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ORIGINAL ARTICLE

Differential effects of nerve growth factor on expression of dopamine 2 receptor subtypes in GH3 rat pituitary tumor cells

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Abstract Nerve growth factor (NGF) can increase expression of dopamine 2 receptors (D2R) in GH3 rat pituitary tumor cells (GH3 cells). As D2R exists in long (D2L) and short (D2S) isoforms, effects of NGF on D2R subtypes have not been accurately evaluated. In this study, we compared mRNA levels of D2R subtypes in GH3 cells treated with or without NGF with real-time RT-PCR. In addition, we also evaluated the relationship between GH3 cell growth after bromocriptine treatment and mRNA levels of D2R subtypes. We found that D2R total, D2L, and D2S mRNA in GH3 cells were significantly increased after NGF treatment, compared with the vehicle group. Moreover, NGF increased the ratio of D2S to D2L. GH3 cell survival rate after bromocriptine treatment was negatively correlated with D2R total mRNA, and D2S may be more potent than D2L in inhibiting cell growth. Cell apoptosis rate was highly elevated in GH3 cells treated with NGF and bromocriptine, compared with the control group or the group treated with NGF or bromocriptine alone. Our data provide preliminary evidence that the effect of NGF was more prominent on expression of D2S than D2L, in addition, D2S might have a greater impact suppressing GH3 cells growth than D2L.

Keywords GH3 cell · Nerve growth factor · Dopamine 2 receptor · Dopamine receptor agonist

Introduction

The dopamine 2 receptor (D2R) exists as two alternatively spliced subtypes, long (D2L) and short (D2S), which differ by an insertion of 29 amino acids in the third intracellular protein loop [1]. This polypeptide region is involved in G protein coupling of the D2R, and the D2R subtypes have been shown to differentially activate intracellular signal transduction pathways [2, 3]. Therefore, D2L and D2S may have different physiological functions.

Dopamine receptor agonists (DAs) have been proven to be effective in treating 70–90 % of prolactinomas [4–6] and about 30 % of clinically nonfunctioning pituitary adenomas (NFPAs) [7, 8]. The clinical response to DA treatment correlates well with D2R expression levels in both prolactinomas [9] and NFPAs [10]. Expression levels of D2R subtypes appear to impact the response to DA treatment, based on the observation that the presence of D2S subtype in pituitary adenomas favors the growth suppressive response to DA [10, 11].

Nerve growth factor (NGF) is the first reported member of the neurotropin family. Previous studies show that NGF induces expression of D2R in GH3 rat pituitary tumor cells (GH3 cells) [12] and human prolactinoma cell lines [13].

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A recent study reveals that NGF up-regulates D2R expression via $p75^{NGFR}$-mediated activation of nuclear factor-κB [14]. However, variation of D2R subtypes expression after NGF treatment has not been accurately evaluated.

In the present study, real-time RT-PCR (rtRT-PCR) was performed to assess mRNA level of D2R subtypes in GH3 cells after NGF treatment. In addition, we also evaluated the relationship between GH3 cell growth suppressive response to bromocriptine and expression levels of D2R subtypes mRNA.

Materials and methods

GH3 cells culture and NGF treatment

GH3 cells purchased from the American Type Culture Collection were cultured in F12 medium (Hyclone, USA) supplemented with 2.5 % fetal bovine serum, 15 % horse serum (Gibco, USA), 100 U/L penicillin, and 100 mg/L streptomycin (Sigma, USA) at 37 °C and 5 % CO_2. GH3 cells were treated with 50 ng/ml NGF (2.5S, mouse, Invitrogen, USA) or vehicle (control) for 4 days. GH3 cells were collected for RNA isolation and received cytotoxicity assay and flow cytometric apoptosis detection on days 0, 2, 4, and 6 after NGF treatment.

