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Apomorphine Up-Regulates NGF and GDNF Synthesis in Cultured Mouse Astrocytes

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Apomorphine, a D_1/D_2 dopamine agonist, is an antiparkinsonian drug. We examined the effects of apomorphine on synthesis of neurotrophic factors in cultured mouse astrocytes. After 24 h incubation with apomorphine, NGF and GDNF contents in the culture medium increased to 122-fold and 1.8-fold of the control, respectively; whereas the BDNF content did not change significantly. In Northern blot analysis, expression of NGF mRNA in astrocytes reached the maximum level at 6 h after addition of the drug. By semiquantitative RT-PCR analysis, the GDNF transcript level was found to reach 2.9-fold of the control level at 15 h. These results suggest that apomorphine may exert neuroprotective effects by stimulation of NGF and GDNF synthesis in astrocytes.

Key Words: **apomorphine; dopamine agonist; NGF; GDNF; BDNF; astrocytes; Parkinson's disease; neuroprotection.**

Parkinson's disease (PD) is associated with progressive degeneration of nigrostriatal dopamine neurons. To slow disease progression by protecting surviving neurons, several approaches are in progress (1). One approach is reducing oxidative stress. Levodopa, a dopamine precursor, has long been used for the standard compensatory treatment of PD. However, free radicals derived from levodopa/dopamine metabolism are thought to contribute to the pathogenesis of loss of nigrostriatal dopamine neurons (2–4). Therapeutic dopamine agonists are thought to reduce oxidative stress (4, 5).

Another approach is administration and/or up-regulation of neurotrophic factors. Protective effects on dopamine neurons have been reported for glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and basic fibroblast growth factor (bFGF), when applied exogeneously (6). In particular, GDNF has prominent neuroprotective and neurorestorative properties (6, 7), and now clinical trials of intraventricular injection of GDNF are in progress (8). On the other hand, contents of some neurotrophic factors including BDNF and bFGF in substantia nigra of PD patients appear to be lower than those of control subjects (9, 10). This finding suggests that pharmacological stimulation of the synthesis of endogenous neurotrophic factors may be useful to suppress the progression of the nigral degeneration. Recently, dopamine was reported to up-regulate nerve growth factor (NGF) synthesis/secretion in cultured mouse astrocytes (11, 12). To extend this finding, here we examined whether apomorphine, a D_1/D_2 dopamine agonist, stimulates expression of NGF, BDNF, and GDNF in cultured mouse astrocytes. The expression levels of mRNA and protein were measured by RT-PCR (reverse transcriptase-polymerase chain reaction) or Northern blot and by enzyme-linked immunosorbent assay (ELISA), respectively.

MATERIALS AND METHODS

Culture of mouse astrocytes. As described previously (13), whole brains of 8-day-old mice (ICR) were dissected out under ether anesthesia, and cut into small pieces. The pieces were washed with CA^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS), then treated with 0.25% trypsin at 37°C for 30 min, and triturated with a Pasteur pipette. The dissociated cells were centrifuged at 200*g* for 5 min. Cells were seeded on 12 cm culture dishes, each containing 10 ml of DMEM supplemented with 10% FCS (Gibco BRL, Life Technologies, Inc., Rockville, MD) and penicillin-streptomycin solution. After confluence had been reached, the cells in each dish were dissociated by trypsin treatment and recultured in new dishes. This procedure was repeated two times. The passaged cells were seeded on 24-well plates (for ELISA assay) or 6–10 cm culture dishes (for RNA preparation) at a density of 2×10^4 cells/cm^2 in the above DMEM. The confluent cells were exposed for about 1 week to FCS-free DMEM containing 0.5% bovine serum albumin (BSA). The cells were then incubated with different concentrations of apomorphine (R(−)-apomorphine hydrochloride, RBI, Natick, MA) for 0–24 h. The control cultures

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received the same volume of DMEM. The culture medium and cells were collected after incubation and stored at −20°C until assessed for growth factor content.

