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Dopamine, Dopamine D2 Receptor Short Isoform, Transforming Growth Factor (TGF)-*β*1, and TGF-*β* Type II Receptor Interact to Inhibit the Growth of Pituitary Lactotropes

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Abstract

The neurotransmitter dopamine is known to inhibit prolactin secretion and the proliferation of lactotropes in the pituitary gland. In this study, we determined whether dopamine and TGF*β*1 interact to regulate lactotropic cell proliferation. We found that dopamine and the dopamine agonist bromocriptine stimulated TGF*β*1 secretion and TGF*β*1 mRNA expression but inhibited lactotropic cell proliferation both *in vivo* and *in vitro*. The dopamine's inhibitory action on lactotropic cell proliferation was blocked by a TGF*β*1-neutralizing antibody. We also found that PR1 cells, which express low amounts of the dopamine D2 receptor, demonstrated reduced expression of TGF*β*1 type II receptor and TGF*β*1 mRNA levels and had undetectable levels of TGF*β*1 protein. These cells showed a reduced TGF*β*1 growth-inhibitory response. Constitutive expression of the D2 receptor short isoform, but not the D2 receptor long isoform, induced TGF*β*1 and TGF*β*1 type II receptor gene expression and recovered dopamine- and TGF*β*1-induced growth inhibition in PR1 cells. The constitutive expression of D2 receptor short isoform also reduced the tumor cell growth rate. These data suggest that a TGF*β*1 system may mediate, in part, the growth-inhibitory action of dopamine on lactotropes.

Hyperprolactinemia is a condition in which plasma prolactin (PRL) levels are elevated above normal, which results in amenorrhea, galactorrhea, and infertility in women and gynecomastia and reproductive dysfunction in men (1). Hyperprolactinemia is often associated with pituitary lactotrope adenomas, which are known as prolactinomas. Treatment with dopamine agonists in some patients reverses hyperprolactinemia (2). This reversal supports the notion, developed from experimental models, that prolactinoma formation results from a disruption in dopamine function (3,4). How dopamine regulates lactotropic cell proliferation and tumor promotion is not well understood.

Dopamine has long been known as a physiological inhibitor of PRL secretion from lactotropes (5). Dopamine binds to D2 receptors that are coupled to pertussis toxin-sensitive Gi/Go proteins (6). The dopamine D2 receptor exists as two alternatively spliced isoforms, short (D2S) and long (D2L), both of which are expressed in lactotropes. The activation of Gi and Go heteromeric proteins causes activation of an inward rectifier potassium channel that leads to the inactivation of voltage-gated calcium channels, reduction in intracellular free calcium, and inhibition of PRL release (7). The activation of Gi/Go also leads to reduced

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activity of adenylyl cyclase and decreased production of cAMP (8), which activate cAMP response element-binding protein to induce PRL gene transcription (9,10).

It has been shown that D2 receptor knockout mice develop prolactinomas with age (11,12); dopamine transporter gene knockout mice show arrested lactotrope development (13), and D2S transgenic mice show lactotropic hypoplasia (14). Estradiol, which increases lactotropic cell proliferation and reduces dopamine receptor function (6), has recently been shown to decrease the release and synthesis of TGFβ1 and its type II receptor (TβRII) in lactotropes (15–17). We have reported that rat lactotropic tumor-derived GH3 cells with low D2 receptor expression have reduced TGFβ1 production and response (17). Additionally, it has been shown recently that developing prolactinomas in D2 receptor-deficient mice have altered expression of the TGFβ1-related family of peptide bone morphogenic protein and noggin that transduce signals, like TGFβ1, through phosphorylated mothers against decapentaplegic (Smad) (18). These results raise the possibility of an interaction between TGFβ1 signaling and dopamine signaling because the peptide growth factor is known to inhibit hormone secretion and proliferation in lactotropes by an autocrine/paracrine mechanism (19) and because a loss of TβRII has been shown to increase the incidence of prolactinomas (20). Here we provide experimental evidence of a signaling cascade for lactotrope growth control involving dopamine, D2S receptor, TGFβ1, and TβRII receptor.

Materials and Methods

Animals

Female Fischer344 rats with a body weight of 160–200 g, obtained from Simonsen Laboratories (Gilroy, CA), were housed in a controlled environment (temperature 22 C; lights on 0500–1900 h) and provided rodent chow meal and water *ad libitum*. Animals were ovariectomized bilaterally and sc implanted with a 1-cm estradiol-17β (Sigma, St. Louis, MO)-filled 1-cm SILASTIC capsule (Dow Corning Corp., Midland, MI) using sodium pentobarbital anesthesia (40–50 mg/kg, ip). Some of these animals were given daily sc injections of bromocriptine (4 mg/kg) or saline (0.1 ml) for 7 d. Animal surgery and care were performed in accordance with institutional guidelines and complied with National Institutes of Health policy. The animal protocol was approved by the Rutgers Animal Care and Facilities Committee.

Primary cultures of anterior pituitary cells

Anterior pituitaries from estradiol-treated ovariectomized Fisher 344 rats were dissociated and maintained in primary cultures as described by us previously (21). In one study, enriched lactotropic cells were prepared and maintained in cultures as previously described (22). Cells were maintained at 37 C in 7.5% CO_2 for 72 h in fetal bovine serum (FBS) containing phenol red-free DMEM (Sigma) and then for 24 h in serum-free DMEM containing human transferrin (100 μM), insulin (5 μM), putrescence (1 μM), and sodium selenite (30 nM) before treatment with the tested agents. For the TGFβ1 release studies, cultures were then treated with dopamine or bromocriptine for various time periods in the presence or absence of 10 nM of estradiol-17β. For detection of TGFβ1 levels, media were changed every 24 h. Media samples were collected and used for determination of TGFβ1 levels. For cell proliferation studies, cultures were maintained for 96 h (media changed every 48 h) with 10 nM of estradiol-17β in DMEM containing human transferrin (100 μM), insulin (5 μM), putrescence (1 μM), and sodium selenite (30 nM). Lactotropic cells in primary cultures do not proliferate without estradiol; therefore, a cell growth-response study could not be conducted in the absence of the steroid. Because lactotropic cells in primary cultures grow at a slow rate, it necessitated the use of a long-lasting dopaminergic agent, in this case bromocriptine, to determine the effect of dopamine on cell proliferation. Some of

these cultures were used to determine the effect of immunoneutralization of TGFβ1 using neutralizing antibody for TGFβ1 (R&D Systems, Minneapolis, MN). A dose of 10 μg/ml of the TGFβ1 antibody was employed because this dose of the antibody was previously used for a immunoneutralization study (22). The control group for the immunoneutralization study received 10 μg/ml of antirabbit γ-globulin (Calbiochem, La Jolla, CA).

Transfected PR1 cell lines

The PR1 cell line was derived from a pituitary tumor of a Fischer-344 ovariectomized rat treated with estrogen for 3 months (17). PR1 cells were stably transfected with an expression vector, pcDNA 3.1 (Invitrogen, Carlsbad, CA), containing cDNA encoding the D2L (accession no. X17458) or the D2S (accession no. M36831) receptors for using the Lipofectamine reagent kit (Invitrogen). The transfection was performed following the manufacturer's directions. Transfectants of PR1 cells containing the D2L or D2S receptors or the vector only were maintained in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM/F-12; Sigma) containing 10% FBS and 800 μg/ml G-418 sulfate (Promega, Madison, WI) for selection of the transfected cells.

