PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA IS A NOVEL TARGET OF THE NERVE GROWTH FACTOR SIGNALING PATHWAY IN PC12 CELLS

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Running title: PPARγ: a novel target of the NGF pathway

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Peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear receptor superfamily, is subject to considerable interest because of its role in adipocyte differentiation, metabolic control, and anti-inflammatory action. PPARγ research in brain cells is presently focused on glial PPARγ because of its potential as a pharmacological target in the treatment of neurodegenerative diseases with an inflammatory component. In neurons, PPARγ function is far from clear and PPARγ agonists–dependent and independent effects on cell survival or differentiation have been reported. We used PC12 cells, a model widely used to study neuronal signaling, such as nerve growth factor (NGF)-induced differentiation and survival, or epidermal growth factor (EGF)-dependent cell proliferation, to dissect PPARγ's possible involvement in these pathways. We show that NGF, but not EGF, increases the transcriptional activity of PPARγ, and modulates the expression of this transcription factor. Since NGF signals through the tyrosine kinase (TrkA) NGF-receptor and/or the p75NTR receptor, we used rescue experiments with a PC12 cell mutant lacking TrkA to show that NGF-induced PPARγ activation is dependent of TrkA activation. Our results point out PPARγ as a novel target of the TrkA-mediated neuronal cell survival and differentiating pathway, and suggest a potential new inflammatory-independent therapeutic approach for pharmacological intervention in neurological disorders.
stimulation of embryonic midbrain cell differentiation into dopaminergic neuronal cells was reported for 15d-PGJ2 (12). In fact, many actions of 15d-PGJ2, which has been ascribed to PPARγ activation, have been shown to be mediated by inhibition of the NF-κB pathway (13,14). Because most PPARγ agonists have low affinity for PPARγ, relatively high agonist concentrations are usually used, making it difficult to truly assess PPARγ-dependent effects. Nevertheless, compelling evidence shows that PPARγ is indeed involved in cell differentiation in non-neural tissues, especially in adipocytes (5,6), and recently, endogenous 15d-PGJ2 have been shown to protect PC12 cells from nitrosative-induced cell death, and to increase PPARγ expression (15). To determine whether PPARγ could be involved in neural cell fate we studied its expression and transcriptional activity in cells exposed to the differentiating effect of nerve growth factor (NGF), using both a PPARγ agonist-dependent and non-dependent approach.

NGF signaling is one of the best characterized neurotrophic and differentiation-inducing pathways in nerve cells (recently reviewed in (16,17)). Neurotrophins, such as NGF, exert their effects by interacting with two structurally unrelated membrane receptors; the tropomyosin-related tyrosine kinase (Trk) receptors and the neurotrophin receptor (p75). Whereas p75 binds all neurotrophins, Trk family members exhibit ligand selectivity, NGF being the preferred ligand of TrkA (18). NGF induces an array of biological responses, mainly elucidated using the rat pheochromocytoma cell line PC12 (19), which expresses both NGF receptors (20,21), and differentiate into sympathetic-like neurons when exposed to NGF (19). A sustained activation of extracellular regulated MAP kinases (MAPK) ERK1 and ERK2 is involved in NGF-induced differentiation of PC12 cells, whereas epidermal growth factor (EGF) induces an opposite proliferative effect, paradoxically also via ERK MAPK, but through a transient activation of this pathway (22). We found that NGF, but not EGF, induces increased PPARγ transcriptional activity and modulates its expression in PC12 neural cells. Rescue experiments using a PC12 cell mutant lacking the TrkA receptor, but expressing p75, show that NGF activates PPARγ through the TrkA receptor. Our results suggest that PPARγ is a novel target gene of the NGF signaling pathway, opening new avenues of research on its potential participation in neuronal differentiation and/or survival pathways.

