitor SB203580. 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

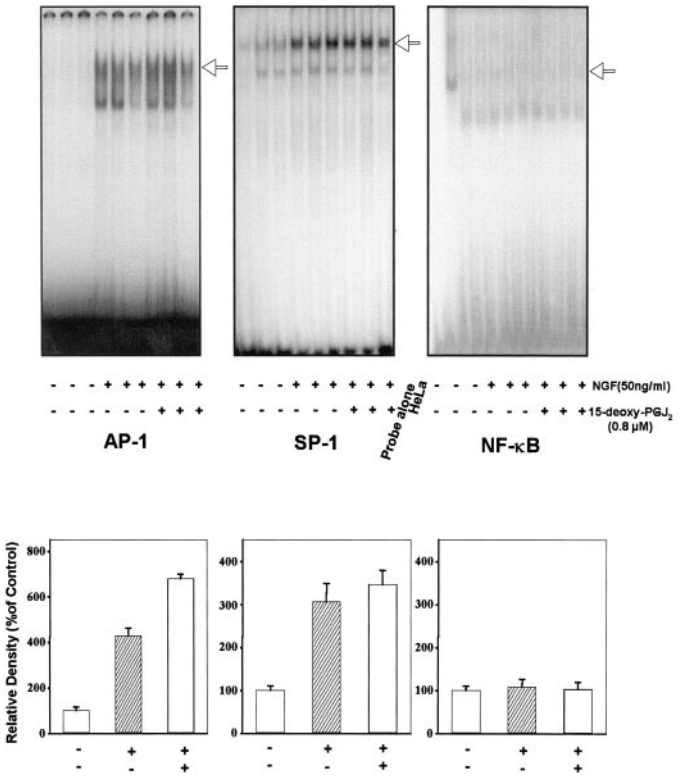

Fig. 7. Effect of 15-deoxy PGJ_2 on the NGF-induced activation of transcription factors AP-1, SP-1, and NF-κB. PC-12 cells cultured with 0.8 μM 15-deoxy PGJ_2 with NGF (50 ng/ml) for 30 min (AP-1) or 1 h (SP-1 and NF-κB). A DNA binding activity of AP-1, SP-1, and NF-κB was determined by gel mobility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

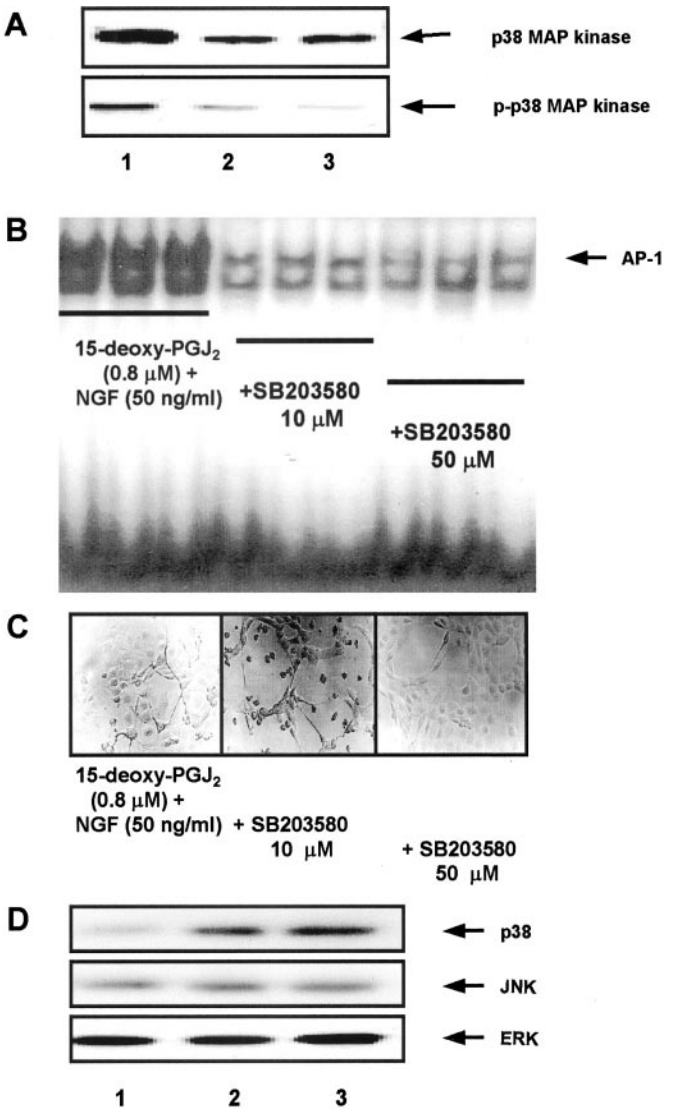

Fig. 8. Effect of SB 203580 on the enhancing effect of 15-deoxy PGJ_2 on NGF-induced activation of p38 MAP kinase (A) and AP-1 (B), and neurite extension (C), and the effect of PD98059 on the activation of the MAP kinase family. Cells were pretreated with 10 or 50 μM SB203580 or 50 μM PD98059 for 30 min and then exposed with NGF (50 ng/ml) and 15-deoxy PGJ_2 (0.8 μM) for 72 h to assay the neurite extension, 24 h to assay the expression of p38 MAP kinase, or 30 min to determine the activation of AP-1. Values are mean ± S.E. 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).

depend upon different stimuli. Similar to the effect of 15-deoxy-PGJ$_2$, morphogenetic protein-2, a member of the transforming growth factor-β 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 seems to be specific for 15-deoxy-PGJ$_2$ because a synthetic PPAR-γ agonist troglitazone (up to 5 μM) neither promotes NGF-induced neurite extension nor the neurofilament expression. Moreover, pretreatment of 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 cell differentiation is likely to depend on cell types. It was also found that similar range concentrations (about 0.5 to 1 μM) of 15-deoxy-PGJ$_2$ were 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$_2$ and 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 PGE$_2$, acting in their biological capacity 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 to promote activity of 15-deoxy-PGJ$_2$ on the NGF-induced neurite extension, and this activity of 15-deoxy-PGJ$_2$ on neurite extension is independent of PPAR-γ and GPCR. PPAR-γ–independent biological activities of 15-deoxy-PGJ$_2$ have 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; Harris et al., 2002; Ward et al., 2002). Moreover, 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 with 15-deoxy-PGJ$_2$ were also found (Vaidya et al., 1999; Li et al., 2001). Further study is needd to determine the precise mechanisms by which 15-deoxy-PGJ$_2$ activates p38 MAP kinase pathways in the promoting activity of 15-deoxy-PGJ$_2$ on the neurite extension. 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 a MAP kinase signaling pathway in colorectal carcinogenesis, a reverse differentiation response (Hsi et al., 2001). In addition, PPAR-γ was down-regulated via a MAP kinase-dependent pathway in the inhibition of adipocyte differentiation (Chan et al., 2001). Moreover, a 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-activated receptor γ-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 be correlated 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 a 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 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 PC-12 cells and other cells. A significant coincidental 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 also demonstrated. A low dose of lead (0.025 to 0.1 μM) activated basal and NGF (50 ng/ml)-induced SP-1 activation during PC-12 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 by the fact that, depending upon the nature and origins of cells and stimuli, transcription factors can be specifically activated. Conclusively, the present study shows that the promoting activity of 15-deoxy-PGJ$_2$ on the NGF-induced neurite extension of PC-12 cells may not be related to the activation of PPAR-γ or GPCR, but its promoting ability may be exerted through activation of the p38 MAP kinase in conjunction with AP-1 signal pathway.

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