Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons

Aiqun Li,*† Hong Guo,* Xiaoying Luo,*† Jiansong Sheng,*† Shuo Yang,*† Yanqing Yin,* Jianwei Zhou,‡ and Jiawei Zhou*,†,1

*Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences and †Graduate School of the Chinese Academy of Sciences, Shanghai, China; and ‡Institute of Public Health, Nanjing Medical University, Nanjing, China

SPECIFIC AIMS

Apomorphine (APO), a potent D1/D2 dopamine receptor agonist, is currently in use as an antiparkinsonian drug. We investigated the molecular mechanism underlying APO-induced survival-promoting activity on nigral dopaminergic neurons in vitro. The present study was designed to investigate the effects of APO on FGF-2 expression and regulation in astrocytes, and furthermore, to identify the underlying signaling mechanism for the effects.

PRINCIPAL FINDINGS

1. Treatment of astrocyte cultures with APO enhanced biosynthesis and extracellular release of FGF-2, which contributed to elevated survival of TH-positive neurons

The conditioned medium (CM) derived from striatal astrocytic cultures increased the number of tyrosine hydroxylase (TH)-positive neurons in the recipient mesencephalic neuronal cultures in a dose-dependent manner. Western blot analysis on the concentrated CM, using a FGF-2 polyclonal antibody, which recognized four FGF-2 isoforms corresponding to higher MW forms (HMW, 24, 22.5, 22 kDa) and lower MW form (LMW, 18 kDa) FGF-2, revealed that in the CM of striatal astrocyte cultures treated with APO for 6 h, the HMW- and LMW FGF-2 were detectable at 18 h and 24 h following the treatment. Conversely, FGF-2 was not detectable in the CM of untreated astrocytic cultures at any time points examined. Incubation of APO-induced CM with FGF-2 antibody (Ab) dramatically attenuated the increase in the number of TH-positive neurons by 55.9%. These data suggest that FGF-2 may be the major contributor that mediated the APO-induced survival-promoting effect.

2. Activation of DA receptor is responsible for APO-modulated FGF-2 biosynthesis

We next asked whether DA receptor activity was required for APO-mediated induction of FGF-2. Treatment of the striatal astrocytes with a variety of concentrations of SAPO (0.02–20 μM), a derivative of R-APO lacking activity of DA receptor agonist, for 6 h did not significantly alter the concentration of FGF-2 biosynthesis. However, striatal astrocytic cultures exposed to the DA receptor agonists SKF-38393 (10 nM), or quinpirole (50 μM) for 6 h showed remarkably increased levels of FGF-2 isoforms. Moreover, preincubation with either D1 receptor antagonists SCH23390 (10 μM), SKF83556 (10 μM) or D2 receptor antagonist haloperidol (1 μM), respectively, significantly blocked APO-stimulated FGF-2 expression. Combination of D1 and D2 receptor antagonists partially abolished APO-stimulated FGF-2 expression. Our data indicated that both D1 and D2 DA receptor is involved in APO-modulated FGF-2 biosynthesis.

3. APO modulates FGF-2 biosynthesis via cAMP and PKC, not phosphoinositide 3-kinase

To determine the mechanism of the DA receptor-mediated FGF-2 induction, we blocked key components of several signal transduction pathways while costimulating the receptors with both D1- and D2-selective agonists. Exposure of the astrocytic cultures to APO resulted in 4.8-fold increase in the concentration of phosphorylation of PKA at 30 min following the treatment (Fig. 1A, B), indicating that APO indeed stimulates the PKA activation. Addition of forskolin (5 μM), into the astrocytic cultures led to an average fourfold

---

1 Correspondence: Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yueyang Rd., Shanghai, 200031, P. R. China. E-mail: jwzhou@sibs.ac.cn doi: 10.1096/fj.05-5510fje
increase of FGF-2 compared to the untreated (Fig. 1C, D). Conversely, pretreatment of the cultures with either hemagglutinin (HA)-100 (30 μM, a PKA/PKC inhibitor) or KT5720 (50 μM, a PKA inhibitor) completely abrogated APO-stimulated FGF-2 expression (Fig. 1C, D). These data suggest that cAMP/PKA signaling pathway play an important role in APO-induced FGF-2 up-regulation.

Moreover, exposure of serum-starved striatal astrocytic cultures to APO phosphor-activated MAPK at all time points (Fig. 1E). Treatment of the astrocytic culture with either GF109203X (2 μM, a PKC inhibitor) or PD98059 (50 μM, a MAPK inhibitor) prior to the addition of APO markedly reduced the up-regulation of FGF-2 protein (Fig. 1C, D). These results suggest that MEK/MAPK signaling pathway is also important for APO-induced FGF-2 up-regulation. In contrast, APO did not alter the levels of phospho-Akt in astrocytic cultures for up to 6 h (Fig. 1E), implicating that APO does not modulate Akt activity.

4. The D1 receptor activates the cAMP/PKA pathway, whereas the D2 receptor mediates the MEK/MAPK pathway following APO stimulation

As shown in Fig. 2, DA receptors and two signaling pathways (cAMP/PKA and MEK/MAPK) were activated contributing to APO-modulated FGF-2 induction. The downstream signaling pathway to corresponding subtype of DA receptor was thus determined. The astrocytic cultures were treated with combination of D2 receptor antagonist haloperidol with APO. Notably, the phosphorylation concentration of PKA was increased, while phosphorylation levels of MAPK were not markedly altered. Likewise, combinational treatment of the astrocytes with D1 receptor antagonist, SCH23390 or SKF83566, and APO produced a significant increment of phosphorylated MAPK levels, but without marked alteration in levels of phosphorylated PKA. Indeed, treatment of astrocytes with SKF38393, a D1 receptor agonist, increased phosphorylation concentration of PKA without marked influence on concentration of phosphorylated MAPK. Moreover, exposure to quinpirole had a completely opposite effect compared with SKF38393. Furthermore, in the presence of APO, treatment of the cultures with either PD98059 or GF109203X abrogated phosphorylation concentration of MAPK having little effect on phosphorylation levels of PKA. Taken together, our data suggest that D1 receptor uses the cAMP/PKA pathway in the presence of APO, whereas the MEK/MAPK pathway is preferably activated by D2 receptor (Fig. 2).
ence of forskolin, which stimulates intracellular concentration of cAMP, APO failed to increase phosphorylation of MAPK, suggesting inhibitory effect of D1 receptor-associated downstream signaling pathway on MEK/MAPK cascade. In contrast, combinational treatment with KT5720 and APO abrogated this inhibitory effect, suggesting that inhibition of MAPK phosphorylation by PKA, but not cAMP, is critical to the inhibitory effect of D1 receptor-associated pathway on D2 receptor pathway (Fig. 2).

CONCLUSIONS AND SIGNIFICANCE

A major finding in the present study is that APO treatment changes subcellular localization of HMW FGF-2 and enhances release of both HMW- and LMW FGF-2 into the cultured medium. The present study has shown that the most abundant forms of FGF-2 in the untreated astrocytes in cultures are the HMW isoforms. However, more HMW FGF-2 was detected in the cytoplasm and the CM of APO-treated astrocytes, suggesting that APO affects FGF-2 release in addition to its synthesis. The released HMW- and LMW FGF-2 may together contribute to promote survival of DA neurons.