Materials and Methods

**Chemicals** - Chemicals and drugs, culture media and sera were obtained from Sigma (St. Louis, MO) and Gibco BRL (Paisley, UK), and rosiglitazone and carbaprostacyclin from Cayman Chemical (Ann Arbor, MI). Stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO) and added to culture medium (0.01% final DMSO concentration).

**Plasmids** - The reporter plasmid containing three tandem repeats of the peroxisomal proliferator response element (PPRE) from the acyl-CoA oxidase gene fused to the herpes virus thymidine kinase promoter upstream of the coding sequence for Luciferase was a kind gift of Dr. R.M. Evans, Howard Hughes Medical Institute, Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA. The expression vector containing only the coding sequence for luciferase under the control of the herpes virus thymidine kinase promoter (Clontech, Palo Alto, CA) was used as control. Co-transfections for reporter gene assays were carried out using 0.3 µg of reporter plasmid and 40 ng of a CMV-β-Gal vector (Clontech, Palo Alto, CA) for normalization. An expression vector containing full-length murine PPARγ cloned in the pCMX vector was also a kind gift of Dr. R.M. Evans. A PPARγ double mutant of Leu468 and Glu471 to alanine was generated by site-directed mutagenesis using QuickChange XL Site-Directed (Stratagene, La Jolla, CA), verified by sequencing, and inserted into the pCMX expression vector. Mutation to alanine of these two highly conserved amino acids in helix 12 of the ligand binding-domain of human PPARγ has been shown to result in a strong constitutive repression of PPARγ (23). An expression vector encoding the full-length rat TrkA cDNA under the control of the cytomegalovirus promoter was obtained from Dr. Philip A Barker, Montreal Neurological Institute, McGill University, Canada.
**Cell culture and transfections** - PC12 and nrr5 cells were maintained at 37°C and 5% CO2 in RPMI 1640 medium containing 10% horse serum (HS), 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin on rat tail collagen-coated plates. Transient transfections of PC12 cells lines were carried out using LipofectAmine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions; PC12 cells were grown until 60–70% confluence in 24-well collagen-coated plates and transfected with a total of 0.26 µg each of the pCMX expression vector alone, the wild type PPARγ-pCMX (WT), the dominant negative PPARγ-pCMX (DN) vectors, or co-transfected with 0.26 µg of both. In all transfections, cells were also co-transfected with the CMV-β-Gal (Clontech, Palo Alto, CA) vector for normalization. For NGF treatment, PC12 cells were cultured in RPMI 1640 medium containing 2% HS.

**Gel mobility shift assay** - PC12 cells were cultured in RPMI 2% HS medium for 3 h in the absence and presence of 100 ng/ml NGF. Nuclear extracts were then prepared as described in (24). In brief, the cultured cells were washed three times with iced-cold phosphate-buffered saline (PBS, pH 7.6), and the pellets were resuspended in 400 µl of cold buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 20% glycerol, 0.2 mM PMSF, and then centrifuged at 11000 x g for 4 min. The pellet were resuspended in 50 µl of a second buffer containing 20 mM HEPES, 20% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 0.2 mM PMSF. After centrifugation at 11000 x g for 6 min, the supernatant containing the nuclear proteins was isolated. Protein content in nuclear fractions was assessed using a microplate modification of the Bradford method (Bio-Rad Bulletin 1177, BioRad Lab., Richmond, CA). Electrophoretic mobility shift assay were performed as follows: 10 µg of nuclear protein was incubated in 9 µl total volume of binding buffer (10 mM Tris HCl, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl2, 4% glycerol, 0.5 mg/ml poly dl/dC) at 25°C for 15 min followed by another 20 min incubation with 100 µCi (γ-32P) ATP-labeled oligonucleotide containing a PPRE binding site. A double stranded 25-bp oligonucleotide probe of PPARγ was synthesized by SynGen (San Carlos, CA, USA) according to the consensus binding site for PPRE (5’GGAACTAGGTCAAAGGTCATCCCCT 3’). For the competition assay, 25x or 50x excesses of unlabeled double-stranded oligonucleotide of the PPRE binding site were used as specific competitor. For supershift assays, 2 µg of antibody against PPARγ was added 30 min prior to the addition of the oligonucleotide probe and incubated at 25°C. Anti-PPARγ was purchased from Affinity Bio-Reagents (Golden, CO. USA.). The DNA-protein binding complex was run on a 4% non-denatured polyacrilamide gel in 0.25X TBE (22.5 mM Tris-Borate and 0.5 mM EDTA) at 220 Volts for 1.5 h. Gels were autoradiographed using Kodak Biomax film.