Real-time RT-PCR

Total RNA was extracted from each group using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, USA) and dNTPs. The mRNA of D2R subtypes was quantified with rtRT-PCR using the SYBR Green Real-time PCR Master Mix (Code No. QPK-201, Toyobo CO., Ltd., Japan) and primers specific for the D2R total (D2RT) and D2L. It was not possible to design a specific and efficient set of primers that only amplified D2S because it required one of the primers to spin the splice site (Ex4/Ex6), where the distribution of this region is GC rich [15]. The primers for D2R total were: 5′-TCAATGGGTCAGAAGGGAAGG-3′ (forward primer) and 5′-CGATGATAAAGATGAGGAGG GT-3′ (reverse primer). The primers for D2L were: 5′-GTA CCCACCCTGAGGACATG-3′ (forward primer) and 5′-CA TCCATTCTCCGCCTGTT-3′ (reverse primer). The reaction mixtures were processed in an ABI Prism 7500 Sequence Detector (Applied Biosystems, CA, USA) with the following temperature profile: initial denaturation at 50 °C for 2 min and 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 62 °C for 1 min. Rat β-actin gene was used as endogenous control and the relative levels of each mRNA to that of β-actin were calculated. Expression of the D2R short subtype mRNA was calculated as D2R total mRNA minus D2R long subtype mRNA according to the previous protocol [16].

In vitro cytotoxicity assay

Cell cytotoxicity assays were performed using a Cell Counting Kit-8 (CCK-8; Donjindo, Japan) according to the manufacturer's instructions. GH3 cells (2×10^4/well) treated with NGF or vehicle were cultured in flat-bottom 96-well microtiter plates and treated with bromocriptine (10 nmol/L; Sigma, USA), then incubated for 24 h. After adding 10 μl CCK-8 in each well, incubation was continued for another 4 h. The absorbance of the cells in each well were measured at 450 nm in sextuplicate. The percentage of GH3 cell survival was calculated as follows: cell survival (%) = [(At − Ab)/(Ac − Ab)] × 100 %, where At, Ab, and Ac were absorbances at 450 nm of treated, blank, and control group, respectively.

Flow cytometric apoptosis detection

Apoptosis detections were performed using annexin V/propidium iodide apoptosis kit (Multisciences, China) according to the manufacturer's instructions. In brief, GH3 cells (1×10^5) after treatment were collected by centrifugation and washed with PBS. Then, GH3 cells were resuspended in 500 μl of 1× annexin-binding buffer. 5 μl of annexin V-FITC and 10 μl of propidium iodide (PI) were added to each tube of GH3 cells. The cells were subsequently incubated at room temperature for 5 min in the dark. Annexin V-FITC binding was evaluated by flow cytometer (Becton–Dickinson, FACS Calibur; Ex = 488 nm; Em = 530 nm) using FITC signal detector and PI staining by the phycoerythrin emission signal detector. Data analysis was performed with the standard Lysis and Cellfit software (Becton–Dickinson).

Statistical analysis

Values represented as mean ± SEM, and statistics were performed by analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc.). Correlations between numerical variables were studied using Spearman's correlation test and multiple linear regression. *P* values less than 0.05 were considered significant.

Results

mRNA levels of D2R subtypes in GH3 cells after NGF treatment

As shown in Table 1, mRNA levels of D2R total (D2RT), D2L, and D2S in GH3 cells were significantly increased

Table 1 D2R subtypes mRNA expression levels of GH3 cells treated with NGF

Day	D2RT	D2L	D2S[a]	D2S/D2L
0	0.80 ± 0.09	0.29 ± 0.07	0.51 ± 0.05	2.56 ± 0.37
2	1.23 ± 0.18	0.31 ± 0.04	0.92 ± 0.16	2.71 ± 0.29
4	7.57 ± 1.01$^{**\#\#}$	1.52 ± 0.22$^{**\#\#}$	6.04 ± 0.86$^{**\#\#}$	4.30 ± 0.39$^{**\#}$
6	6.01 ± 0.60$^{**\#\#}$	1.39 ± 0.20$^{**\#\#}$	4.62 ± 0.61$^{**\#\#}$	4.13 ± 0.41$^{*\#\#}$