There is controversy as to which intracellular signaling pathways correspond to D1 and D2 receptors in neuronal cells. A recent study has shown that D1 receptor induces phosphorylation of PKA, while D2 receptor induces phosphorylation of MAPK and cAMP-responsive element binding protein (CREB) in dopaminergic neurons. In contrast, D1 receptor also can phosphorylate and activate MAPK and CREB. However, very little information is available on the roles of DA receptor-mediated signaling pathways in astroglial cells. We demonstrate here in the present study that PKA was phosphorylated and activated preferentially by D1 receptor following treatment of either APO or selective D1 receptor agonist SKF38393. Similarly, MAPK is a target found to be activated by D2 receptor in response to APO stimulation in this study. We conclude that cAMP/PKA and MEK/MAPK controls the inductive effect of APO on FGF-2 in astroglia by distinctly different mechanisms from that used in neuronal cells. It is plausible that activation of cAMP/PKA signaling pathway enhance FGF-2 gene expression via the activation of cis-regulatory elements located in the FGF-2 promoter. Because APO administrations increase concentration of phosphorylated CREB, APO may thus affect the transcription of the FGF-2 gene by inducing the binding activity of transcription factors to cis-elements located in the FGF-2 promoter. Importantly, we found that the activation of PI-3K pathway did not contribute to FGF-2 induction in this study.

The induction of FGF-2 in astrocytes evoked by APO may be a common mechanism elicited by catechol-derived compounds. Our data imply the dual actions of DA, a neurotransmitter with property of DA receptor agonist, i.e., mediation of neurotransmission and modulation of FGF-2 and other neurotrophic factors expression in astrocytes. Both effects are highly dependent on activation of DA receptors. Dysfunction of DA nigrostriatal pathway in PD leads to deregulation of FGF-2 in this neural pathway, which, in turn, affects the survival and function of nigral DA neurons. On the basis of these considerations, we can speculate that APO, by restoring appropriate levels of endogenous trophic factors, may prevent loss of midbrain DA neurons. The clinical benefits of APO administration can be interpreted as consequence of sustained agonism on DA receptor. It may also result from either APO-induced neuroprotection or modulation of synaptic activity. This therapeutical strategy may open up a new avenue of research for preventing or delaying the onset/progression of PD.
Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons

Aiqun Li,*† Hong Guo,* Xiaoying Luo,*† Jiansong Sheng,*† Shuo Yang,*† Yanqing Yin,* Jianwei Zhou,‡ and Jiawei Zhou*†,†,1

*Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences and †Graduate School of the Chinese Academy of Sciences, Shanghai, China; and ‡Institute of Public Health, Nanjing Medical University, Nanjing, China

ABSTRACT Apomorphine (APO), a potent D1/D2 dopamine receptor agonist, is currently used as an antiparkinsonian drug. We have shown previously that APO stimulates synthesis and release of multiple trophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), in both mesencephalic and striatal neurons, thereby effectively preventing dopaminergic neuron loss in vitro. The present study was designed to investigate the effects of APO on fibroblast growth factor-2 (FGF-2) expression and regulation in astrocytes, and furthermore, to identify signaling mechanisms underlying these effects. Here, we show that FGF-2 expression is robustly induced in cultured astrocytes in response to APO. FGF-2 expression was proportional to APO concentration and time-dependent. Conversely, treatment with S-APO, a derivative of R-APO lacking DA receptor agonist activity, did not alter FGF-2 levels. APO treatment resulted in enhanced cytosol FGF-2 immunoreactivity, export of high MW forms of FGF-2 to the cytoplasm from the nucleus and increased extracellular release of FGF-2. Interestingly, both high and low MW forms of FGF-2 were detectable in conditioned medium of APO-treated cultures. This APO-induced effect was correlated with activation of D1 and D2 receptors, as it could be either mimicked by dopamine receptor agonists (SKF38393, quinpirole) or partially blocked by antagonists (SCH23390, SKF83566, haloperidol). Activation of the D1 receptor preferentially increased PKA activity, whereas activation of the D2 receptor only promoted phosphorylation of MAPK. Importantly, APO-modulated FGF-2 expression was independent of Akt/phosphoinositide 3-kinase signaling. These data suggest that APO can enhance biosynthesis and release of FGF-2 through activation of dopamine receptors in striatal astrocytes. Both cAMP/PKA and MEK/MAPK signaling cascades are major steps mediating this process.—Li, A., Guo, H., Luo, X., Sheng, J., Yang, S., Yin, Y., Zhou, J., and Zhou, J. Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons. FASEB J. 20, E574–E586 (2006)

Key Words: striatum • neurotrophic factor • PD • signal transduction

Parkinson’s disease is a common movement disorder characterized by selective and progressive loss of nigral dopaminergic (DA) neurons (1). Although currently there is no cure for this devastating disease, several therapies provide effective relief from the symptoms. Levodopa therapy remains the gold standard of symptomatic treatment for PD. However, most patients develop levodopa-associated motor fluctuations and dyskinesia within a few years of treatment and ultimately develop disability due to difficulty with balance and cognition. Currently, there is great interest in therapeutic use of dopamine agonists in addition to the use of levodopa, a combination that might result in a better long-term outcome. Apomorphine (APO), a mixed D1/D2 dopamine receptor agonist, provides symptomatic relief by directly stimulating postsynaptic striatal DA receptors, although it causes gastrointestinal side effects. The mechanism of APO action and why it is effective against PD remain poorly understood. For many years, APO has been known to preferentially control excessive apoptotic rates and reduce excess nitric oxide (NO), therefore functioning as a potent antioxidant (2) and affecting DA function.

To address mechanisms underlying the neuroprotective action of APO, our previous studies demonstrated that APO stimulates synthesis of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in rat mesencephalic, as well as striatal neuronal cultures (3). APO has also been found to increase neuronal and astroglial expression of neurotrophic factors, including nerve growth factor (NGF) and GDNF (4). These factors released from both neurons and astrocytes can efficiently protect DA neurons against degeneration both in vitro and in vivo.

1 Correspondence: Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yueyang Rd., Shanghai 200031, P. R. China. E-mail: jwzhou@sibs.ac.cn
These observations indicate that increased synthesis and release of trophic factors may be important mechanisms mediating APO’s antiparkinsonian effect, in addition to its antioxidative activity as a free-radical scavenger (5). However, the modulatory effect of APO on other neurotrophic factors in astrocytes is unknown.

One molecule that could play an important role in this context is fibroblast growth factor-2 (FGF-2). FGF-2 belongs to a large family of growth factors, which currently comprises at least 23 structurally related members (6). FGF-2 protein is predominantly synthesized by astrocytes, whereas other family members, e.g., FGF-5, FGF-8, and FGF-9, are primarily synthesized by neurons (7). FGF-2 is an important modulator of cell growth and differentiation under both physiological and pathological conditions, such as in embryogenesis, morphogenesis, wound healing, cardiovascular disease, and neovascularization (8, 9). It has also been shown that FGF-2 exerts a potent neuroprotective action on nigral DA neurons (10, 11).

Astrocytes, the largest cell population in the central nervous system, play multiple roles in maintaining homeostasis of the neural extracellular environment. Astrocytes are involved in provision of metabolic substrates for neurons, maintenance of the extracellular ionic environment and pH and uptake of neurotransmitters. They also play important roles in creation and maintenance of the blood-brain barrier. In addition to these common features, earlier studies indicate that the biological function of astrocytes varies from region to region in the nervous system (12). This difference may be associated with diverse characteristics of regional astrocytes and distinct interactions between astrocytes and local neurons. The expression of DA receptors on astrocytes may contribute to this diversity. Accumulated evidence suggests that both dopamine D1/D2 receptors are expressed in striatal and mesencephalic astrocytes (13–15). Their biological function in these cells, however, is not fully understood.