**RNA extraction, Northern Blot, real time PCR, immunoblotting, and immunofluorescence studies** - Total RNA extraction has been described previously (25,26). For northern blots, total PC12 RNA samples were fractionated by denaturing agarose electrophoresis, and transferred to nylon membranes (Gene Screen Plus, NEN, Boston, MA, USA). Membranes were then cross linked by UV irradiation and pre-hybridized in ULTRAHyb (Ambion, Austin, TX, USA) 2 h at 42°C. Hybridizations were done using specific cDNA probes for PPARγ, and were radio labeled using Klenow amplification in the presence of 32P deoxythymidine. Hybridization was done at 48°C for 14 h. The probes were synthesized by PCR amplification of cDNAs. The following primers were used: PPARγ, forward: 5’-CCA-AGT-GAC-TCT-GCT-CAA-GT-3’, reverse: 5’-GAT-GGG-CTT-CAC-GTT-CAG-CA.-3’ (301 bp amplification product); rat GAPDH , forward: 5’-TCC-CTC-AAG-ATT-GTC-AGC-AA-3’, reverse: 5’-AGA-TCC-ACA-ACG-GAT-ACA-TT-3’ (308 bp amplification product; Gibco BRL Paisley, UK). For quantitative real-time PCR amplifications we used the Light Cycler instrument from Roche (Penzberg, Germany). The same primer sets used for northern blot were employed. The Light Cycler fast start DNA Master SYBR Green I was purchased from Roche (Penzberg, Germany). Thermal cycling conditions were as follows: for PPARγ, pre incubation with Fast
start Taq DNA polymerase (Roche) at 95°C for 10 min, followed by 35 cycles of amplification at 95°C for 15 s, 58°C for 1 s, 72°C for 12 s. For GAPDH, pre-incubation with Fast start Taq DNA polymerase at 95°C for 10 min followed by 25 cycles of amplification at 95°C for 15 s, 54°C for 1 s, 72°C for 12 s. The normalized expression of PPARγ with respect to GAPDH was computed for all samples. For immunoblotting analysis, cells were lysed in high-salt lysis buffer, as previously described (25,26). The protein concentration of each sample was determined using a protein assay kit (Bio-Rad, Hercules, CA), and 30 µg of protein was analyzed by SDS-polyacrylamide electrophoresis and then transferred to a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech, Bucking Hampshire, UK). PPARα, PPARβ and PPARγ antibodies were from Affinity Bio-Reagents (Golden, CO). The PPARγ antibody recognizes an epitope extending from amino acids M (284) to L (298). A second PPARγ antibody, directed to a different epitope, in the C-terminal region of PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA) was also used, in order to confirm the subcellular localization observed with the Affinity Bio-reagents antibody, and western blots. Both antibodies recognize a band of about 60 kDa in PC12 cells and rat adipose tissue, used as positive control. The 60 kDa band was not recognized by a PPARγ2 specific antibody (Affinity Bio-Reagents, Golden, CO). Unless otherwise stated, results using the Affinity Bio-Reagents anti-PPARγ antibody are presented. Anti β-actin was from ABCAM (Cambridge, UK). For immunofluorescence studies, PC12 cells plated on polylysine-coated covers were immunostained using PPARs antibodies followed with a secondary fluorescein-labeled antibody. Nuclei was stained using the DNA staining TO-PRO-3 (Molecular Probes, Eugene, OR). Covers were mounted and analyzed using a Zeiss Confocal microscope.