ELISA for NGF. A two-site ELISA for NGF was performed as follows: The microtiter plates (Costar, Cambridge, MA) were coated for 1 h at room temperature with 100 μl of 0.25 μg/ml anti-NGF-β monoclonal antibodies (Boehringer Mannheim, Germany) diluted with 10 mM sodium carbonate-bicarbonate buffer, pH 9.3. The non-specific binding sites were saturated with 200 μl of the sodium carbonate-bicarbonate buffer containing 0.5% BSA. The sample and the standard solution (0–500 pg/ml of 2.5S NGF-β) diluted with sample buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, 1% BSA, 0.1% Triton X-100; pH 7.0) were added, and the microtiter plates were incubated for 1 h at room temperature. After three washings with BSA-free sample buffer, 100 μl of biotinylated anti-NGF-β monoclonal antibody conjugate was added; and the plate was then incubated for 1 h at room temperature. After three more washings, the plate was incubated for 1 h at room temperature with streptavidin-linked HRP (Zymed Laboratories, San Francisco, CA) diluted 1000-fold. Then after three final washings, the reaction was developed for 20 min with TMB and H_2O_2 (Kirkegaad & Perry Laboratories, Gaithersburg, MD), and stopped with 100 μl of 1 M H_3PO_4. The plates were read on a plate reader (Molecular Dynamics, Menlo Park, CA) at 450 nm with 620 nm correction for plastic interference.

Biotinylated anti-NGF antibody conjugate was prepared with 5-(N-succinimidyl-oxycarbonyl) pentyl-D-biotinamide (Dojindo, Kumamoto, Japan) (14). The standard assay curve showed a quantitative relationship in a range of 3–500 pg/ml for mouse NGF. The specificity of the NGF ELISA was confirmed by testing BDNF, GDNF, and NT-3 which did not show any crossreactivity at concentrations up to 2 μg/ml.

Preparation of anti-BDNF antisera and ELISA for BDNF. Anti-BDNF antisera were raised in immunizing chickens by subcutaneous administration of the human recombinant BDNF (PeproTec EC Ltd., London, UK) (10 μg/injection) emulsified with an equal volume of Freund's complete adjubant (Difco Laboratories, Detroit, MI) once a week for 6 weeks. The anti-BDNF antisera were further purified by affinity chromatography on a rabbit IgG anti-chicken IgG-linked Sepharose 4B column and then used as the primary antibody. Anti-BDNF antisera were also raised in rabbits and further purified by affinity chromatography on a human recombinant BDNF-linked Sepharose 4B column. Affinity-purified anti-BDNF antibody IgG was biotinylated as described above (14).

A two-site ELISA for BDNF protein measurement was performed in a way similar to that for NGF. Briefly, the microtiter plates were coated with 100 μl of 1 μg/ml affinity purified anti-BDNF antibody. Recombinant BDNF (0–1000 pg/ml) was used as the standard solution. The standard curve showed a quantitative relationship in a range of 10–1000 pg/ml of BDNF.

Preparation of anti-GDNF antisera and ELISA for GDNF. Anti-GDNF antisera were raised by immunizing rabbits with the human recombinant GDNF (50 μg/injection, Amgen Inc., CA). The immunoglobulin fraction of the antiserum was further purified on a protein G-Sepharose column (Pharmacia, LKB, Uppsala, Sweden) and used as the primary antibody and also was biotinylated as described above (14).

ELISA for GDNF protein measurement was performed similarly as that for NGF. Briefly, the microtiter plates were coated with 100 μl of 5 μg/ml anti-GDNF antibody IgG. Human recombinant GDNF (0–500 pg/ml) was used as the standard solution. The standard curve showed a quantitative relationship in a range of 5–500 pg/ml of human GDNF.