The present study was designed to investigate the effects of APO on FGF-2 expression and regulation in astrocytes, and furthermore, to identify the underlying signaling mechanisms mediating these effects. We demonstrate here that treatment of astrocytes with APO results in increase in FGF-2 expression, both at the mRNA and protein levels, in striatal astrocytes. These results provide a potential mechanism for the antiparkinsonian action of APO.

**MATERIALS AND METHODS**

**Mesencephalic cell culture**

Rat fetal ventral mesencephalon (VM) were obtained at embryonic day 14 (E14, where E0 is the day of the vaginal plug), and immediately afterward, rats were killed with an overdose of pentobarbital sodium. VM cultures were prepared as described previously (5). Cells were incubated at 37°C in a 95% air-5% CO₂ humidified atmosphere and maintained for 24 h after switching to serum-free medium. All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Primary astrocytic culture**

Astrocytes were prepared from the brains of 1- to 2-day-old neonatal Sprague-Dawley rat pups, as described previously by Menet et al. (16). Briefly, the striata were trypsinized, dissociated by gentle trituration, and plated at a density of 5 x 10⁷ cells per 75 cm² flask (Corning, Corning, CT, USA) in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 (1:1 v/v, Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated FBS. Cells were maintained in complete culture medium for 7 days. Between the 8th and 12th days, cultures were shaken to remove the top layer of cells sitting over the astroglial monolayer to yield mainly type-1 astrocytes with a flat morphology.

Before experimental treatments, cultures of astrocytes were passaged twice. Cells were allowed to reach 90% confluence. To investigate the effects of APO and other compounds on FGF-2 expression, astrocytes were maintained in serum-free DMEM/F12 and treated with various drug concentrations for the indicated incubation times. Nontreated cells were included as controls in all experiments.

**Preparation of conditioned medium**

To prepare conditioned medium (CM), postnatal striatal and nigral astrocytes were plated on a poly L-lysine-coated 35 mm-diameter dishes (Nunc, Roskilde, Denmark) and grown in DMEM/F12 supplemented with N2 (Invitrogen, Carlsbad, CA, USA) at a cell density of 5 x 10⁷/cm² for 3 days. During that time, cultures were treated twice with APO. The CM was collected, centrifuged at 10,000 g for 5 min, and stored at −20°C until use.

**Experiment treatment**

R(−)-apo (referred to here as “APO”), S(+)-apo, forskolin (Sigma, St. Louis, MO, USA), and DA receptor agonists (SKF-38395, quinpirole, Sigma) were added to cultures starting the day after plating out the culture (day 0). DA receptor antagonists (haloperidol and SCH-23390, Sigma) and hemagglutinin (HA)-100, LY294002, PD98059, KT5720 and GF109203X (Sigma) were applied to cultures at least 2 h before APO was added. Antibodies against BDNF, NT-3 (a kind gift of X. F. Zhou, Flinders University, Adelaide, Australia), or GDNF (PeproTech, London, UK) were applied to VM cultures 4 h before APO-induced CM was added.

**Protein precipitation with acetone**

Striatal astrocytes were cultured in DMEM/F12 without serum and containing 2 μM APO for 6 h, 18 h, and 24 h. After incubation, media were prepared by centrifugation at 10,000 g for 5 min. Proteins were precipitated by using 4 times the sample volume of prechilled acetone and maintaining samples at −20°C for 30 min, followed by centrifugation at 15,000 g for 15 min. Subsequently pellets were subjected to Western immunoblot analysis using FGF-2 antibody (Ab).

**Subcellular fractionation**

Cell cultures were washed with ice-cold D-Hanks buffer twice and scraped in 1 ml ice-cold D-Hanks buffer with 50× proteinase inhibitor cocktail (Sigma). Cells were then trans-
ferred to an ice-cold Eppendorf tube and centrifuged at 1,000 g at 4°C for 2 min. The supernatant was removed followed by addition of Solution A (50 mM HEPES, pH 7.9, 10 mM potassium chloride, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate) at a 1:1 (v/v) ratio without resuspending the pellet. Centrifugation was performed and the supernatant was removed. Solution A was then added to the cell pellet at a 1:5:1 ratio. The pellet was briefly resuspended, incubated on ice for 10 min, and centrifuged at 6,000×g at 4°C for 30 s following addition of 0.5% Triton X-100. The supernatant was collected to obtain a cytoplasmic extract, and the pellet was resuspended to obtain nuclear proteins. Fractions were then stored immediately at −20°C.

RNA isolation and RT-polymerase chain reaction

Cultured striatal astrocytes exposed to either APO (2 µM) or H₂O were rinsed with 0.01 M PBS, harvested in denaturing solution at various time points and stored at −70°C until use. Total RNA was isolated using a TOTALLY RNA isolation kit (Ambion, Austin, TX, USA). Specific oligonucleotide PCR primers were designed to amplify regions of coding sequence of FGF-2 (forward: 5'-CACGTCTCCAAAGCAGAAGAGA-3'; backward: 5'-TGCCAGTTGCTTAGC-3') and hypoxanthine phosphoribosyltransferase (HPRT, forward: 5'-CTTGCTACATTAACAGC-3'; 5'-CCTGAAAGTGCTCATATTAGTC-3') genes. Negative control templates for each set of PCR reactions included H₂O. Detailed procedures for performing hot-labeled RT-PCR are provided elsewhere (3).

Immunocytochemical staining and cell counts

Striatal astrocyte or VM neuronal cultures were fixed in PBS containing 4% paraformaldehyde followed by incubation with rabbit anti-FGF-2 polyclonal antibody (pAb) (1:700, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit antityrosine hydroxylase (TH) pAb (Chemicon, Temecula, CA, USA), respectively. After washing, sections were incubated with a biotinylated anti-rabbit IgG as the secondary Ab. Immune complexes were detected by avidin-linked horseradish peroxidase (HRP) and diaminobenzidine/hydrogen peroxide.

Numbers of FGF-2-positive cells with immunoreactivity in cytoplasm in astrocytic cultures and in TH-positive neurons in the recipient VM cultures were calculated in eight randomly chosen fields under ×200 magnification and expressed as the percentages of immunoreactive cells found in paired cultures treated with H₂O (used as the reference value, i.e., 100%). Controls included samples without primary antibodies.