Cell proliferation response

Primary cultures of pituitary cells were mixed cells and required identification of cell proliferation in PRL-secreting cells. Hence, lactotropic cell proliferation was determined by identifying cells that displayed both bromodeoxyuridine (BrdU) and PRL immunoreactivities as described by us previously (21). BrdU is a marker of DNA synthesis; therefore, double-stained cells were considered proliferating lactotropes. Four hours before fixation with 99% ethanol, cultures were treated with 0.1 mM BrdU. Cells were incubated at 4 C overnight with BrdU monoclonal mouse IgG (1:200; Becton Dickinson Immunocyto-chemistry Systems, San Jose, CA) and stained using the Vectastain ABC kit (Vector Inc., Burlingame, CA) with diaminobenzadine as the chromagen. The cells were then incubated with PRL antibody [1:100,100 PRL-S9; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)] at 4 C overnight and stained using the Vectastain ABC-AP kit (Vector). Negative controls were conducted by exposing cells to 3% normal serum from the host species rather than the primary antibody and also by preabsorbing the antibody with 100-fold excess antigen. Two investigators independently performed cell counts that involved counting five separate areas in each coverslip with approximately 500 total cells/area. Data were calculated as the percentage of total cells that were proliferating and are presented (see Figs. 2A, 4A, and 5, A and C).

PR1 cells were all lactotrope-derived cells and did not require identification using PRL immunostaining. Therefore, in experiments in which only PR1 cells were used, proliferation of these cells was determined using [^{3}H]thymidine incorporation methods (see Fig. 8). Previously we have shown that both the BrdU and [^{3}H]thymidine incorporation methods essentially identified similar changes of cell growth in lactotropes (23). The tritiated thymidine assay was conducted as we have previously described (23). In brief, PR1 cells containing vector only (V), D2S, and D2L cells (100,000/well) were grown in 96-well plates in 10% dextran-coated charcoal extracted FBS containing DMEM F12 medium. After 24 h of plating, the cells were treated with various concentrations of bromocriptine (0.01–1 μM) and incubated for 96 h. The cells were pulsed with 0.5 μCi [methyl-^{3}H]thymidine (specific activity 82.2 C/mM; Amersham Corp., Arlington Heights, IL) per well during the last 10–12 h. Cells were harvested using a cell harvester (Packard Bioscience, Meriden, CT) and counted in a liquid scintillation counter. Each experiment was conducted in duplicate or triplicate and repeated three times. The mean counts per minute ± SEM value (n = 6) of control cultures were 40096 ± 201 for V cells, 37083 ± 379 for D2S cells, and 40509 ± 324 for D2L cells.

Assays of PRL, TGFβ1, and tissue total proteins

PRL levels in plasma were measured by RIA using NIDDK RIA kits, and the protocols have been previously described (17). The standard used was rPRL-RP-3. TGFβ1 release from primary anterior pituitary cells and transfected PR1 cells was determined after incubating these cells in media containing serum supplement with or without a dopaminergic substance. The levels of TGFβ1 protein in the pituitary tissue extracts or in the cell culture media were determined using a Quantakine ELISA kit (R&D Systems). The pituitary tissue samples were extracted with 4 mM HCl, 50 μg/ml bacitracin, 1 KU/ml trysalol, and 1% BSA before assay. The media samples were directly used in the assay. Total protein concentrations in cell lysates and pituitary extracts were determined using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) to calculate expression of total protein per microgram and used to normalize hormone values in the pituitary and culture samples. In some experiments, cell extracts were used for DNA estimation (23), and the values were used to normalize hormone secretion from the cells in cultures.

Detection of TGFβ1, TGFβ3, D2S, and D2L mRNA expression

The gene expression patterns of TGFβ1, TGFβ3, and D2 receptor mRNA were initially screened using the RT-PCR method. cDNA was prepared using random hexamer primers and the Superscript reverse transcriptase kit (Invitrogen) and using the methods described by the manufacturers. The sequence of the 5′ forward primer of D2 receptor was TTCGAGCCAACCTGAAGACACCA, and the sequence of the 3′ reverse primer was GCTTTCTGCCGCTCATCGTCTTAA. The sequence of the 5′ forward primer of TGFβ1 was GAATACAGGGCTTTCGCTTCA, and the sequence of the 3′ reverse primer was CAG-GAAGGGTCGGTTCATGT. The sequence of the 5′ forward primer of TGFβ3 was CACAGCACACAGTCCGCTACTT, and the sequence of the 3′ reverse primer was CAGGCGTCCTCCCCAGAT. Measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression as an internal standard for calibration was performed using a control reagent (PerkinElmer Applied Biosystems, Foster City, CA). The amplification conditions for PCR were described by us previously (21).

Measurement of TGFβ1 and TβRII mRNA expression

Expression levels of TGFβ1 and TβRII in cultured transfected cells were measured using quantitative real-time RT-PCR on an ABI PRISM 7700 sequence detector (PerkinElmer Applied Biosystems) with the florigenic 5′ nuclease assay. This particular assay is based on the 5′ nuclease activity of *Taq* DNA polymerase for fragmentation of a dual-labeled fluoro genic hybridization probe (24) and has been described by us previously (25). Total RNA (1 μg) was reverse transcribed into cDNA using the Superscript first-strand synthesis system for RT-PCR (Invitrogen) and then subjected to real-time PCR. The sequences of gene-specific primers were used as follows: 5′-FAM (carboxyfluorescein)-TCAGTCCCAAACGTCGAGGTGACCTG-TAMRA as a TaqMan probe for TGFβ1, GAATACAGGGCTTTCGCTTCA as a 5′ forward primer for TGFβ1, and CAGGAAGGGTCGGTTCATGT as a 3′ reverse primer for TGFβ1; 5′-FAM-CAACCACAATACGGAACTGCTGCCC-TAMRA (carboxytetramethylrhodamine) as a TaqMan probe for TβRII, CTCTACGTGCGCCAACAACA as a 5′ forward primer for TβRII receptor, and CTGCTTCAGCTTGGCCTTGTA as a 3′ reverse primer for TβRII receptor. Relative quantification of mRNA samples was performed using the standard curve method as described by the manufacturer (PerkinElmer Applied Biosystems). The standard curves were prepared by a 5-fold serial dilution of the cDNA templates (25–0.008 ng total RNA). The threshold cycles were plotted against the log of the initial amount of the templates, which generated the linear standard curve. PCR were performed in a total volume of 25 μl with 1× TaqManuniversalPCRmix,0.2 μM each primer and probe, and 2 μl (5 ng) of the cDNA template. The reaction conditions were one cycle of a sequential incubation at 50

C for 2 min and 95 C for 10 min with 60 subsequent cycles of a consecutive incubation at 95 C for 15 sec and 60 C for 1 min. For the pituitary cells and pituitary tissues, the quantity of each sample was normalized to its GAPDH mRNA level, and for the PR1 cells, each was normalized to 18S ribosomal. Both were measured using the TaqMan control reagents (PerkinElmer Applied Biosystems).

Dopamine receptor binding

Cell membranes were prepared from enriched lactotropes (23) or PR1 cells stably transfected with pcDNA (vector only) or pcDNA containing D2L or D2S plasmids. Briefly, cell membranes were prepared by harvesting cells in 50 mM Tris-HCl (pH 7.5) and then homogenizing the cells using glass-glass homogenizer. Homogenate was centrifuged for 20 min at 4 C at 39,000 × *g*, and membrane pellets were resuspended in binding buffer [Tris-HCl (pH 7.5)]. Dopamine receptor binding assays were performed in duplicate using various concentrations of ^{3}H-Spiperone (specific binding 118 Ci/mmol; Amersham Biosciences, Piscataway, NJ) as a radioligand and 1 μM butaclamol to define nonspecific binding. After a 1-h incubation at room temperature, reaction was terminated using rapid filtration through GF/C filters using a cell harvester (Packard Bioscience). The filters were air dried and counted in a β-counter. Receptor binding data were analyzed by nonlinear regression using Prism 4.0 software (GraphPad, San Diego, CA).