GH3 cells were treated with 50 ng/ml NGF for 4 days. D2R subtypes mRNA expression levels were evaluated by rt-RT-PCR. Rat β-actin gene was used as endogenous control. D2RT, D2L, and D2S were the total, long, and short subtypes of D2R mRNA, respectively. Values represented the mean ± SEM. Compare to 0 day, * $P < 0.05$, ** $P < 0.01$; compare to 2 d, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$

[a] D2S was calculated as D2RT minus D2L. 0, 2, 4, and 6 day meant the days 0, 2, 4, and 6 after NGF treatment, respectively

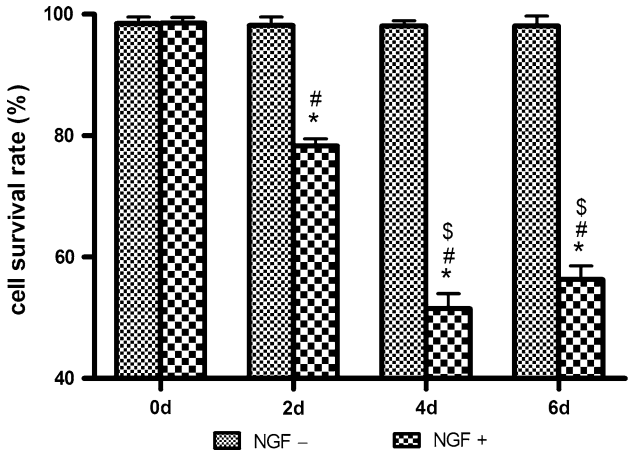

Fig. 1 Cell survival rate after bromocriptine treatment (10 nmol/l, 24 h) in GH3 cells treated with or without NGF (50 ng/ml, 4 day). 0, 2, 4, and 6 day meant the days 0, 2, 4, and 6 after NGF treatment, respectively. Values represented the mean ± SEM. Compare to GH3 cells treated without NGF, * $P < 0.01$; compare to 0 day, $^{\#}$ $P < 0.01$; compared to 2 day, $^{\$}$ $P < 0.01$

after NGF treatment. About 9.5-fold increases in D2RT mRNA on day 4, and 7.5-fold increases on day 6 were observed after NGF treatment compared to vehicle-treated GH3 cells ($P < 0.01$). About fivefold increases in D2L mRNA on day 4 and 6 were found after NGF treatment ($P < 0.01$). D2S mRNA levels were also increased to about 12-folds on day 4, and ninefolds on day 6 after treatment ($P < 0.01$). In addition, a 1.6-fold increase in the ratio of D2S to D2L was detected in GH3 cell treated with NGF on day 4 and 6, compared to the vehicle group ($P < 0.01$, $P < 0.05$, respectively).

Cell survival rate after bromocriptine treatment in GH3 cells treated with NGF

As shown in Fig. 1, bromocriptine treatment had no effect on growth of GH3 cells without NGF treatment. In contrary, cell survival rate after bromocriptine treatment significantly reduced to 78.3 ± 1.1 % ($P < 0.01$) on 2 day and 51.5 ± 2.4 % ($P < 0.01$) on 4 day, and 56.3 ± 2.2 % ($P < 0.01$) on 6 day after NGF treatment, compared to the vehicle group. Compared to 2 days after NGF treatment, combination therapy of NGF and bromocriptine showed more suppressive effects on GH3 cell growth on 4 and 6 day ($P < 0.01$).

Relationship between mRNA levels of D2R subtypes and GH3 cells in response to bromocriptine

First, we performed univariate correlation analysis between survival rate of GH3 cells after bromocriptine treatment and mRNA levels of D2RT, D2L, and D2S. As shown in Fig. 2, there was a negative correlation between GH3 cell survival rate after bromocriptine treatment and mRNA levels of D2R subtypes. Spearman (nonparametric) correlation coefficients were: $r = -0.865$ ($P < 0.0001$) for D2RT; $r = -0.802$ ($P < 0.0001$) for D2L; and $r = -0.857$ ($P < 0.0001$) for D2S. Because of the known interrelationships among several variables, we next performed a multiple linear regression. We included GH3 cell survival rate after bromocriptine treatment (as a dependent variable) and D2L and D2S (as independent variables). We found that only D2S associated with GH3 cell survival rate ($P = 0.007$), whereas D2L had no independent association with GH3 cell survival rate ($P = 0.259$).