Western blot analysis and semiquantification

Cultures were rinsed with 0.1 M PBS three times, and cells were scraped in lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease-inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) at 4°C. Lysates were incubated on ice for 30 min and homogenized by vortexing. The suspension was centrifuged at 14,000 rpm for 10 min and the supernatant was mixed with loading buffer followed by heating at 95°C for 5 min. Samples were subjected to SDS-PAGE, followed by transfer to nitrocellulose membranes (Pharmacia, Uppsala, Sweden). Blots were blocked with 5% fat-free milk in 10 mM Tris-buffered saline (pH 8.0) for 30 min and incubated for 2 h at room temperature with one of following antibodies: (a) rabbit anti-FGF-2 pAb (1:700 dilution, Santa Cruz); (b) mouse anti-α-tubulin monoclonal antibody (mAb) (1:4,000 dilution, Sigma); (c) goat anti-α-actin pAb (1:1,000 dilution, Santa Cruz); (d) mouse antiproliferating cell nuclear antigen (PCNA) mAb (1:500 dilution, Santa Cruz); (e) phospho-p44/42 MAPK Ab (1:1,000 dilution, Cell Signaling Technology Inc., Beverly, MA, USA); (f) p44/42 MAPK Ab (1:1,000 dilution, Cell Signaling); (g) phospho-Akt (Ser473) Ab (1:1,000 dilution, Cell Signaling); (h) Akt Ab (1:1,000 dilution, Cell Signaling). The membrane was washed four times for 15 min with Tris-buffered saline solution containing 0.05% Tween 20 and incubated for 1 h at room temperature with the corresponding secondary Ab: (a) HRP-conjugated goat anti-rabbit IgG; (b) HRP-conjugated goat antimouse IgG (1:10,000 dilution, Jackson Immunoresearch Lab); or (c) HRP-conjugated donkey anti-goat IgG (1:2,000 dilution, Jackson Immunoresearch Laboratories, West Grove, PA, USA). Peroxidase activity was detected with SuperSignal West Pico® Chemiluminescent substrate (Pierce, Rockford, IL, USA) and visualized with Kodak BioMax film. Bands were digitized and optical densities were analyzed using ImageMaster™ 2D Platinum (Version 5.0, Amersham Biosciences, Piscataway, NJ, USA). FGF-2 protein isoform levels and other protein levels, quantified by computer analysis as the ratio between each immunoreactive band and the levels of α-tubulin, were expressed as a percentage of untreated cultures.

Protein kinase A (PKA) phosphorylation assay

The phosphorylation assay to detect cAMP-dependent protein kinase was performed according to the manufacturer’s (Promega, Madison, WI, USA) instructions. Briefly, astrocytes stimulated with or without 2 μM APO were lysed at different time points in cold PKA extraction buffer (25 mM Tris·HCl, pH 7.4, containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol and 50× protease inhibitor cocktail). Supernatants were assayed for PKA activity using the PepTag nonradioactive PKA assay kit (Promega). The mixture containing PKA reaction buffer, Al peptide and PKA activator solution were incubated in a 30°C water bath for 1 min, and samples were added. Following a 30-min incubation at room temperature, the reaction was stopped by 95°C heating. Samples were loaded onto 0.8% agarose gels and electrophoresed. Resulting bands were digitized and their optical densities were analyzed using ImageMaster 2D Platinum software. Phosphorylated PKA protein, quantified by computer analysis as the ratio between each band and the levels of α-tubulin, was expressed as a percentage of untreated cultures.

Scanning electron microscopy

Scanning electron microscopy (EM) of striatal astrocytes was undertaken using standard techniques. In brief, astrocytes were fixed with 2.5% glutaraldehyde in culture dishes, post-fixed with 1% OsO₄, and dehydrated with ethanol. Samples were then dried with a critical-point drier, glued onto stubs, sputter-coated with gold under a vacuum, and observed under a scanning electron microscope (S-450, Hitachi, Japan) at 2–50 kV.

Statistical analysis

Statistical analysis was carried out using GraphPad software (GraphPad Prism v4.0, GraphPad Software, San Diego, CA, USA). Data corresponding to means (SEM of 3–12 samples per condition) were submitted to one/two-way ANOVA (ANOVA). Either the Dunnet test or Student-Newman-Keuls test (as a post hoc test) was used to compare data samples
from the untreated group with different treatment groups, or between pairs of groups. Differences were considered significant when $P$ values were $< 0.05$.

**RESULTS**

**FGF-2 in APO-induced conditioned medium contributes to increased survival of TH-positive neurons**

Our previous studies demonstrated that CM derived from APO-treated fetal VM cultures (donor) could enhance survival of embryonic DA neurons in recipient VM cultures. This effect may be correlated with increased levels of BDNF and GDNF in the CM following APO treatment. It is known that DA receptors are expressed in astrocytes. We therefore hypothesized that APO may exert a similar effect on striatal and nigral astrocytes, resulting in production of CM that promotes survival of DA neurons. Astrocytic cultures from postnatal striatum and substantia nigra were thus prepared and treated with APO (2 μM). CM from either APO-stimulated striatal or nigral astrocytic cultures was collected 3 days following treatment and various percentages (10%, 20%, 50%, 80%, and 100%) of CM were used as supplements to culture DA neuron-enriched recipient VM cells. Cultures were maintained for 1 day and then fixed for TH immunohistochemical staining. As shown in Fig. 1A, CM derived from either VM or striatal astrocytic cultures increased the number of TH-positive neurons in recipient VM neuronal cultures in a dose-dependent manner. The number of TH-positive neurons in these cultures increased up to 7.8–8.2-fold when exposed to CM (100%) derived from either VM or striatal astrocytic cultures compared to untreated CM. These results indicate that CM from both VM and striatal astrocytic cultures incubated with APO may contain soluble factors responsible for the elevated number of TH-positive neurons.

We next asked what those soluble factors might be in the hope of identifying the mechanism underlying survival-promoting activity of CM from cultures treated with APO. FGF-2 has been shown to stimulate growth and differentiation of several neuronal cell types, including DA neurons. To determine whether FGF-2 is present in the CM of APO-treated striatal astrocytic cultures, the CM was concentrated with the addition of acetone, and precipitates were subjected to Western blot analysis using FGF-2 antibodies. As shown in Fig. 1B, both HMW- and LMW forms of FGF-2 were released into the media following APO treatment. Recipient VM cultures were treated with one of the antibodies (1:500 dilution) followed by the addition of APO-induced CM and then fixed and stained for TH immunohistochemistry 1 day after plating. Additions of individual Ab against each protein reduced APO-mediated increase in the number of TH-expressing neurons in recipient VM cultures. Data represent the means ± sem from three to four independent experiments performed in triplicate. $*P < 0.05$, compared with that of APO-induced CM without addition of Ab.

**FGF-2 Recipient VM cultures were treated with one of the antibodies (1:500 dilution) followed by the addition of APO-induced CM and then fixed and stained for TH immunohistochemistry 1 day after plating. Additions of individual Ab against each protein reduced APO-mediated increase in the number of TH-expressing neurons in recipient VM cultures. Data represent the means ± sem from three to four independent experiments performed in triplicate. $*P < 0.05$, compared with that of APO-induced CM without addition of Ab.**

**Figure 1.** FGF-2 is an important component in conditioned medium contributing to survival of TH-positive neurons. A) Dose-response curve of the effect of APO-induced CM from donor striatal or VM cultures on the number of TH-positive neurons. The CM was prepared from donor striatal and mesencephalic astrocytic cultures by incubating cultures with or without APO (2 μM). The CM was added to recipient cultures of E14 VM plated at $10^5$ cells/cm$^2$. Cultures were fixed 24 h after media were switched to serum-free medium, and the number of TH-positive neurons was counted. Data represent means ± sem from five to seven independent experiments performed in triplicate. $*P < 0.05$, compared with untreated. B) Both HMW- and LMW forms of FGF-2 were released into the media following APO treatment. CM derived from donor striatal or mesencephalic astrocytic cultures treated with or without APO (2 μM) was concentrated and subjected to Western blot analysis using FGF-2 antibodies. C) Inhibition of the trophic activity derived from APO-induced CM by antibodies against either BDNF, NT-3, GDNF, or from the untreated group with different treatment groups, or between pairs of groups. Differences were considered significant when $P$ values were $< 0.05$. **RESULTS**

**FGF-2 in APO-induced conditioned medium contributes to increased survival of TH-positive neurons**

Our previous studies demonstrated that CM derived from APO-treated fetal VM cultures (donor) could enhance survival of embryonic DA neurons in recipient VM cultures. This effect may be correlated with increased levels of BDNF and GDNF in the CM following APO treatment. It is known that DA receptors are expressed in astrocytes. We therefore hypothesized that APO may exert a similar effect on striatal and nigral astrocytes, resulting in production of CM that promotes survival of DA neurons. Astrocytic cultures from postnatal striatum and substantia nigra were thus prepared and treated with APO (2 μM). CM from either APO-stimulated striatal or nigral astrocytic cultures was collected 3 days following treatment and various percentages (10%, 20%, 50%, 80%, and 100%) of CM were used as supplements to culture DA neuron-enriched recipient VM cells. Cultures were maintained for 1 day and then fixed for TH immunohistochemical staining. As shown in Fig. 1A, CM derived from either VM or striatal astrocytic cultures increased the number of TH-positive neurons in recipient VM neuronal cultures in a dose-dependent manner. The number of TH-positive neurons in these cultures increased up to 7.8–8.2-fold when exposed to CM (100%) derived from either VM or striatal astrocytic cultures compared to untreated CM. These results indicate that CM from both VM and striatal astrocytic cultures incubated with APO may contain soluble factors responsible for the elevated number of TH-positive neurons.