RT-PCR. Total RNA was extracted from the cells with TRIzol reagent (Gibco BRL, Life Technologies, Inc.), which extraction was followed by DNase I (GibcoBRL, Life Technologies, Inc.) treatment. First-strand cDNA was synthesized from the RNA by using random hexamers and AMV reverse transcriptase (Promega, Madison, WI.). Aliquots of cDNA (corresponding to 120 ng RNA for GDNF, 80 ng for NGF, and 40 ng for GAPDH) were amplified in 25 μl of PCR cocktail containing a 200 nM concentration of each specific primer and Taq DNA polymerase (TAKARA, Shiga, Japan). The sets of primers were the following: NGF, 5′-CAACAGGACTCACCGGAGCA-3′ and 5′-GGCTGCAGGCAAGTCAGCCT-3′ (nt 384-403 and nt 784-765, GenBank Accession No. M17298) (15); GDNF, 5′-TATCCTGACCAGTTTGATGA-3′ and 5′-TCTAAAAACGACAGGTCGTC-3′ (nt 169-188 and nt 575-556, GenBank D49921) (16); GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-AACGGATTTGGCCGTATT-3′ and 5′-ACTGTGGTCATGAGCCCTT-3′ (nt 65-82 and 573-555, GenBank M32599) (17). One cycle of PCR comprised 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were electrophoresed in agarose gel containing ethidium bromide. For semiquantitative RT-PCR, intensity of the amplified band was analyzed with National Institute of Health Image software (NIH Image 1.59). A linear correlation between intensity and cycle numbers was found with 31–34 cycles for GDNF, and with 20–23 cycles for GAPDH. Intensities of GDNF bands relative to the intensity of the control (no addition of the drug) were standardized with respect to the band intensity of GAPDH.

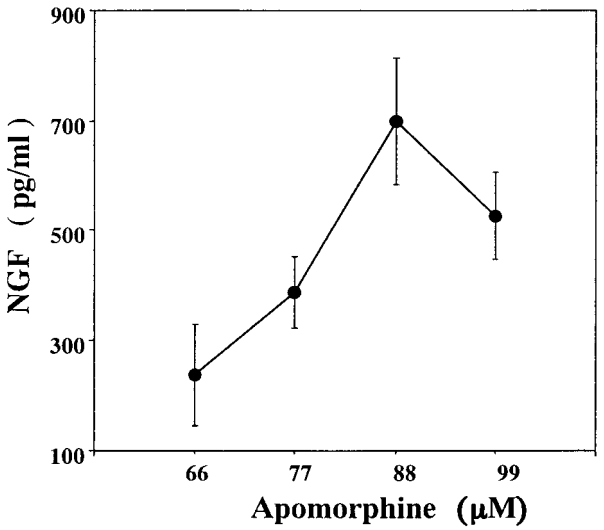

FIG. 1. Effect of apomorphine on NGF expression in mouse astrocytes. Results are the mean ± S.D. of five plates.

Northern blot analysis. Twenty micrograms of total RNA per lane were electrophoresed by the glyoxal/DMSO method and transferred onto positively charged nylon membranes (Gene Screen Plus, NEN Life Science Products, Inc., Boston, MA). A fluorescein-labeled NGF probe was prepared by PCR amplification as described previously (18) with a slight modification. In brief, the RT-PCR product of mouse NGF fragment (401 bp) was re-amplified in PCR cocktail containing fluorescein-N6-dATP and the same set of NGF primers as described above. Thirty-five cycles of PCR (94°C, 15 s; 50°C, 15 s; 60°C, 4 min) were performed. Hybridization was done at 65°C overnight. Detection of the hybridized probes was done by the chemiluminescence method using alkaline phosphatase-conjugated anti-fluorescein antibody (NEN Life Science Products, Inc., Boston, MA) and CDP-Star nucleic acid chemiluminescence reagent (Tropix Inc., NEN Life Science Products, Inc., Boston, MA).

RESULTS

We developed highly sensitive sandwich-type ELISA systems for measuring NGF, BDNF, and GDNF.