RESULTS

NGF induces PPARγ transcriptional activity - Using a gene reporter assay with a plasmid containing the isoform-shared PPAR response element (PPRE), we found that NGF induces PPAR transcriptional activity (Fig.1A). Transcriptional activity is maximal between 3 and 8 h, and starts decreasing at 8 h, time point at which a change in the luciferase product accumulation time-course slope is observed. To seek the PPAR isoform responsible for this effect, PC12 cells were transfected with the reporter gene and incubated for 16 h in the presence of PPAR agonists and antagonists (Fig. 1B). In the absence of NGF, only the PPARγ agonist rosiglitazone (RGZ), a TZD, induced a significant increase in transcriptional activity, which was suppressed by GW9662, a PPARγ antagonist. Wy14643 and carbaprostacyclin, agonists of PPARα and PPARβ, respectively, were without effect. When NGF was present, a 5-fold increase in PPAR basal transcriptional activity was observed, as expected from previous data; PPARα and PPARβ agonists were without effect, whereas a supplementary boost in transcriptional activity was observed in the presence of RGZ that was suppressed by GW9662. Similar results were obtained with citiglitazone, another TZD (not shown). The NGF effect was highly reproducible and observed in PC12 cells clones obtained from two other laboratories. These observations suggest that PPARγ is responsible for the NGF-induced PPAR transcriptional activity in PC12 cells.

To independently assess this conclusion we used PC12 cells transiently transfected with the PPAR reporter gene and plasmids coding for a PPARγ dominant negative protein (DN), a mouse wild type PPARγ (WT), or the vector alone (pCMX; see the Experimental section). To assess the effectiveness of transfections, we first determined the effect of increasing RGZ concentrations in PPAR transcriptional activity in transfected cells (Fig.1C). WT transfected cells presented increased RGZ-induced transcriptional activity in a RGZ concentration-dependent manner, when compared to non-treated cells. The effect was almost completely suppressed, and below control values, in DN transfected cells, whereas transfection with both WT and DN resulted in partial inhibition of the WT-induced transcriptional activity. As shown in Fig.1D, in the presence of NGF, cells transfected with the vector alone show the expected augmented transcriptional PPAR activity, when compared with controls in the
absence of NGF. Transcriptional activity is further and significantly increased by NGF in WT-transfected PC12 cells, whereas in DN-transfected cells the effect is almost abolished. Simultaneous transfection with both WT and DN represses the inductive effect of WT, and is significantly diminished when compared to controls. These observations strongly suggest that NGF induces increased transcriptional activity of PPARγ. To further validate this conclusion we used gel electro mobility shift assay (EMSA) to determine whether PPARγ indeed binds to DNA in nuclear protein extracted from control PC12 cells and cells cultured for 3 h in the absence and presence of 100 ng/ml NGF (Fig. 2). Binding of PPAR to DNA was observed in nuclear extracts from control cells and was suppressed in the presence of unlabelled oligonucleotide of PPRE. NGF treatment resulted in an apparent increase of the PPAR DNA binding activity that was reproducible, but it was difficult to quantify because there are not obvious control for nuclear protein loading. The DNA binding band was supershifted with anti-PPARγ antibody indicating that the DNA–protein complex is PPARγ. This result show that PPARγ binds to DNA in both control and 3h NGF-treated cells, when transcriptional activity start increasing, supporting our previous conclusion.

NGF modulates PPARγ expression - Next, we examined whether NGF induces changes in PPARγ expression. Since it has been previously reported that PC12 cells have no detectable PPARγ by western-blot (9), we used two different antibodies directed against different epitopes of the PPARγ protein (see experimental section); both antibodies detected a 60 kDa band in PC12 cells and in adipose tissue, used as positive control (not shown). This divergence might be due to different experimental conditions, particularly the culture conditions, or to different sources or batches of PPARγ antibodies. It is worth noting that PPARγ has been recently detected by western blot in PC12 cells (15).