Cell apoptosis rate after bromocriptine treatment in GH3 cells treated with NGF

As shown in Table 2, treatment with bromocriptine or NGF alone had no effect on GH3 cells apoptosis. In contrary, cell apoptosis rate was significantly elevated in GH3 cells treated with NGF and bromocriptine ($P < 0.01$), especially on 4 and 6 day after NGF treatment. Furthermore, cell apoptosis rate was negatively correlated with cell survival rate performed by cell cytotoxicity assay in GH3 cells treated NGF and bromocriptine ($r = -0.786$, $P < 0.0001$).

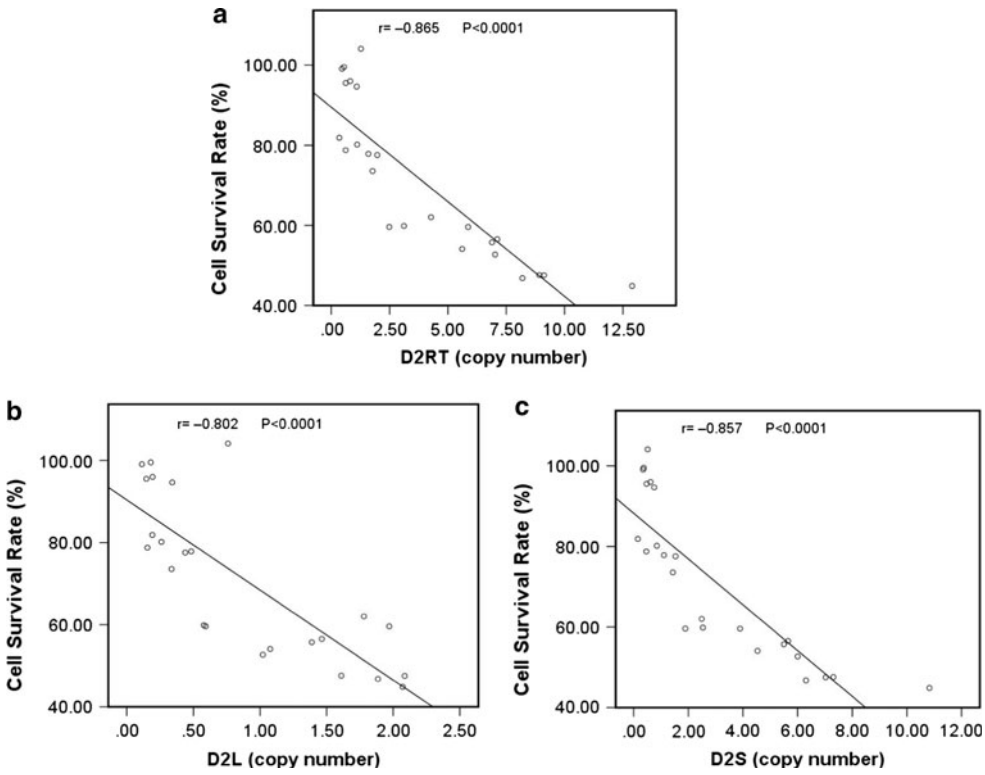

Fig. 2 Correlations between GH3 cell survival rate after bromocriptine treatment (10 nmol/l, 24 h) and D2R subtypes mRNA levels. D2RT (**a**), D2L (**b**), and D2S (**c**) represented the total, long, and short subtypes of D2R mRNA, respectively. D2S was calculated as D2RT minus D2L. Rat β-actin gene was used as endogenous control. Correlations between numerical variables were studied using Spearman's correlation test

Table 2 Cell apoptosis rate after bromocriptine treatment in GH3 cells treated with NGF

Day	Control	BRC	NGF	NGF + BRC
2	6.86 ± 1.64	8.35 ± 2.32	7.21 ± 1.98	26.13 ± 6.36*#$
4	8.57 ± 3.06	10.43 ± 3.15	8.13 ± 2.35	42.06 ± 9.28*#$&
6	8.26 ± 2.60	10.08 ± 3.25	9.22 ± 2.57	37.10 ± 8.29*#$&