Statistical analysis

The data shown in the figures and text are mean ± SEM. Comparisons between two groups were made using *t* tests. Data comparisons between multiple groups were made using one-way ANOVA. Student-Newmann-Keuls test was used as a *post hoc* test. A value of $P < 0.05$ was considered significant.

Results

Dopamine and bromocriptine increase the release of TGFβ1 in pituitary cells in primary cultures

We determined the effect of various concentrations of dopamine on TGFβ1 release from pituitary cells in primary cultures. Treatment with dopamine at concentrations range of 0.05 and 5 μM (which is within the physiological dose range) (26,27) for a period of 24 h dose-dependently increased TGFβ1 release (Fig. 1A). Dopamine also increased TGFβ1 release after 48 h of treatment, although the TGFβ1 response to the highest dose of dopamine was lower than that after 24 h of treatment. The catecholamine also increased TGFβ1 release during a 2-h treatment period but with less potency (TGFβ1, ng/ml; n = 5–6; control, 0.76 ± 0.08; 0.5 μM dopamine, 0.58 ± 0.1; 5.0 μM dopamine, 1.3 ± 0.01; $P < 0.05$, control *vs.* 5.0 μM dopamine). The long-lasting dopaminergic agent bromocriptine also increased TGFβ1 release from the pituitary cells in a concentration-dependent manner between 24 and 96 h after the treatment (Fig. 1B). Estradiol, which is known to reduce dopamine receptor function (25) and TGFβ1 production in lactotropes (23), reduced the bromocriptine's ability to increase TGFβ1 release. These results suggest that dopaminergic agents are potent stimulators of TGFβ1 release from the lactotropes.

Dopaminergic agent bromocriptine's inhibition of lactotropic cell proliferation is associated with increased expression of TGFβ1 in pituitary cells in primary cultures

Whether dopamine and TGFβ1 interact to regulate lactotropic cell growth was studied *in vitro* using primary cultures of pituitary cells. Using a bromocriptine concentration of 0.1 μM, known to reduce estradiol's cell proliferation action on lactotropes (28) and increase TGFβ1 secretion from pituitary cells in primary cultures (Fig. 1B), we found that treatment

with this concentration of bromocriptine for a period of 96 h reduced the number of proliferating lactotropes (Fig. 2A). We also measured the changes in mRNA levels of TGFβ1 and TβRII after bromocriptine treatment in pituitary cells in primary cultures using real-time RT-PCR assay. Using this assay, we found that bromocriptine increased mRNA levels of both TGFβ1 (Fig. 2B) and its receptor TβRII in pituitary cells (Fig. 2C). These data suggest that dopamine may interact with the TGFβ1 system to control lactotropic cell proliferation.

Bromocriptine's inhibition of lactotropic cell growth is associated with increased levels of TGFβ1 and TβRII in the anterior pituitary

We further investigated TGFβ1 and dopamine interaction on lactotropes *in vivo*, using a previously established animal model in which bromocriptine has been shown to inhibit the estradiol-induced increase in pituitary weight and plasma PRL (both of these are indirect measures of the pituitary lactotrope growth) in Fischer-344 rats (29). Consistent with these findings, we demonstrated that bromocriptine treatment reduced the plasma levels of PRL (control, 195 ± 48 ng/ml; bromocriptine, 22 ± 3 ng/ml; $P < 0.001$) and lowered the weights of the pituitaries in estradiol-treated rats (Fig. 3A). Bromocriptine treatment also increased the pituitary levels of TGFβ1 (Fig. 3B) and TGFβ1 mRNA (Fig. 3C) and TβRII mRNA (Fig. 3D). These *in vivo* data also suggest the possibility of involvement of TGFβ1 in dopamine-regulated lactotropic cell growth.

Antiproliferative effect of bromocriptine on lactotropic cells is prevented by the TGFβ1-neutralizing antibody in primary cultures

To determine whether TGFβ1 mediates dopamine's antiproliferative action on lactotropes, we determined the effect of a TGFβ1 neutralizing antibody on bromocriptine's action on cell growth *in vitro*. As shown in Fig. 4A, treatment with 0.1 μM of bromocriptine reduced the percentage of proliferating lactotropes. A polyclonal antibody that neutralizes TGFβ1 did not affect the basal cell proliferation but did prevent bromocriptine's antiproliferative effect on lactotropes. Control cultures treated with antirabbit γ globulin did not significantly affect the bromocriptine-inhibitory action on the growth of lactotrope. These data suggest that TGFβ1 may mediate dopamine's antiproliferative effect on lactotropes.

Antiproliferative effects of dopamine and TGFβ1 are lost in TGFβ1-deficient PR1 cells

To further determine dopamine-TGFβ1 interaction in lactotropes, the actions of the dopaminergic agent bromocriptine on PRL release and on cell proliferation were determined in TGFβ1-deficient PR1 cells. These cells are PRL secreting but express very low or undetectable quantity of TGFβ1 protein and TGFβ1 mRNA (17) and reduced amounts of TβRII mRNA and protein (21). The cell growth-reducing responses to bromocriptine and TGFβ1 in PR1 and pituitary cells were compared. As expected, bromocriptine concentration-dependently inhibited the estradiol-induced cell growth of lactotropes in pituitary cells in primary cultures (Fig. 5A). However, the same doses of bromocriptine that inhibited cell growth in primary pituitary cells failed to alter PR1 cell growth in the presence or absence of estradiol. The estradiol-induced growth of lactotropes was dose-dependently inhibited by TGFβ1 in primary cultures of pituitary cells (Fig. 5B). However, TGFβ1 failed to inhibit the growth of PR1 cells in the presence or absence of estradiol. The parallel loss of the dopamine response and the TGFβ1 response on cell growth in PR1 cells is consistent with the dopamine and TGFβ1 interaction in the regulation of lactotropic cell proliferation.

Reduced TGFβ1 and TβRII expression is associated with low expression of dopamine receptors in PR1 cells

Previously we have shown that TGFβ1 is produced in lactotropes and acts to inhibit the growth of these cells via TβRII receptors (17,21). However, PR1 cells do not produce TGFβ1, and they show low levels of the TβRII receptor (21). Whether the reduced expression of TGFβ1 and its receptors is associated with altered expression of dopamine D2 receptors was studied. The dopamine D2 receptor exists as two alternatively spliced isoforms, D2S and D2L, both of which are expressed in lactotropes (30). Determination of D2S and D2L mRNA transcript expression using RT-PCR indicated that primary pituitary cells express significant levels of both D2S and D2L transcripts, whereas PR1 cells show low or undetectable expression of these dopamine D2 receptor transcripts (Fig. 6A). The maximal binding capacity (Bmax) and dissociation constant (Kd) values for dopamine D2 receptors in PR1 cells with a control vector were 38.4 ± 4 fmol/mg protein (n = 3) and 0.04 ± 0.02 nM (n = 3), and for enriched lactotropes they were 356 ± 29 (fmol/mg; n = 2) and 0.5 ± 0.1 (nM; n = 2), suggesting that the PR1 cells have very low amounts of D2 receptors.