A transient decrease in PPARγ protein between at 8- and 16 h after NGF addition was observed (Fig. 3A, upper panel; quantification in lower panel), which is consistent with the reported degradation of this receptor upon activation (27); PPARγ mRNA expression does not change significantly after 8 h NGF treatment, but start increasing at 16 h up to a maximum of about 3-fold in 24 and 48 h NGF-treated PC12 cells, as determined by northern blot (Fig.3B, upper panel) or real time PCR (Fig.3B, lower panel). The NGF-induced PPARγ mRNA increased expression follows down-regulation of PPARγ protein and maximal transcriptional activation. No changes were observed in PPARα or PPARβ mRNA expression upon NGF treatment (not shown). To determine whether these changes are reflected in PPARγ intracellular distribution, we used immunofluorescence against PPARγ (Fig. 4). In untreated and 3 h NGF-treated PC12 cells, PPARγ presents a cytoplasmic-nuclear distribution, while in 8 h NGF-treated cells, the PPARγ signal has partially disappeared from the nucleus. After 16 h NGF treatment there is almost no PPARγ presence in the nucleus., whereas in 24 and 48 h NGF-treated cells, PPARγ is present mostly in the cell body, and its presence in the nucleus has partially recovered, when compared with 16 h NGF-treated cells. The fact that the luciferase product in the reporter gene assay (previously shown in Fig.1A) still accumulates after 16 h NGF- treatment may be explained in terms of a delay between mRNA and protein synthesis. Immuno-fluorescence experiments agree with the down regulation of PPARγ protein, as determined by western blot, and with the increased mRNA PPARγ expression that follows protein down-regulation. Similar PPARγ intracellular localization was observed using antibodies directed against a different epitope of the PPARγ protein (see the experimental section). In contrast to PPARγ, no apparent changes were observed for both PPARα and PPARβ, which showed a clear nuclear localization in NGF-treated cells, as well as in untreated cells, for the 48 h treatment period (not shown). As a whole, these results strongly suggest a cross-talk between the NGF and PPARγ signaling pathways.

NGF-induced PPARγ transcriptional activity is dependent on the TrkA receptor - To investigate whether TrkA or p75 were involved in activating PPARγ, we used nmr5 cells, a PC12 mutant deficient in TrkA that does not differentiate in response to NGF, but that
expresses p75 (28). In agreement with published data (28), nnr5 cells transfected with a plasmid containing the full length TrkA receptor rescued NGF-induced neurite outgrowth (Fig. 5A). PC12 and nnr5 cells express similar PPARγ levels (Fig.5A). As shown in Fig. 5C, in nnr5 cells RGZ but not NGF induces PPAR transcriptional activity, which is suppressed by a PPARγ antagonist, suggesting that TrkA is necessary for NGF-induced PPARγ transcriptional activity. Moreover, TrkA-transfection of nnr5 cells results in increased basal PPAR transcriptional activation and rescues the NGF-dependent effect. RGZ induces an additional boost of luciferase activity, which is abrogated by a PPARγ antagonist as occurs in PC12 cells. These results show that NGF-mediated TrkA activation induces PPARγ transcriptional activity.

DISCUSSION

In this study we show that NGF increases PPARγ transcriptional activity in PC12 cells, strongly suggesting that this nuclear transcription factor is a novel target of the NGF signaling pathway. Two independent methods, using PPARγ agonists and antagonists, or PPARγ over-expression or repression, and also EMSA analysis sustain this conclusion. Disappearance of PPARγ from the nucleus between 8 and 16 h after NGF treatment is also consistent with this conclusion, with the down-regulation of PPARγ protein, as detected by western blot, and with the reported degradation of this receptor upon activation (27). Furthermore, rescue experiments with nnr5 cells lacking the TrkA receptor show that NGF-induced TrkA activation is essential for increasing PPARγ transcriptional activity.