We next asked what those soluble factors might be in the hope of identifying the mechanism underlying survival-promoting activity of CM from cultures treated with APO. FGF-2 has been shown to stimulate growth and differentiation of several neuronal cell types, including DA neurons. To determine whether FGF-2 is present in the CM of APO-treated striatal astrocytic cultures, the CM was concentrated with the addition of acetone, and precipitates were subjected to Western blot analysis using FGF-2 pAb. In the CM of striatal

**Figure 1.** FGF-2 is an important component in conditioned medium contributing to survival of TH-positive neurons. A) Dose-response curve of the effect of APO-induced CM from donor striatal or VM cultures on the number of TH-positive neurons. The CM was prepared from donor striatal and mesencephalic astrocytic cultures by incubating cultures with or without APO (2 μM). The CM was added to recipient cultures of E14 VM plated at $10^5$ cells/cm$^2$. Cultures were fixed 24 h after media were switched to serum-free medium, and the number of TH-positive neurons was counted. Data represent means ± sem from five to seven independent experiments performed in triplicate. $*P < 0.05$, compared with untreated. B) Both HMW- and LMW forms of FGF-2 were released into the media following APO treatment. CM derived from donor striatal or mesencephalic astrocytic cultures treated with or without APO (2 μM) was concentrated and subjected to Western blot analysis using FGF-2 antibodies. C) Inhibition of the trophic activity derived from APO-induced CM by antibodies against either BDNF, NT-3, GDNF, or from the untreated group with different treatment groups, or between pairs of groups. Differences were considered significant when $P$ values were $< 0.05$. **RESULTS**

**FGF-2 in APO-induced conditioned medium contributes to increased survival of TH-positive neurons**

Our previous studies demonstrated that CM derived from APO-treated fetal VM cultures (donor) could enhance survival of embryonic DA neurons in recipient VM cultures. This effect may be correlated with increased levels of BDNF and GDNF in the CM following APO treatment. It is known that DA receptors are expressed in astrocytes. We therefore hypothesized that APO may exert a similar effect on striatal and nigral astrocytes, resulting in production of CM that promotes survival of DA neurons. Astrocytic cultures from postnatal striatum and substantia nigra were thus prepared and treated with APO (2 μM). CM from either APO-stimulated striatal or nigral astrocytic cultures was collected 3 days following treatment and various percentages (10%, 20%, 50%, 80%, and 100%) of CM were used as supplements to culture DA neuron-enriched recipient VM cells. Cultures were maintained for 1 day and then fixed for TH immunohistochemical staining. As shown in Fig. 1A, CM derived from either VM or striatal astrocytic cultures increased the number of TH-positive neurons in recipient VM neuronal cultures in a dose-dependent manner. The number of TH-positive neurons in these cultures increased up to 7.8–8.2-fold when exposed to CM (100%) derived from either VM or striatal astrocytic cultures compared to untreated CM. These results indicate that CM from both VM and striatal astrocytic cultures incubated with APO may contain soluble factors responsible for the elevated number of TH-positive neurons.

We next asked what those soluble factors might be in the hope of identifying the mechanism underlying survival-promoting activity of CM from cultures treated with APO. FGF-2 has been shown to stimulate growth and differentiation of several neuronal cell types, including DA neurons. To determine whether FGF-2 is present in the CM of APO-treated striatal astrocytic cultures, the CM was concentrated with the addition of acetone, and precipitates were subjected to Western blot analysis using FGF-2 pAb. In the CM of striatal
astrocyte cultures treated with APO for 6 h, this Ab recognized four FGF-2 isoforms corresponding to higher MW form (HMW, 24, 22.5, and 22 kDa) and lower MW forms (LMW, 18 kDa) FGF-2 (Fig. 1B). The concentration of FGF-2 protein was further increased at 18 h and declined 24 h after treatment. Conversely, FGF-2 was not detectable in the CM of untreated astrocytic cultures at any time point examined (Fig. 1B). To exclude the possibility that increased levels of FGF-2 resulted from lysis of astrocytes following APO treatment, astrocyte viability was examined using trypan-blue staining at 6, 18, and 24 h after APO treatment. However, only a few positively stained cells were observed, and there was no difference between APO-treated or control medium (data not shown), suggesting that most astrocytes were intact. These findings thus indicate that the observed robust increase in FGF-2 levels in the extracellular media was likely derived from cultured astrocytes in response to APO treatment. Consistent with this finding, we did observe by scanning EM a doubled number of vesicles on the cell surface of astrocytes from APO-treated cultures compared to control (Fig. 2), consistent with the release of factors from these cells. To verify whether FGF-2 contributes to APO-induced survival-promoting activity, APO-induced CM was incubated with a specific Ab to FGF-2 for 4 h to neutralize its biological activity prior to applying it to VM recipient cultures. As shown in Fig. 1C, the increase in the number of TH-positive neurons was dramatically attenuated by 55.9% when the CM was pretreated with FGF-2 Ab. We also asked whether antibodies to other neurotrophic factors such as BDNF, NT-3, and GDNF had similar effects and found that, consistent with our previous observations, the addition of these antibodies to the CM reduced the APO-induced increase in TH-positive neurons by 16.1, 17.7, and 23.4%, respectively (Fig. 1C). These data suggest that FGF-2 may be the major contributor to the APO-induced survival-promoting effect, while BDNF, NT-3, and GDNF may also play important roles in mediating the process.

To determine how APO regulates production of FGF-2, we examined both FGF-2 protein and mRNA levels in cultured astrocytes treated with APO. Western blot analysis revealed that levels of four FGF-2 isoforms were increased up to 2.7–5.1-fold after exposure to APO (2 μM) for 6 h, as compared to untreated controls (Fig. 3A, B), and then decreased from 12 h onward. The increase of FGF-2 protein in APO-treated astrocytic cultures was dose-dependent. The maximal increase in FGF-2 was observed at 2 μM APO (Fig. 3C, D). Higher concentrations of APO (e.g., 20 μM) did not result in higher production of FGF-2. Thus, 2 μM APO was used in further studies. Moreover, semiquantitative RT-PCR analysis revealed significant elevation in FGF-2 transcripts 10 min after addition of APO. mRNA levels returned to basal levels at 2 h and thereafter (Fig. 3E). Taken together, our data provide direct evidence that treatment with APO modulates transcription of FGF-2 and enhances extracellular release of FGF-2.