Ligand-independent TGFβ1 production was observed in PR1 cells stably transfected with D2S receptor

Studies were conducted to determine the role of D2 isoforms in dopamine-induced TGFβ1 production by stably transfecting PR1 cells with an expression vector containing D2S or D2L. After selection by G418, two stable cell lines, PR1-D2S and PR1-D2L, were established. Transfected cells expressed the gene that had been introduced (Fig. 6A). The D2 receptor levels in PR1 cells with control V or D2S or D2L gene were compared. D2S and D2L cells expressed 7- to 8-fold higher levels of D2 receptors than the control V cells (Fig. 6B and Table 1), suggesting that these cells were expressing D2 receptors.

The expressions of mRNA transcripts of two TGFβ isoforms, TGFβ1 and TGFβ3, which are known to express in lactotropes(22), were screened using RT-PCR in V, D2S, and D2L cells. The mRNA transcripts for TGFβ1 and TGFβ3 were very low in PR1 cells transfected with the control vector or the D2L gene (Fig. 7A). The mRNA transcript levels of TGFβ1 but not TGFβ3 were elevated in PR1 cells expressing D2S. Quantitation of TGFβ1 mRNA levels using realtime-RT-PCR also showed significantly elevated expression of this gene in PR1 cells expressing D2S (Fig. 7B). TGFβ1 levels were measured in the culture media of each transfected cell, and onlyD2S-transfected PR1 cells secreted TGFβ1 (Fig. 7C). The TβRII gene expression in D2S-transfected cells was higher than in control V-transfected cells and D2L-transfected cells (Fig. 7D). D2S-transfected cells also had higher basal TGFβ1 mRNA levels than did D2L and vector-transfected cells (Fig. 7D). Bromocriptine significantly increased TGFβ1 gene expression and TGFβ1 secretion in D2S-transfected cells but not D2L-transfected or control V-transfected cells.

Recovery of TGFβ1's growth-inhibitory responses in PR1 cells stably transfected with D2S receptor

The aforementioned study using a TGFβ1 neutralizing antibody suggested the possibility of TGFβ1 mediation of dopamine's action on lactotropic cell proliferation. Additional evidence resulted from the above demonstrations that PR1 cells with reduced D2 receptors also had abnormal functioning of the TGFβ1 system and a decrease in TGFβ1's and dopamine's growth-inhibitory responses. Hence, we tested whether the recovery of the TGFβ1 and TβRII gene expression after D2S transfection results in recovery of the dopamine and TGFβ1 actions on cell growth. The D2S-transfected cells, but not the control and D2L-transfected cells, showed negative growth responses to bromocriptine (Fig. 8A) and to TGFβ1 (Fig. 8B). Additionally, D2S-transfected cells showed lower cell growth rates (Fig.

9). Together these data suggest that D2S receptor activation leads to increased production and action of TGFβ1, and possibly decreased cell growth rate in PR1 cells.

Discussion

Dopamine plays a key role in maintaining the normal function of lactotropes in the pituitary gland. Abnormalities in dopamine receptors and dopamine transporter function lead to lactotropes hyperplasia and tumors (11–14). The cellular mechanism by which dopamine controls lactotropic cell proliferation is of interest because prolactinomas frequently occur in humans. Data presented in this study show, for the first time, that dopamine and the dopamine agonist bromocriptine stimulated TGFβ1 expression and secretion *in vivo* and *in vitro*. We have also shown that dopamine's inhibitory action on lactotropic cell growth was blocked by a TGFβ1-neutralizing antibody and was lost in transformed lactotropes (PR1 cells) in which TGFβ1 and its receptor expressions were repressed. Additionally, data are presented to show that constitutive expression of the dopamine D2S receptor up-regulated TGFβ1 expression and action. It also caused a reduction in the cell proliferation rate in transformed lactotropes. These data suggest that dopamine's inhibitory action on lactotropic cell growth may be mediated partly by TGFβ1 in lactotropes.

Dopamine and the dopaminergic agent bromocriptine produced dose-response release of TGFβ1 from pituitary cells. The TGFβ1-inducing response of the high dose of dopamine in pituitary cells was reduced after continuous exposure of the neurotransmitter for a period of 48 h. This reduction in the TGFβ1-inducing response of the high dose of dopamine may not be due to decrease in dopamine's half-life, which is more than 48 h (31). TGFβ1-inducing response of bromocriptine from pituitary cells was also reduced after 96 h of exposure. Hence, the reduced TGFβ1-releasing response of dopaminergic agents after long-term treatment might be related to desensitization of D2 receptors on lactotropes (32).

Data presented here show that dopaminergic agent inhibition of lactotropic cell growth is reduced by a TGFβ1-neutralizing antibody. These data are in agreement with those showing that combined administration of maximal doses of TGFβ1, and dopamine in rat anterior pituitary cells does not lead to greater suppression of lactotropic hormone secretion when compared with doses of dopamine alone (33). This suggests the possibility that both dopamine's and TGFβ1's inhibitory actions on lactotropes share a common mechanism.

Our study with stably transfected PR1 cells with D2S and D2L cDNA identified D2S receptor-specific TGFβ1 signaling. D2S-transfected cells also had higher basal TGFβ1 mRNA levels. As described by an extended allosteric ternary complex model of G protein-coupled receptor activation, receptors spontaneously isomerize between active and inactive conformations, so receptors can modulate signaling pathways in the absence of an agonist. In addition, significant constitutive activity of recombinant D2S receptors expressed in mammalian cells has been described previously (34). These data suggest that ligand-independent TGFβ1 production was due to constitutively activated D2S receptors in transfected cells. PR1 cells that lacked functional D2 receptors and had a dysfunctional TGFβ1 system also showed significant recovery of the TGFβ1 function after D2S transfection. The D2L-transfected cells showed either partial or no recovery of TGFβ1 function. These data support a preference of D2S over D2L in TGFβ1 activation.

It is well known that estradiol inhibits dopamine release from the hypothalamus and down-regulates dopamine receptor activity in lactotropes (4,6,25). Data presented in this report indicate that TGFβ1 levels were elevated by dopamine. Also, TGFβ1 and TβRII levels decreased after estradiol treatment (16,17). Thus, by inhibiting dopamine secretion and thereby inhibiting D2 receptor activation, estradiol could inhibit TGFβ1 expression and

action. Interestingly, estradiol increases the D2L to D2S ratio in lactotropes and the lactotrope-derived MMQ cell line (24,35). Similarly, ethanol treatment increases lactotropic cell proliferation, increases the D2L to D2S ratio and decreases TGFβ1 expression (24). This reported evidence is in agreement with our present data that D2S overexpression enhances TGFβ1 production and release and also increases TβRII receptor expression.

Dopamine D2 receptor activation in lactotropes leads to the alteration of G protein coupling, inhibition of adenylyl cyclase, and reduction of intercellular cAMP (36,37). Although the sequence of the TGFβ1 promoter contains no element similar to the cAMP-responsive element core sequence, the TGFβ1 promoter contains several activator protein-2-like sequence elements that could potentially mediate the cAMP response (38). Additionally, cAMP analogs inhibit TGFβ1 gene transcription in the pituitary (39). Hence, it is possible that cAMP-dependent mechanisms may be involved in dopamine regulation of TGFβ1. Studies involving the cAMP and its analogs actions on TGFβ1 and D2 receptors may provide further understanding of dopamine's regulation of TGFβ1 release.

In addition to inhibition of the cAMP system, dopamine receptors have been shown to regulate other transduction pathways that lead to alteration of intracellular calcium, protein kinase C, and the MAPK pathway (6,14,36,37,40). The data presented here identify a role of D2S receptor-specific action on the TGFβ1 system and the cell growth cycle in lactotropes. It has recently been shown that transgenic mice, overexpressing D2S but not D2L, show pituitary hypoplasia; the D2S-overexpressing mice also showed increased phosphorylated MAPK (14). The MAPK system has also shown to be involved in TGFβ1-activated signaling in various cell types (41,42). Hence, the possibility that the MAPK pathway is involved in dopamine-TGFβ1 interaction mechanisms needs to be investigated.