Two main questions arise from our observations: the molecular mechanism by which NGF induces increased PPARγ transcriptional activity and the identity of the PPARγ-controlled genes. The delayed increase in PPARγ mRNA induced by NGF, which follows protein down regulation, strongly suggests that NGF induces the transcriptional activity of pre-existent PPARγ. The disappearance of nuclear PPARγ after 16 h NGF treatment further supports this view, as already pointed out. It is tempting to speculate that TrkA activation results in the generation of endogenous PPARγ ligands. In PC12 cells, TrkA activation induces a rapid release and catabolism of arachidonic acid through the lipoxygenase pathway (29), which is required for neurite outgrowth (30). Several eicosanoids, products of lipoxygenase action, have been proposed as PPARγ ligands, (31) a view supported by the requirement of this enzyme in some PPARγ responses in vitro (32). Moreover, it has been recently shown that nitrosative-induced stress in PC12 cells results in elevated production of endogenous 15d-PGJ2 and increased PPARγ expression (15).

Alternatively, phosphorylation-dephosphorylation mechanisms could also play a role in the NGF-induced PPARγ transcriptional activity. PPARγ is phosphorylated by ERK2 and other MAPKs (33), resulting in either inhibition or activation of PPARγ transcriptional activity (34,35). In human urothelial cells, PPARγ agonist-induced cell differentiation is maximal under conditions in which the EGF receptor signaling pathway is inhibited and results from dephosphorylation of PPARγ and its translocation to the nucleus (36). On this basis, it is reasonable to hypothesize that in NGF-activated PC12 cells, the EGF signaling pathway is inactive and activation of PPARγ by dephosphorylation might occur. Both possibilities can be tested experimentally and deserve further research.

Concerning the identity of PPARγ-controlled genes, activation of ERK MAPKs is essential for PC12 cell differentiation and survival induced by NGF, but also for EGF-induced cell proliferation (22). The question of how different growth factors elicit distinct biological outcomes through common signal transduction elements has provoked considerable interest resulting in controversial literature, even though it is now accepted that in PC12 cells, cellular responses are determined by the duration of ERK activation (22,37,38). NGF induces sustained activation of this signaling pathway, resulting in cell differentiation and survival, whereas EGF produces only a short-lived activation, inducing cell proliferation. Since we show that NGF but not EGF, induces increased PPARγ transcriptional activity, our
results add a new significant difference between the signaling pathways activated by these two opposed growth factors, and suggest that PPARγ signaling could be controlling genes related to cell differentiation and/or cell survival in neurons. In agreement with this proposal, we have found that activation of PPARγ by three different thiazolidinediones protects rat hippocampal neurons against β-amyloid-induced neurodegeneration and from the excitotoxic β-amyloid-induced rise in bulk-free Ca2+. Further, PPARγ activation results in inhibition of glycogen synthase kinase-3β, a target gene of the NGF survival pathway (Inestrosa et al., Exp. Cell. Res., in press).

At present, research interest in PPARγ function in brain cells is mainly centered in glia. PPARγ activation in glial cells, resulting in diminished production of glial-derived pro-inflammatory molecules, has been involved in the protective effect of TZDs and anti-inflammatory drugs in several neurodegenerative diseases (1,3,39). This assumption is further sustained on the fact that PPARγ agonist inhibit β-amyloid-stimulated proinflammatory responses and neurotoxicity (1). On the other hand, Alzheimer’s disease patients, treated for type 2 diabetes with rosiglitazone, showed improved verbal memory after six months of treatment (40), and pioglitazone, a TZD, prevents experimental autoimmune encephalomyelitis (41).