Figure 2. Scanning electron microscopy analysis of striatal astrocytes grown in the absence (A and C) or presence (B and D) of APO. E) Quantitative data showing the number of vesicles on the cell membrane in 10 randomly chosen fields at ×5,000 magnification. Data represent the means ± SEM of three independent experiments. *P < 0.05, compared with untreated.

APO treatment alters subcellular localization of high MW FGF-2

Previous studies indicated that HMW FGF-2 proteins were localized in the nucleus, while a fourth 18-kDa isoform of FGF-2 was mostly cytosolic (8). As expression levels of both FGF-2 mRNA and protein were upregulated following APO treatment and subsequently FGF-2 release was enhanced, we hypothesized that subcellular localization of FGF-2 isoforms may be altered following APO treatment. To address this issue, astrocytic cultures were immunostained with FGF-2 Ab. FGF-2 immunoreactivity was primarily localized to the nucleus in the majority of control astrocytes, although some cells showed weak cytoplasmic immunoreactivity (Fig. 4A). Following treatment with APO (0.02–20 μM),
cytosol FGF-2 immunoreactivity, however, was enhanced (Fig. 4B). The number of cells with positively stained cytoplasm increased up to threefold over controls in cells exposed to 2 μM APO (Fig. 4C), suggesting enhanced translocation of FGF-2 to the cytoplasm of astrocytes in response to APO stimulation.

To determine which isoform(s) of FGF-2 were enriched in cytoplasm of astrocytes, nuclear and cytoplasmic extracts of cells stimulated by APO were prepared and subjected to Western blot analysis using antibodies against FGF-2, PCNA (a nuclear marker) and/or α-tubulin (a cytoplasmic marker). Western blotting of PCNA and α-tubulin confirmed that nuclear and cytoplasmic proteins were separated and showed little or no cross contamination (Fig. 4D). Consistent with other reports, Western blotting analysis of FGF-2 revealed that the nuclear fraction of untreated cells contained HMW FGF-2 proteins with no detectable LMW FGF-2 (Fig. 4D). In these untreated astrocytes, cytoplasmic fractions contained both HMW- and LMW FGF-2. Following APO treatment, however, the intensity of the band representing 24-kDa FGF-2 increased up to threefold (Fig. 4D, E), suggesting selective export of HMW FGF-2 from nucleus to the cytoplasm of astrocytes induced by APO.

**Activation of the DA receptor is responsible for APO-modulated FGF-2 biosynthesis**

We next asked whether DA receptor activity was required for APO-mediated induction of FGF-2. Treatment of the striatal astrocytes with 0.02–20 μM S-APO, a derivative of R-APO lacking DA receptor agonist activity, for 6 h did not significantly alter the concentration of FGF-2 biosynthesis (Fig. 5A, B). However, striatal astrocytic cultures exposed to the DA receptor agonists SKF-38393 (10 μM) or quinpirole (50 μM) for 6 h showed remarkably increased levels of FGF-2 isoforms (Fig. 5A, B), indicating that activation of both D1 and D2 receptors mimics APO-mediated FGF-2 induction. Moreover, preincubation with either the D1 receptor antagonists SCH23390 (10 μM), SKF83566 (10 μM) or the D2 receptor antagonist haloperidol (1 μM) significantly blocked APO-stimulated FGF-2 expression (Fig. 5C, D). Treatment with combinations of D1 and D2 receptor antagonists almost abolished APO-stimulated FGF-2 expression (Fig. 5C, D). This data indicate that both DA D1 and D2 receptors are involved in APO-modulated FGF-2 biosynthesis.

**APO modulates FGF-2 biosynthesis via cAMP and PKC, not PI-3K**

To determine the mechanism underlying DA receptor-mediated FGF-2 induction, we blocked key components of several signal transduction pathways while stimulating receptors with both D1- and D2-selective agonists. Three intracellular signaling pathways, i.e., cAMP/cAMP-dependent protein kinase (PKA), protein kinase C (PKC)/MAPK, and phosphoinositide 3-kinase (PI-3K)/Ser-Thr kinase B (Akt), have been reported to mediate D1 and D2 receptor downstream cell signaling (17–22). We therefore asked whether activation of these pathways is associated with APO-stimulated FGF-2 expression. Exposure of astrocytic cultures to APO...
Figure 4. Treatment of astrocytic cultures with APO alters FGF-2 expression pattern. A, B) Photomicrographs showing subcellular localization of FGF-2 immunoreactivity in untreated (A) and APO-treated cultures (B). Rat striatal astrocytes exposed to APO (2 μM) were immunostained with an FGF-2 Ab 6 h after treatment. FGF-2 immunoreactivity was visualized using 3, 3′-diaminobenzidine as the chromogen. Typical astrocytes with strong FGF-2 immunoreactivity in the cytoplasm are indicated by arrowheads. Scale bar: 20 μm. C) Quantitation of A and B. The number of astrocytes with strong FGF-2 immunoreactivity in the cytoplasm was determined in eight randomly chosen fields at x200 magnification. The data are expressed as a percentage of astrocytes with strong FGF-2 immunoreactivity in the cytoplasm relative to the entire cell population. Data were from 3 independent experiments. D) Fractionation analysis revealed that stronger HMW FGF-2 bands were detected in the cytoplasm following APO treatment. PCNA and α-tubulin serve as markers of nuclear and cytoplasmic proteins, respectively. Note that the nuclear fraction only shows PCNA but not α-tubulin staining, confirming the high purity of the fractions. E) Quantitation of D. PCNA and α-tubulin serve as markers and loading controls of nuclear and cytoplasmic fractions, respectively.

Figure 5. Activation of dopamine receptors is involved in APO-modulated FGF-2 biosynthesis. A) Dopamine receptor agonists, but not S-APO, mimic the effect of APO. Striatal astrocytes were stimulated with either APO (2 μM), S-APO (0.02–20 μM), SKF83893 (10 μM, D1 receptor agonist), or quinpirole (50 μM, D2 receptor agonist). Six hours later, cells were harvested and FGF-2 levels were assayed by Western blot analysis. Bottom) Levels of α-tubulin are shown as a loading control. B) Quantitation of A. *P < 0.05, compared with untreated. C) Dopamine receptor antagonists can block the effect of APO. Striatal astrocytes were stimulated with APO (2 μM) for 6 h after a 2-h treatment with SCH23390 (10 μM, D1 receptor antagonist), SKF83893 (10 μM, D1 receptor antagonist), or haloperidol (1 μM, D2 receptor antagonist), either alone or together. After a 6-h incubation, FGF-2 levels were assayed by Western blot analysis. Bottom) Levels of α-tubulin are shown as a loading control. D) Quantitation of C. *P < 0.05, compared with untreated.
resulted in a 4.8-fold increase in the concentration of phosphorylation of PKA 30 min after treatment (Fig. 6A, B), indicating that APO indeed stimulates PKA activation. Addition of forskolin (5 μM), a cAMP activator, to astrocytic cultures led on average to a 4-fold increase in FGF-2 compared to controls (Fig. 6C, D). Conversely, pretreatment of cultures with either HA-100 (30 μM, a PKA/PKC inhibitor) or KT5720 (50 μM, a PKA inhibitor) completely abrogated APO-stimulated FGF-2 expression (Fig. 6C, D). These data suggest that cAMP/PKA signaling pathways function in APO-induced FGF-2 up-regulation.