Estrogen is known to reduce both the levels of dopamine and dopamine D2 receptor activity in lactotropes (4,25,43,44). Thus, by inhibiting dopamine, estrogen could inhibit TGFβ1 and its receptor expression. Indeed, this association has been observed in our previous study with long-term estrogen treatment (17). In addition, we have observed that GH3 cells that have reduced functional dopamine D2 receptors have a low TGFβ1 response and low TGFβ1 and TβRII production (17). In the present study, PR1 cells, which did not respond to TGFβ1 and did not express detectable TGFβ1 or TβRII, showed a lack of dopamine response and dopamine receptor binding. Hence, we propose that during sustained exposure, estrogen cancels the inhibitory effect of dopamine and thereby down-regulates TGFβ1's inhibitory effect on cell proliferation. This may cause an alteration in the balance between positive and negative regulators of cell growth, resulting in abnormal lactotropic cell proliferation.

In summary, this report provides the first direct evidence for the involvement of TGFβ1 and its receptor TβRII in dopamine's growth-suppressing action on lactotropes. This report also shows for the first time that the dopamine D2 receptor's splice variant D2S activates TGFβ1-TβRII signaling to inhibit lactotropic cell proliferation (Fig. 10). Although the mechanism by which D2S receptor activation increases TGFβ1 production and TβRII receptor activation is not determined, the literature suggests a possible involvement of a negatively coupled cAMP system in this process. The identification of TGFβ1 mediation of dopamine action provides a novel possibility to consider the TGFβ1-TβRII signaling as a molecular target for treating prolactinomas.

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Abbreviations

Bmax	Binding capacity
BrdU	bromodeoxyuridine
D2L	D2 receptor long isoform
D2S	D2 receptor short isoform
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Kd	dissociation constant
PRL	prolactin
TβRII	TGFβ type II receptor
V	PR1 cells containing vector only

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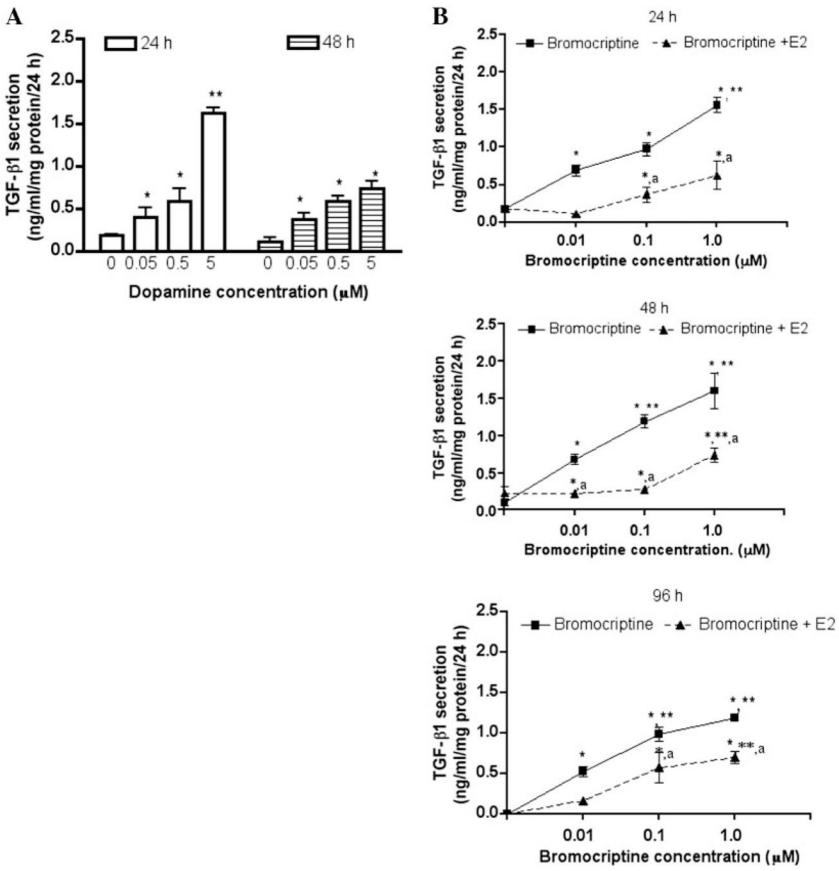

Fig. 1.

Dopamine and bromocriptine effects on TGFβ1 release from pituitary cells in primary cultures. A, Pituitary cells in primary cultures were treated with different doses of dopamine (0–50 μM) for 24 or 48 h. Media samples were collected at 24-h intervals, and media levels of TGFβ1 were determined by ELISA (n = 4/group). *, $P < 0.05$, compared with the 0 μM dose. **, $P < 0.05$, compared with other groups. B, Pituitary cells in cultures were treated with various concentrations of bromocriptine (0–10 μM) with or without estradiol (E2, 10 nM) for 24, 48, or 96 h. Media samples were collected at 24-h intervals and used for measurements of TGFβ1 (n = 4/group). *, $P < 0.05$, compared with the 0 μM dose. **, $P < 0.05$, compared with the group treated with a 0.01 μM concentration. a, $P < 0.01$, compared with the respective dose of bromocriptine alone.

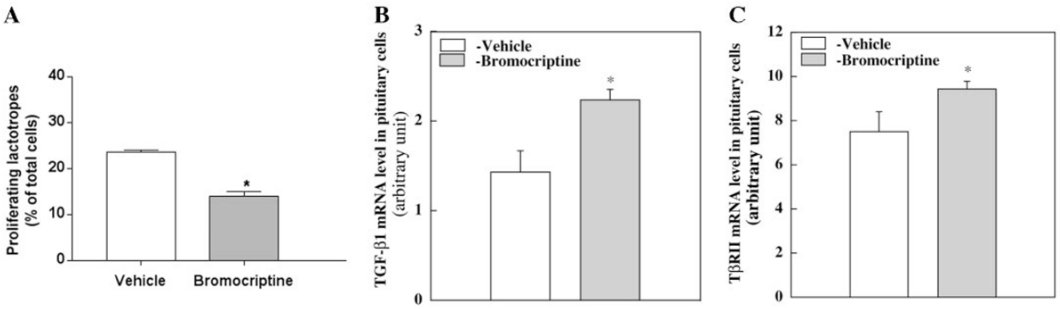

Fig. 2.

Stimulatory effects of the long-acting dopaminergic agent bromocriptine on the TGFβ1 system in pituitary cells in primary cultures. A, Cells were treated with 0.1 μM bromocriptine or vehicle for 96 h, and proliferating lactotropes were determined using the BrdUrd assay (n = 6/group. *, $P < 0.05$, compared with vehicle-treated group. B and C, Primary cultures of pituitary cells were treated with 0.1 μM bromocriptine or vehicle for 96 h and assayed for TGFβ1 mRNA (B) and TβRII mRNA (C) using the real-time RT-PCR (n = 4–5/group). *, $P < 0.05$, compared with vehicle.