GH3 cells were treated with 50 ng/ml NGF for 4 days, then 10 nmol/l bromocriptine for 24 h at designated time-point. Control, BRC, NGF, and NGF + BRC were the group with control, group treated with bromocriptine alone, group treated with NGF alone, and group treated with NGF and bromocriptine, respectively. 2, 4, and 6 day meant the days 2, 4, and 6 after NGF treatment, respectively. Values (%) represented the mean ± SEM. Compare to group with control, * $P < 0.01$; compare to group treated with bromocriptine alone, # $P < 0.01$; compare to group treated with NGF alone, $ $P < 0.01$; compare to 2 day, & $P < 0.01$

Discussion

Previous studies show that NGF increases expression of D2R in GH3 cells [12] and human prolactinoma cell lines [13]. In the present study, we confirmed these previous findings. In addition, we also found that mRNA levels of D2R isoforms, D2L and D2S, in GH3 cells were significantly increased on day 4 and 6 after NGF treatment, which is to the best of our knowledge, the first study to quantitatively assess the effect of NGF on expression of D2R subtypes mRNA by real-time RT-PCR. Moreover, we found that the ratio of D2S to D2L in GH3 cells was significantly increased after NGF treatment, suggesting that NGF more favorably affected D2S expression, compared to that of D2L. Fiorentini et al. [14] found that exposure of bromocriptine-resistant prolactinoma cells to NGF resulted in selective induction of only D2S mRNA, which was inconsistent with our results. This difference may be due to different cell lines, or a low level of D2L mRNA which is difficult to detect by routine RT-PCR.

A recent study [17] on HEK293 cells reveals that polypyrimidine tract-binding protein 1 (PTBP1) regulates the alternative splicing of D2R, as the overexpression of PTBP1 reduces the expression of D2S, and the knockdown of PTBP1 increases the expression of D2S. However, whether PTBP1 involves differential effects on expression profiles of D2R subtypes after NGF treatment remains to be further investigated.

Treatment of prolactinomas with dopamine agonists is very efficacious [4–6]. Many studies show that D2R expression levels correlate well with the clinical response to DA treatment in both prolactinomas [9, 18, 19] and

NFPAs [10]. A recent study shows that median D2R mRNA expression in responsive prolactinomas is about 10 times higher than in resistant ones by quantitative real-time RT-PCR [9]. We found that GH3 cell survival rate after bromocriptine treatment correlated well with D2R total mRNA in vitro, which was consistent with the previous reports as described above.

Several studies observe that the presence of D2S subtype in pituitary adenomas may favor the growth suppressive response to DA [10, 11]. In this report, we found that D2S might have a greater impact on suppressing GH3 cell growth in vitro. A recent in vivo study [20] shows that the apoptosis of lactotropes induced by DA is mediated by D2S, but not D2L, which may explain the phenomena stated above.

In the present study, we found that cell apoptosis rate was significantly elevated in GH3 cells treated with NGF and bromocriptine, and negatively correlated with cell survival rate. These results suggested that growth suppression of GH3 cells after treatment with NGF and bromocriptine was mainly due to cell apoptosis.

An interesting phenomenon occurred in our experiments, the response of GH3 cells to bromocriptine was pronounced after 2 days of NGF treatment, whereas there was no significant change in D2R mRNA expression. This inconsistent result may be due to two reasons as below. First, the D2S mRNA level of GH3 cells increased by about 2 times after 2 days of NGF treatment compared to GH3 cell without NGF treatment, despite no significant difference, and second D2S might have a greater impact suppressing GH3 cell growth [10, 11, 20].

In conclusion, we demonstrate that NGF has a greater effect on expression of D2S than D2L. In addition, GH3 cell response to bromocriptine correlates well with D2R total mRNA level, and D2S may be more potent than D2L in inhibiting GH3 cells growth. NGF and bromocriptine inhibited the growth of GH3 cells mainly by inducing cell apoptosis.

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Conflict of interest The authors have no conflicts of interest.

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