Next, we examined the role of the MEK/MAPK signaling pathway in APO-modulated FGF-2 protein expression. Exposure of serum-starved striatal astrocytic cultures to APO phospho-activated MAPK at all time points (Fig. 6E). Treatment of cultures with either GF109203X (2 μM, a PKC inhibitor) or PD98059 (50 μM, a MAPK inhibitor) before the addition of APO markedly reduced up-regulation of FGF-2 protein (Fig. 6C, D). These results suggest that the MEK/MAPK signaling pathway is also important for APO-induced FGF-2 up-regulation. In contrast, APO did not alter the levels of phospho-Akt in astrocytic cultures for up to 6 h (Fig. 6E), indicating that APO does not modulate Akt activity. Indeed, application of LY294002 (20 μM, a PI-3K inhibitor) did not attenuate APO-induced FGF-2 up-regulation (Fig. 6C, D).

APO enhances expression of neurotrophic factors, such as BDNF, NT-3, GDNF and FGF-2 (3, 4, 23). We asked whether these factors could bind to their respective receptors and activate signaling pathways leading to FGF-2 induction. We thus investigated whether FGF-2 or BDNF released from APO-treated astrocytes was sufficient to up-regulate biosynthesis of FGF-2 in striatal astrocytes. FGF-2 levels were assayed using Western blotting 6 h after the addition of either exogenous FGF-2 (1 ng/ml, 10 ng/ml) or exogenous BDNF (1 ng/ml, 10 ng/ml). The lower concentration of BDNF (1 ng/ml) used here was much higher than levels of endogenous BDNF detected in APO-stimulated VM cultures (50.8 pg/ml) (3). Either concentration of BDNF (1 or 10 ng/ml) or the lower dose of FGF-2 (1 ng/ml) had no effect on concentration of FGF-2 protein, while only the higher concentration of FGF-2 (10 ng/ml) stimulated expression of 18 kDa FGF-2 in the culture (Fig. 6F).

The D1 receptor activates the cAMP/PKA pathway, whereas the D2 receptor mediates the MEK/MAPK pathway following APO stimulation

As shown in Figs. 5 and 6, DA receptors and two signaling pathways (cAMP/PKA and MEK/MAPK) were activated contributing to APO-modulated FGF-2
induction. Thus we determined the signaling pathway activated downstream of either DA receptor. To do so, astrocytic cultures were treated with the D2 receptor antagonist haloperidol plus APO. Notably, phosphorylation levels of PKA were increased (Fig. 7A, Lane 5, Fig. 7B), while phosphorylation levels of MAPK were not altered (Fig. 7C, Lane 5). Likewise, combinational treatment of astrocytes with D1 receptor antagonists SCH23390 or SKF83566 plus APO produced a significant increase in phosphorylated MAPK levels (Fig. 7A, B, lanes 3 and 4), but levels of phosphorylated PKA were not markedly altered (Fig. 7C, D, lanes 3 and 4). Indeed, treatment of astrocytes with SKF38393, a D1 receptor agonist, increased phosphorylation levels of PKA (Fig. 7A, B, lane 6) without markedly influencing levels of phosphorylated MAPK (Fig. 7C, D, lane 6). Moreover, exposure to quinpirole, a D2 receptor agonist, had an effect opposite to SKF38393 (Fig. 7A–D, lane 7). Furthermore, in the presence of APO, treatment of cultures with either PD98059 or GF109203X abrogated phosphorylation levels of MAPK (Fig. 8C, D, lanes 4 and 7), having little effect on phosphorylation levels of PKA (Fig. 8A, B, lanes 4 and 7). Taken together, our data suggest that the D1 receptor uses the cAMP/PKA pathway in the presence of APO, whereas the MEK/MAPK pathway is preferentially activated by the D2 receptor (Fig. 9).

Inhibitory effect of the cAMP/PKA pathway on the MEK/MAPK cascade following D1 receptor activation

As shown in Fig. 5A, B, the levels of FGF-2 isoforms induced by either SKF38393 or quinpirole were
similar to those induced by APO alone, suggesting that the extent of FGF-2 induction evoked by activation of either DA receptor subtype is comparable to that produced by activation of both subtypes. We thus postulated that, in the presence of APO, there may be inhibitory interactions between D1- and D2-mediated downstream signaling pathways. To test this hypothesis, we took a pharmacological approach in combination with Western blot analysis. As shown in Figs. 7 and 8, the phosphorylation states of PKA and MAPK in the astrocytic cultures served as indicators for activation of D1 or D2 receptor signaling pathways, respectively.

APO alone elevated phosphorylation levels of both PKA and MAPK (Fig. 8A, B, lane 2). However, in the presence of forskolin, which stimulates intracellular levels of cAMP, APO failed to increase phosphorylation of MAPK (Fig. 8A, C, lane 8), suggesting an inhibitory effect of the D1 receptor-associated downstream signaling pathway on the MEK/MAPK cascade. In contrast, combinational treatment with KT5720, a PKA inhibitor, and APO abrogated this inhibitory effect (Fig. 8C, D, lane 5), suggesting that inhibition of MAPK phosphorylation by PKA, but not cAMP, is critical for the inhibitory effect of the D1 receptor-associated pathway on the D2 receptor pathway (Fig. 9).

**DISCUSSION**

In the present study, we demonstrate that APO increases the survival of mesencephalic DA neurons by enhancing biosynthesis and release of FGF-2 in astrocytes. Modulation of FGF-2 biosynthesis by APO involves activation of the dopamine D1 and D2 receptors, leading to activation of cAMP/PKA and MEK/MAPK signaling pathways, respectively. We present novel evidence that APO can modulate FGF-2 biosynthesis and release in astrocytes through distinct signaling pathways, thereby promoting survival of DA neurons.

**APO-induced extracellular secretion of FGF-2 protein**

A major finding in the present study is that APO treatment changes the subcellular localization of HMW FGF-2 and enhances release of both HMW- and LMW FGF-2 into the culture medium. FGF-2 is produced naturally in several forms (HMW- and LMW FGF-2) due to alternative splicing of translation initiation sites of a single mRNA (24). The subcellular localization and apparent functions of HMW- and LMW FGF-2 differ. Most LMW FGF-2 is cytoplasmic, but it can also be exported to the cell surface where it is localized to the basement membrane or extracellular matrix in association with matrix heparins and heparans. By contrast, most cellular HMW FGF-2 is likely directly exported from the nucleus to the cytoplasm because of arginine-glycine repeats found within the amino-terminal region (8, 25, 26). Although HMW isoforms usually act through intracellular pathways independent of cell surface receptors (8), there is evidence that similar to 18-kDa FGF-2, these isoforms also possess neurotrophic activity for mesencephalic DA neurons in a paracrine mode (27). The results obtained by Western blot and immunohistochemical analysis (Fig. 3A, C, Fig. 4) show that the most abundant forms of FGF-2 in untreated astrocytes in culture are the HMW isoforms. In contrast, more HMW FGF-2 was detected in the cytoplasm and CM of APO-treated astrocytes, suggesting that APO affects FGF-2 release in addition to its synthesis. Secreted HMW- and LMW FGF-2 may together promote the survival of DA neurons observed in this study.