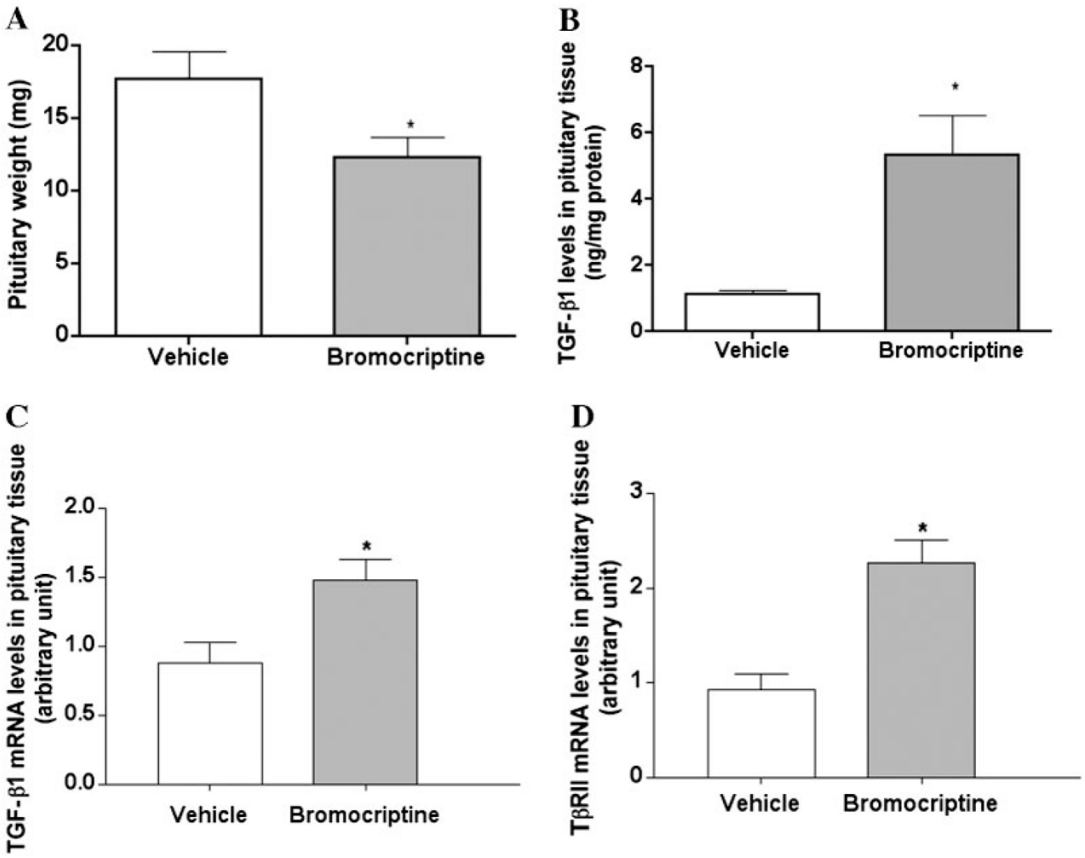

Fig. 3.

Bromocriptine's stimulatory effects on the pituitary TGFβ1 system *in vivo*. Ovariectomized and estradiol-treated rats were given daily sc injections of bromocriptine (4 mg/kg) or saline (0.1 ml) for 7 d. Pituitary weight (A) and anterior pituitary levels of TGFβ1 protein (B), TGFβ1 mRNA (C), and TβR-II mRNA (D) were determined using the real-time RT-PCR assay (n = 4–6 rats/group). *, $P < 0.05$, compared with vehicle.

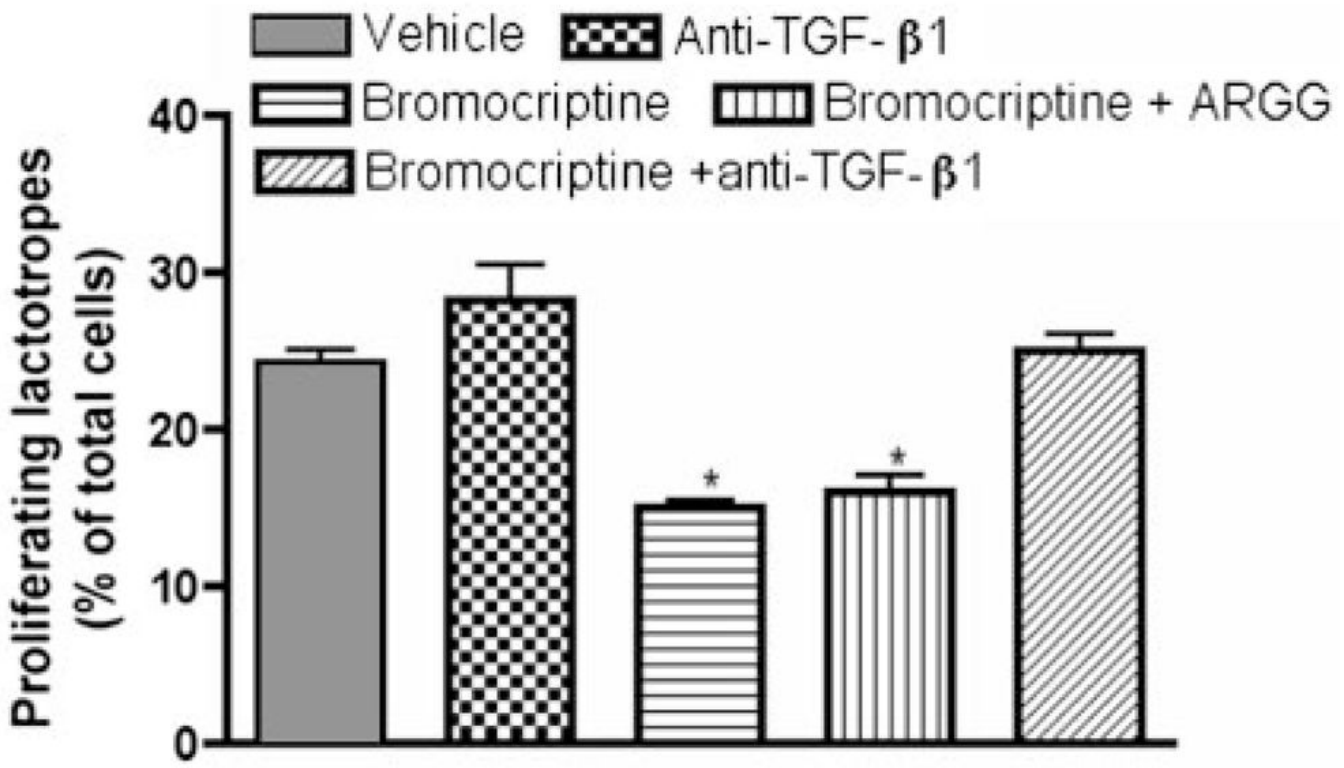

Fig. 4.

TGFβ1's mediation of bromocriptine inhibition of lactotropic cell proliferation in primary cultures. Cultures were treated with vehicle alone, 10 μg/ml TGFβ1-neutralizing antibody (anti-TGFβ1), 1 μM bromocriptine, 1 μM bromocriptine + 10 μg/ml antirabbit γ globulin (ARGG), or 1 μM bromocriptine + 10 μg/ml anti-TGFβ1 for a period of 96 h to study cell proliferation (n = 6–9/group). *, $P < 0.01$, compared with the rest of the groups.

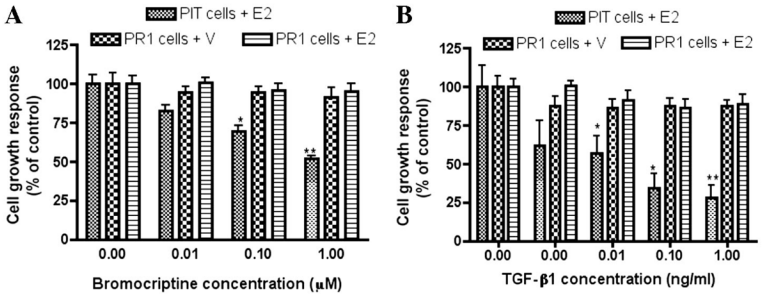

Fig. 5.