Our data suggest the possibility of a direct effect of neuronal PPARγ activation in modulating neuron survival and/or differentiation. Since NGF is also a target in the treatment of neural degeneration (42), understanding the molecular mechanism underlying the cross-talk between the NGF and PPARγ signaling pathways in neurons could have important clinical implications in the pharmacological approach to neurodegenerative diseases.

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FIGURE LEGENDS

Fig. 1. NGF induces PPAR\gamma transcriptional activity in PC12 cells. Results are presented as mean ± SD of at least three independent experiments. (*) P<0.01 or less; student’s t test. A) NGF induces PPAR transcriptional activity. PC12 cells were transfected with a PPRE-containing luciferase-reporter plasmid (PPRE-Tk-luc). NGF (100 ng/ml) or vehicle was added at time 0 (arrow). In all incubations, controls were run with PC12 cells transfected with a plasmid lacking PPRE but containing the luciferase reporter.
In all cases luciferase activity was well below control values either in the absence or presence of NGF, as illustrated in the inset for the 24 h determination (RLA: relative specific activity). B) Effect of PPAR agonists and a PPARγ antagonist on NGF-induced PPAR transcriptional activity. PC12 cells transfected with PPRE-Tk-luc were cultured with or without NGF (100 ng/ml) for 12 h, and then supplemented with either the vehicle alone, the PPARα agonist WY-14643 (50 µM); the PPARβ agonist carbaprostacyclin (CPC; 50 µM); the PPARγ agonist rosiglitazone (RGZ; 1 µM); the PPARγ agonist GW9662 (10 µM) or RGZ plus GW9662, during 8 h. EGF addition (100 ng/ml) had no effect on transcriptional activity. Lower EGF concentrations (10 and 50 ng/ml) were also without significant effect (not shown). C) RGZ concentration-dependence of PPARγ transcriptional activity in transiently transfected PC12 cells. Cells were transfected with the PPRE-Tk-luc, and the pCMX expression vector alone (pCMX; ●); the wild type PPARγ-pCMX (WT; ○), the dominant negative PPARγ-pCMX vector (DN; Δ), or co-transfected with both the WT and DN-PPARγ-pCMX (WT+DN; □). Transcriptional activity is expressed relative to the cells transfected with the vector alone, in the absence of RGZ. D) NGF-induced PPAR transcriptional activity is increased in PC12 cells transiently over-expressing PPARγ (WT), and is reversed by co-transfection with a PPARγ dominant negative (DN). Cells were cultured with or without NGF for 16 h.

**Fig. 2.** PPARγ binds to DNA in control and NGF-treated PC12 cells. PC12 cells were cultured for 3 h in the absence and presence of NGF and DNA binding activity of PPAR was determined by gel mobility shift assay (EMSA) as described in the experimental section. Lane 1: control cells; Lanes 2 and 3: competition assay with unlabelled oligonucleotides of PPRE (25 and 50 excess, respectively). Lane 4: only probe; Lane 5 and 6: PC12 cultured for 3 h in the absence (lane 5) and presence (lane 6) of 100 ng/ml NGF. An apparent increase in bound PPAR is observed in NGF treated cells. Lane 7 and 8: supershift assay in the presence of 2 µg of PPARγ antibody for PC12 cells cultured for 3 h in the absence (lane 7) or presence (lane 8) of NGF (100 ng/ml). A similar result was obtained in three different set of experiments.

**Fig. 3.** NGF modulates PPARγ expression. A) Western blot against PPARγ from cells incubated for 3, 8, 16, 24, and 48 h with NGF are shown (upper panel). Quantification of bands, normalized with β-actin, is shown in the lower panel. B) NGF enhances PPARγ mRNA expression in PC12 cells. RNA extracted from cells incubated for 3, 8, 16, 24 and 48 h with NGF were submitted to northern blot against PPARγ (upper panel), or to real time PCR using specific PPARγ primers (lower panel). PPARγ mRNA increased 2-3 folds at 24h and 48h in both experimental procedures. Similar results were obtained in two other northern blot experiments. Results are presented as mean ± SD of three independent experiments. * p< 0.01 or less (student’s t test), for real time PCR.