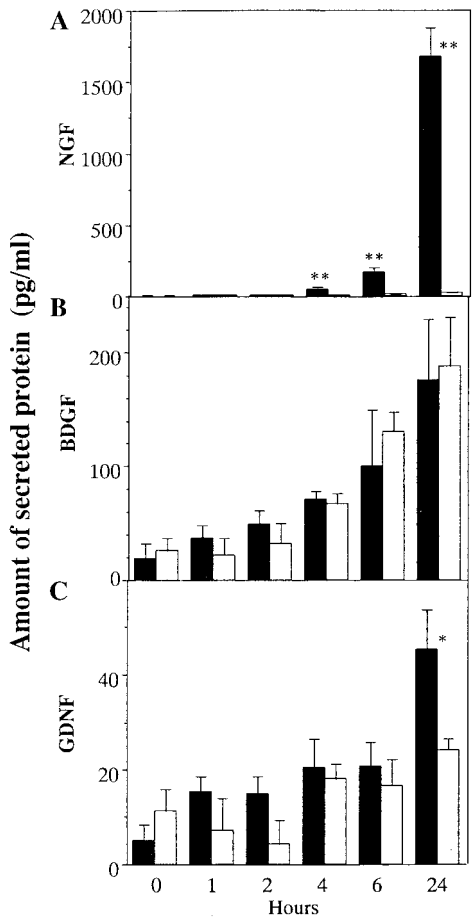

FIG. 2. Time course of induction of NGF (A), BDNF (B), and GDNF(C) in mouse astrocytes. Cells were treated with 88 μM apomorphine followed by measurement of NGF, BDNF, and GDNF contents in the culture medium 0, 1, 2, 4, 6, and 24 h later. These levels were determined by the ELISA described in the text, and expressed in comparison with the control value at each time. Results are the mean ± S.D. of five plates. Closed columns: apomorphine treated, open columns: control. Asterisk represents significant difference (*$P < 0.005$, **$P < 0.001$, Student's *t*-test).

Mouse astrocytes were incubated with various concentrations of apomorphine for 17 h, and the NGF content was measured directly in each culture medium by use of our ELISA. Apomorphine showed a bell-shaped dose-response curve with a maximal NGF induction at 88 μM (Fig. 1).

We next examined the time course of NGF secretion, as well as that of BDNF and GDNF, by mouse astrocytes incubated with 88 μM apomorphine (Fig. 2). The NGF content in the culture medium was not affected by apomorphine up to 2 h, but increased rapidly to 20-fold of the control at 4 h, 50-fold at 6 h, and 122-fold (1681 pg/ml, $P < 0.001$, Student's *t*-test) at 24 h (Fig. 2A). The BDNF content was not significantly altered by apomorphine, compared with the control value throughout the period of time examined (Fig. 2B). The GDNF content remained at the control level up to 6 h, but increased significantly to 1.8-fold (42 pg/ml, $P < 0.005$, Student's *t*-test) by apomorphine at 24 h (Fig. 2C).

To rule out that the increase in NGF and GDNF contents might have been due to leakage from the cells, we investigated apomorphine-induced expression of NGF and GDNF gene transcripts. By Northern blot analysis, the NGF transcript was undetectable in control. After the addition of apomorphine, the hybridized band was clearly detected at 2 h, reached its maximal intensity at 6 h. The NGF transcript decreased at 24 h, but was still detectable (Fig. 3).

Because of the minute level of GDNF mRNA, GDNF transcripts were analyzed by semiquantitative RT-PCR (Fig. 4). The ratio of GDNF transcript/GAPDH transcript relative to the control was calculated and plotted. Expression of GDNF mRNA increased with time, reaching the maximum level at 15 h (2.9-fold of the control) after the addition of apomorphine before subsequent decrement at 24 h (Fig. 4).

DISCUSSION

In PD, nigral dopaminergic neurons are progressively depleted, but the surrounding astrocytes remain intact. Thus, these astrocytes may promote neuronal survival by secreting neurotrophic factors in response to dopaminergic therapy (19). To address this possibility, we examined whether apomorphine, a dopamine agonist, may up-regulate synthesis/secretion of neurotrophic factors in cultured mouse astrocytes. Furukawa *et al.* have previously shown that dopamine, epinephrine, and other catechol-containing molecules in fact enhance synthesis and secretion of NGF in cultured mouse astrocytes (11, 12, 20). In this study, apomorphine showed a 122-fold increase of NGF secretion in cultured astrocytes within 24 h. This increase

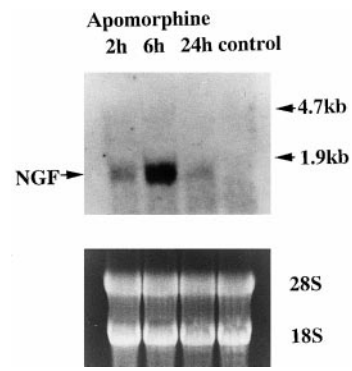

FIG. 3. Northern blot analysis of NGF mRNA expression in mouse astrocytes treated with apomorphine (88 μM). Total RNA was extracted from the cells with (2, 6, and 24 h) or without (control) apomorphine treatment. Each lane contained 20 μg of total RNA. The blot was hybridized with fluorescein-labeled NGF probe. Equal loading was confirmed by use of 28 S and 18 S ribosomal RNA stained with SYBR Green II (Molecular Probes, Inc. FMC BioProducts, Rockland, ME).