Reduced dopamine and TGFβ1 response of PR1 cells. A, Comparison of the cell growth response of primary cultures of pituitary (PIT) cells and PR1 cells to various concentrations of bromocriptine treated for 96 h in the presence and/or absence of 10 ng/ml of estradiol-17β (E2; n = 6/group). *, $P < 0.05$, compared with the 0 μM dose. **, $P < 0.05$, compared with other groups. B, Comparison of the cell growth response of PIT cells and PR1 cells to various doses of TGFβ1 treated for 96 h in the presence and/or absence of 10ng/ml of estradiol-17β (E2) (n = 6/group). *, $P < 0.05$, compared with the 0 μM dose. **, $P < 0.05$, compared with other groups.

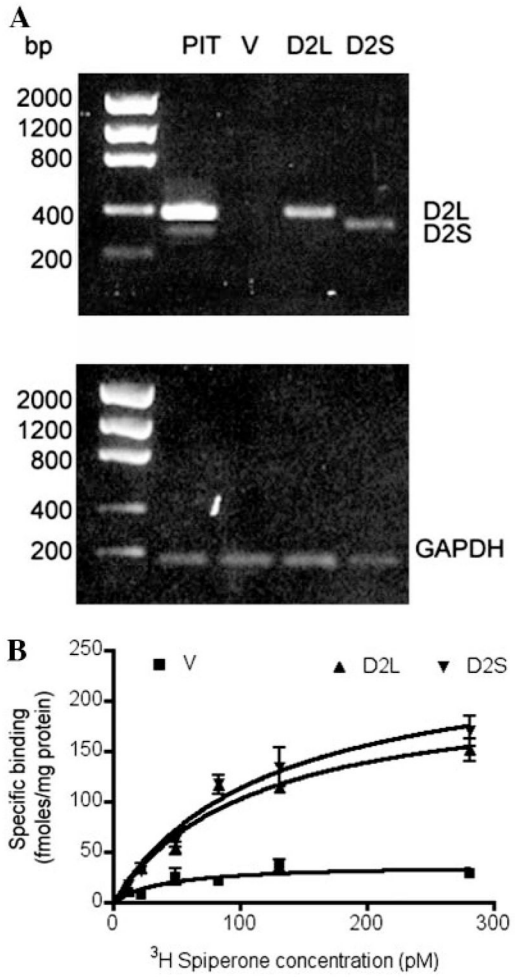

Fig. 6.

Low expression of dopamine D2 receptor isoforms in PR1 cells. A, Representative gel figure showing low or undetected levels of D2S and D2L mRNA in PR1 cells as detected by RT-PCR. For comparison, the expression of D2S and D2L in pituitary cells (PIT) and PR1 cells stably transfected with the vector pcDNA 3.1 (V) or vectors containing a sequence that encodes the dopamine D2S or D2L are shown. For the loading controls, the levels of GAPDH mRNA were measured in the same cell samples and are shown in the *bottom* gel figure. The transcripts for D2L, D2S, and GAPDH are indicated on the *right*. The molecular-weight marker in base pairs is shown on the *left* of each gel. B, Saturation curves showing the ^{3}H-Spiperone binding to the dopamine receptor in V, D2L, and D2S cells. Membrane preparation from V, D2L, and D2S cells was used for saturation binding of ^{3}H-Spiperone. Data points are average of three separate experiments. Nonlinear regression analysis of curves yielded Kd and Bmax values presented in Table 1.

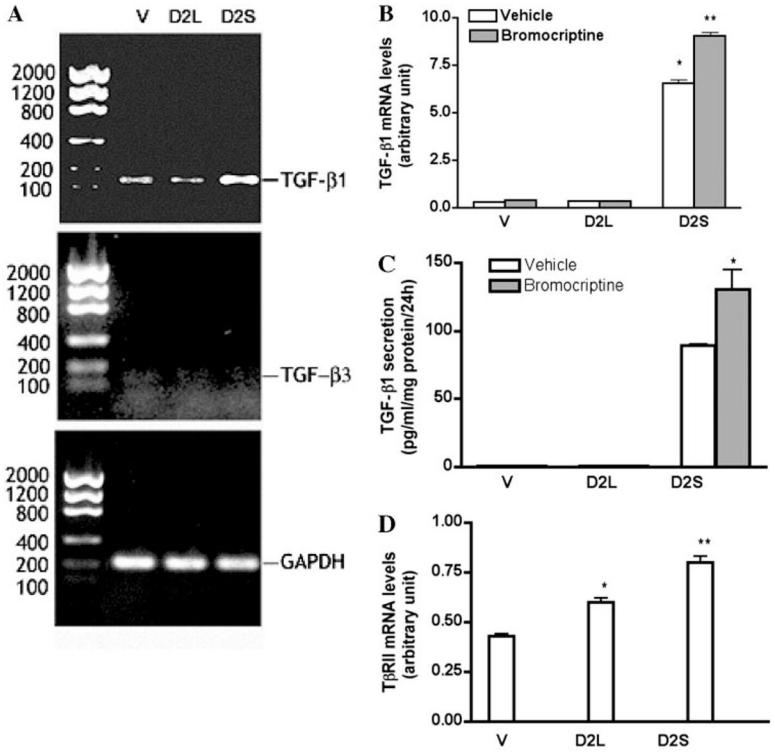

Fig. 7.

Ligand-independent TGFβ1 production in PR1 cells stably transfected with the D2S receptor. TGFβ1-deficient PR1 cells were stably transfected with the vector pcDNA 3.1 (V) or vectors containing a sequence that encodes the dopamine D2S or D2L. These cells were maintained in serum-free defined medium at a cell density of 5×10^5 for 24 h before experimentation. Cultures were then fed fresh medium and maintained for an additional 24 h in the presence or absence of 1 μM of bromocriptine to determine TGFβ1 and TβRII mRNA expression and protein secretion. Whether D2 receptor expression alters other isoforms of TGFβ was also tested by determining the mRNA levels of TGFβ3 in D2 receptor or vector gene-transfected PR1 cells. A, Representative gel figure showing the levels of TGFβ1 mRNA (*top*), TGFβ3 mRNA (*middle*), and GAPDH (*bottom*) in the same sample of PR1 cells containing V, D2S, or D2L as detected by RT-PCR. B, Detection of TGFβ1 mRNA levels using quantitative real-time PCR in V, D2S, and D2L cells with or without 1 μM of bromocriptine for 24 h (n = 6). *, $P < 0.05$, significantly different from the rest of the groups; **, $P < 0.05$, significantly different from the vehicle-treated D2S group. C, Detection of TGFβ1 levels using ELISA in the media collected from V, D2S, and D2L cells treated with or without 1 μM of bromocriptine for 24 h. The level of TGFβ1 was detectable only in D2S cells. *, $P < 0.05$, significantly different from the vehicle-treated D2S group. D, Measurement of TβRII mRNA levels using quantitative real-time PCR in V, D2S, and D2L cells. *, $P < 0.05$, significantly different from V cells; **, $P < 0.05$, significantly different from D2L cells.