**Fig. 4.** NGF-induced PC12 cell differentiation modifies PPARγ intracellular distribution. PC12 cells were cultured for 0, 3, 8, 16, 24, and 48 h in the presence of NGF (100 ng/ml), fixed and processed for immunofluorescence with antibodies against PPARγ followed with a secondary fluorescein-labeled antibody, and analyzed by confocal microscopy. Nuclei were stained with To-Pro-3, as described in the experimental section. Bar represents 20 µm. Co-localization (merge) is also presented. Representative pictures of PC12 cells cultured in the presence of NGF (100 ng/ml) during the indicated time periods are shown. In control cells, PPARγ is present in both the cytoplasm and nuclei. After 8 h, and more evident after 16 h, in the presence of NGF, most cells present only cytoplasmic or peri-nuclear staining, whereas in 24 and 48 h treated-cells PPARγ is present in the cell body and its presence in the nuclei is partially recovered, when compared to 8 or 16 h NGF-treated cells. Similar results were obtained with a PPARγ antibody directed to a different epitope of the PPARγ protein (Santa Cruz Biotechnology; not shown).
Fig. 5. TrkA transfection of a PC12 cell mutant deficient in TrkA (nnr5 cells) rescues NGF-induced PPARγ transcriptional activity. A) TrkA transfection rescues NGF-induced neurite outgrowth in nnr5 cells. Cells were transfected with a full length TrkA-expressing plasmid (+TrkA) or the vector alone (-TrkA), and cultured 48 h with NGF (100 ng/ml). Representative pictures are shown. B) PC12 and nnr5 cells express similar PPARγ levels, as determined by western blot. Relative PPARγ expression of 0.61 ± 0.16 and 0.73 ± 0.14 was determined respectively, for nnr5 and PC12 cells (mean ± SD of three independent experiments). C) RGZ but not NGF induces PPAR transcriptional activity in nnr5 cells, and effect which is suppressed by the PPARγ antagonist GW9662 (GW). TrkA transfection rescues NGF-induced transcriptional activity, which is further increased by RGZ, and sensitive to the PPARγ antagonist GW9662 (GW). Results are presented as mean ± SD of three independent experiments. * p<0.01 or less (student's t test). ** p<0.01 from NGF alone.
FIGURE 1

A

Relative Luciferase activity

- NGF

+ NGF

Time (h)

0 3 8 16 24

B

Relative Luciferaser activity

VEH

Wy14643

CPC

RGZ

RGZ + GW

GW

- NGF

+ NGF

C

Relative Luciferase activity

WT

WT+DN

pCMX

DN

Rosiglitazone (µM)

0 0.5 1.0

D

Relative Luciferase activity

Vector

WT

DN

WT+DN

0 5 10

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FIGURE 2
FIGURE 3

A
Upper panel: Western blot

PPARγ
β Actin
Time (h) 0 3 8 16 24 48

Lower panel: Western blot quantification

Relative area

Time (h) 0 3 8 16 24 48

B
Upper panel: Northern blot

Relative expression

Time (h) 0 3 8 16 24 48

Lower panel: Real time PCR

Relative expression

Time (h) 0 3 8 16 24 48
FIGURE 4

| Time (h) | PPARγ | Nuclei | Merge |
|----------|-------|--------|-------|
| 0        | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| 3        | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| 8        | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| 16       | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| 24       | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| 48       | ![Image](image16) | ![Image](image17) | ![Image](image18) |
FIGURE 5

A

- TrkA

+ TrkA

B

PC 12

nrr5

PPARγ

β Actin

C

Vector

NGF

RGZ

RGZ+GW

GW

Vector

NGF

RGZ

RGZ+GW

GW

Relative luciferase activity

0 6 12
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J. Biol. Chem. published online January 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M409447200

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