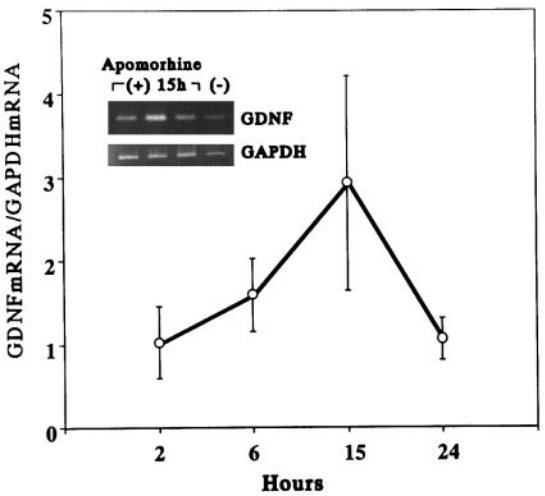

FIG. 4. Semiquantitative RT-PCR analysis of GDNF mRNA in mouse astrocytes treated with apomorphine (88 μM). Total RNA was extracted from the cells with (2, 6, 15, and 24 h) or without (control) apomorphine treatment. RT-PCR bands of GDNF (34 cycles) and GAPDH (23 cycles) amplified from three experimental plates and the control at 15 h were indicated. Intensities of the amplified bands of GDNF and GAPDH were measured by NIH Image 1.59. The GDNF and GAPDH intensities relative to those of control were calculated. The ratio of (relative GDNF)/(relative GAPDH) was used to indicate the relative amount of GDNF gene expression. Results are the mean ± S.D. of three plates.

was much greater than NGF secretion induced by dopamine (13.3-fold) (11). Apomorphine is a catechol-containing dopamine D_1/D_2 agonist whose affinities to D_1-like (D_1 and D_5) and D_2-like (D_{2A}, D_{2B}, D_3, and D_4) receptors are 10 times higher or more than those of dopamine (21). These findings indicate that stimulatory effect on NGF by apomorphine may be partly due to some mechanism involving its catechol structure. At present, however, whether apomorphine up-regulates NGF by its dopamine agonist action remains unknown. NGF has been reported to have no protective effects on nigral dopaminergic neurons (22); however, the factor is necessary to maintain brain grafts of pheochromocytoma (PC12) cells or adrenal chromaffin cells (23, 24). Therefore, apomorphine- or dopamine-induced NGF secretion in astrocytes may be a useful piece of information for the therapeutic view of PD.

In light of a recent notion that GDNF and BDNF have neuroprotective and neurorestorative effects on nigrostriatal neurons (6–8), we have extended our study to include GDNF and BDNF, in addition to NGF. There was no indication that apomorphine may affect BDNF secretion in astrocytes. In contrast, apomorphine significantly increased GDNF secretion in astrocytes (1.8-fold over the control). This was accompanied by an increase in expression of GDNF mRNA (2.9-fold over the control). At present, how synthesis and secretion of GDNF are stimulated by apomorphine remains unknown. However, up-regulation of GDNF induced by dopaminergic medications may have a significance in relation to the treatment of PD with GDNF (6–8). Apomorphine has been reported to protect PC12 cells from cell death induced by 6-hydroxydopamine (25). This neuroprotective effect of apomorphine may be exerted by stimulation of GDNF expression in astrocytes, in addition to its anti-oxidant action (25).

To our knowledge, this is the first demonstration of up-regulation of endogenous NGF and GDNF by apomorphine in astrocytes. In view of the current clinical application of GDNF to PD, the results of the present study may be considered to be a clue to account for symptomatic therapeutic efficacy of dopaminergic medications in PD.

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