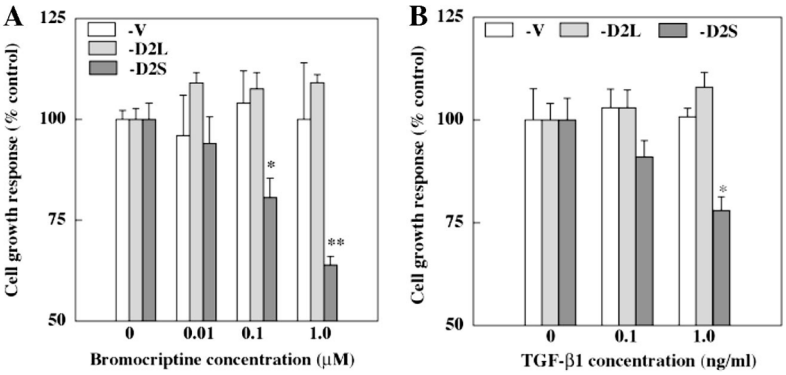

Fig. 8.

Recovery of dopamine's and TGFβ1's growth-inhibitory responses in PR1 cells stably transfected with the D2S receptor. PR1 cells were stably transfected with the native vector pcDNA 3.1 (V) or a vector containing a sequence that encodes the D2S or D2L dopamine receptor. The PR1 cells were maintained in serum-free defined medium at a cell density of 5×10^5 for 24 h before experimentation. A, Effect of different doses of bromocriptine treatment for 96 h on growth of V, D2L, and D2S cell lines. Data are mean ± SEM values of six cultures. *, $P < 0.05$, significantly different from the vehicle-treated D2S group; **, $P < 0.05$, significantly different from the 0.01 and 0.1 bromocriptine-treated D2S group. B, Effects of different doses of TGFβ1 treatment for 96 h on growth of V, D2L, and D2S cell lines. Data are mean ± SEM values obtained from six cultures. *, $P < 0.05$, significantly different from the vehicle-treated D2S group.

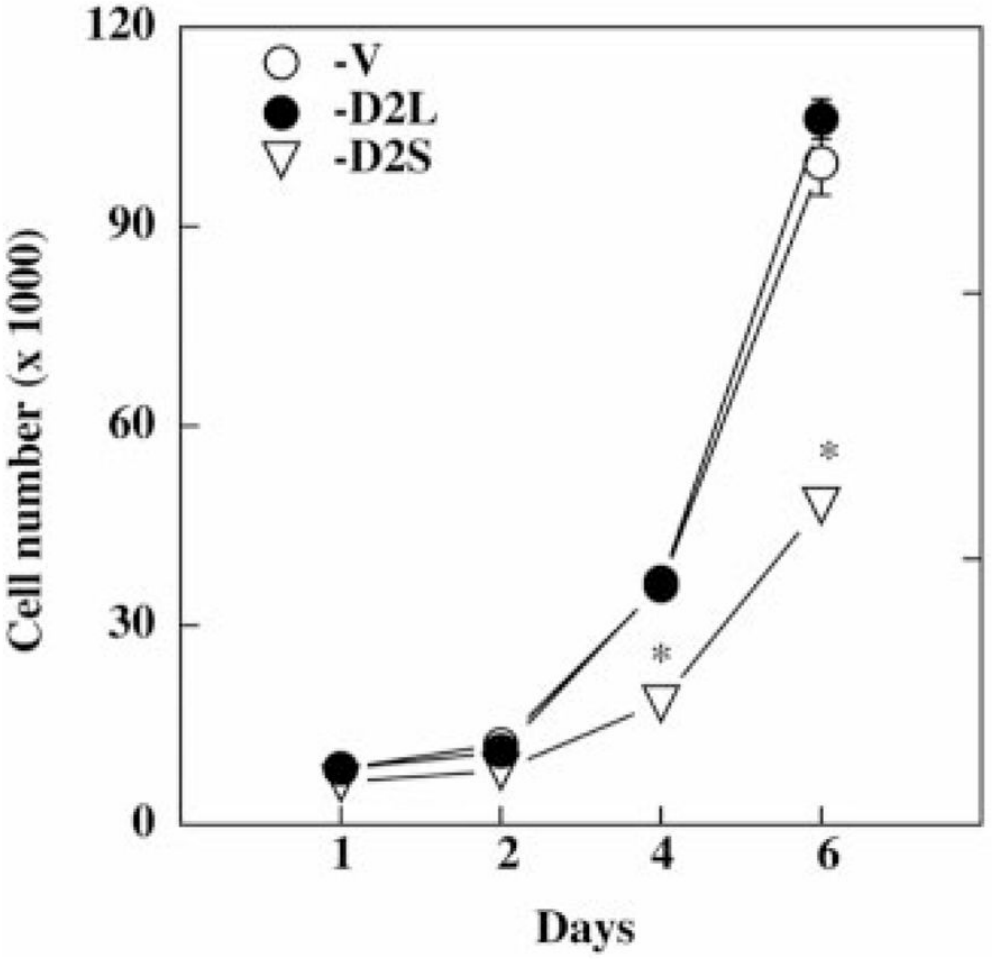

Fig. 9.

Reduced cell growth rate in PR1 cells stably transfected with the D2S receptor. Comparison of the cell growth rates of V, D2S, and D2L cell lines. *, $P < 0.05$, significantly different from the V and D2L groups on the same day. Data are mean ± SEM values of six cultures.

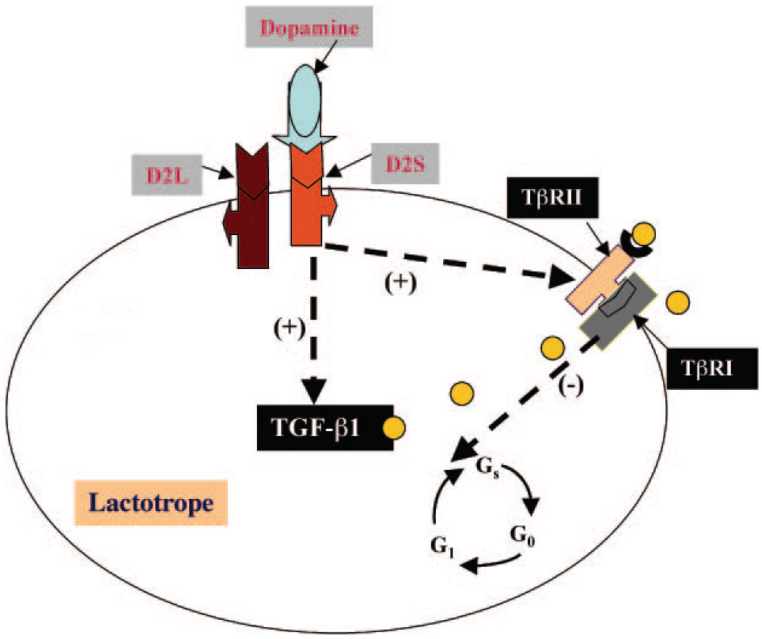

Fig. 10.

A diagram summarizing the postulated role of TGFβ1 signaling in mediation of dopamine action on lactotropic cell proliferation. It is hypothesized that dopamine activates dopamine D2 receptor's splice variant D2S to increase the production of TGFβ1 and the production and activation of TβRII. Activated TβRII by dimerizing with TGFβ receptor type I (TβRI) induces cell signaling to inhibit cell cycle progression and lactotropic cell proliferation.

TABLE 1

3H-Spiperone binding to the dopamine receptor in V, D2L, and D2S cells

Sample	Bmax (fmol/mg protein)	Kd (nM)
V	38.41 ± 4.2	0.0
D2L	220 ± 19.6	0.08 ± 0.02
D2S	255 ± 16.2	0.09 ± 0.02