How is FGF-2, a protein lacking a signal peptide, secreted into extracellular space? Exocytosis is one likely mechanism, since we observed a larger number of vesicles at the surface of astrocytes in response to APO treatment by the scanning electron microscope. This agrees with a report that the 18-kDa form of FGF-2 can be translocated through the plasma membrane and quantitatively exported in an energy-dependent manner (28). Moreover, there is evidence that FGF-2 is detected in CM of different cell lines, such as SK-Hepl hepatoma cells, Ad-AH nasopharyngeal cells and bovine arterial endothelial cells (9, 29, 30). Piotrowicz et al. (29) have demonstrated that 27-kDa heat shock protein facilitates release of both HMW- and 18-kDa FGF-2 into the extracellular space from endothelial cells. Although FGF-2 is apparently undetectable in CM.
of normal or activated astrocytes (31), it was indeed detected in the CM of APO-treated astrocytes in our study. Thus, it is likely that APO-treated astrocytes use the same mechanisms for secretion of FGF-2 as endothelial cells do. In addition, NF-κB signaling has been shown to play an important role in extracellular release of 18-kDa FGF-2 protein (9). We demonstrate here that after APO treatment, the MAPK signaling pathway is activated. It is thus conceivable that one of its targets, NF-κB signaling, may also be activated, leading to increased release of 18 kDa FGF-2 protein. The precise pathway mediating APO-stimulated FGF-2 secretion requires further investigation.

**cAMP/PKA and MEK/MAPK signaling cascades are key components involved in modulation of FGF-2 expression elicited by APO**

There is controversy as to which intracellular signaling pathways correspond to D1 and D2 receptors in neuronal cells. A recent study has shown that D1 receptor induces phosphorylation of PKA (32), while the D2 receptor induces phosphorylation of MAPK and the cAMP-responsive element binding protein (CREB) in dopaminergic neurons (33). In contrast, the D1 receptor also can phosphorylate and activate MAPK (34, 35) and CREB (36–38). However, little information is available on the roles of DA receptor-mediated signaling pathways in astroglial cells. We demonstrate here that PKA is phosphorylated and activated preferentially by D1 receptor activity following treatment with either APO or the selective D1 receptor agonist SKF38393. Similarly, MAPK is a target activated by the D2 receptor in response to APO stimulation. We conclude that cAMP/PKA and MEK/MAPK control the inductive effect of APO on FGF-2 in astroglia by distinctly different mechanisms from those employed in neuronal cells. These findings agree with published reports by Moffett et al. (20, 21) that FGF-2 expression is up-regulated in astrocytes following treatment with forskolin or with PKC-stimulating PMA. It is plausible that activation of the cAMP/PKA signaling pathway increases FGF-2 gene expression via cis-regulatory elements located in the FGF-2 promoter (23). Because APO administration increases levels of phosphorylated CREB (39), APO may thus affect transcription of FGF-2 by inducing the binding activity of transcription factors to cis-elements located in the FGF-2 promoter. Furthermore, in contrast to previous reports we found that activation of the PI-3K pathway does not contribute to FGF-2 induction in this study. For example, in cultured primary striatal neurons Brami-Cherrier et al. (40) showed that both D1 and D2 agonist treatments rapidly increase phosphorylation levels of Akt at a residue required for kinase activity. Moreover, activation of the PI-3K pathway leading to the phosphorylation of Akt also contributed to FGF-2 induction (18, 19, 22). These discrepancies probably arise from use of different tissues or cell culture lines. Determining which specific signal transduction event is involved in APO activity may require development of specific pharmacological agents or genetic animal models.

We demonstrated here that potency of FGF-2 induction by either D1 or D2 receptor agonists alone is similar to that of APO alone. This suggests that unlike other events, such as activation of immediate early gene expression and locomotion in the DA-depleted striatum (41, 42), in which D1/D2 synergy is required, independent activation of either the D1 or D2 receptor is sufficient to elicit ample FGF-2 induction. Moreover, our observation that increasing levels of APO could not further elevate FGF-2 expression also suggests that in the presence of APO, inhibitory interactions between D1 and D2 receptors and downstream signaling pathways may occur. It is generally believed that although APO is a nonselective DA receptor agonist, its activation of the D2 receptor is much stronger than on the D1 receptor, based on the fact that APO has a Ki for the D2 receptor of ~5 nM and a Ki for the D1 receptor of ~500 nM (43). Thus, it is conceivable that APO activates both D1 and D2 receptors but preferentially the latter. However, because of the relatively weak binding of APO to the D1 receptor, less potent activation of the downstream cAMP/PKA signaling pathway leads to only partial suppression of APO-induced D2-activated downstream pathways (i.e., MEK/MAPK). This would explain increased phosphorylation and activation of both cAMP/PKA and MEK/MAPK pathways in the presence of APO and that production of FGF-2 induced by APO is similar to that of either D1 or D2 receptor agonists alone.

APO-evoked induction of FGF-2 in astrocytes may represent a common mechanism shared by other catechol-derived compounds. Our data demonstrating remarkable modulation of FGF-2 expression by APO and other D1 or D2 receptor agonists imply dual actions of DA, a neurotransmitter with DA receptor agonist activity. It is likely that DA mediates neurotransmission and also modulates expression of FGF-2 and other neurotrophic factors in astrocytes. Both effects are highly dependent on activation of DA receptors. Dysfunction of DA nigrostriatal pathways in PD leads to deregulation of FGF-2, which, in turn, affects survival and function of nigral DA neurons. PD has been associated with impaired dopaminergic function. Loss of neuronal density and depletion of FGF-2 have been found postmortem in midbrains of PD patients (44), suggesting that PD is associated with decreased or lack of trophic support. On the basis of these considerations, we speculate that APO, by restoring appropriate levels of endogenous trophic factors, may prevent loss of midbrain DA neurons. Indeed, previous studies have shown that there are numerous activated astrocytes in the nigrostriatum of neurotoxin 6-hydroxydopamine or MPTP-treated rodents (45, 46). Administration of APO may elevate FGF-2 production in these glial cells, consequently leading to more effective rescue of injured neurons. The clinical benefits of APO administration can be interpreted as a consequence of sustained agonist activity on the DA receptor. It may also
result from either APO-induced neuroprotection or modulation of synaptic activity.

It is worth noting that the present study was performed on cultured astrocytes derived from newborn animals, while the astrocytes from aged brains of PD patients react differently in vitro. Whether the astrocytes in aged brain respond to APO treatment in the same way in vivo as reported in the present study needs to be investigated in the future. Moreover, the concentration of APO (2 μM, i.e., 0.625 μg/ml) used in cell culture is much lower than the levels in plasma (range 250–600 μg/ml), and perhaps in the brain as well, of PD patients receiving s.c./i.v. infusion of APO, suggesting feasibility of intracerebral FGF-2 modulation by APO in vivo (47). In conclusion, this study demonstrates up-regulation of FGF-2 expression in striatal astrocytes after APO treatment and suggests that FGF-2 may be an important component contributing to the APO-mediated neuronal survival. We have established, using classical inhibitors of various signal transduction events, that D1 and D2 receptors can signal via cAMP/PKA and MEK/MAPK pathways to modulate FGF-2 induction. This study provides further insight into the molecular mechanisms underlying modulation of FGF-2 expression in astrocytes. The unique effect of APO on astrocytes shown here suggests that APO is a promising pluripotent neuroprotective compound and may be useful in the development of new methods of treatment of PD.

We thank Dr. X. C. Zhen for his critical reading of the manuscript. This work was supported by grants from the Chinese Ministry of Science & Technology (No. 2004AA221130), Shanghai Metropolitan Fund for Research and Development (No. 04BZ14005), Natural Science Foundation of China (Nos. 30470540 and 30525041), State Key Program for Basic Research of China (No. 2006CB500704) and the Key Laboratory of Functional Human Genomics of Jiangsu Provinces, China (No. Hg004).

REFERENCES


E858

APOMORPHINE INCREASES FGF-2 EXPRESSION


Received for publication November 30, 2005.
Accepted for publication January 24, 2006.