

NOTE

Effect of Cabergoline, a Dopamine Agonist, on Estrogen-Induced Rat Pituitary Tumors: *In Vitro* Culture Studies

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Abstract. Cabergoline (CG) is a dopamine agonist that inhibits secretion of prolactin (PRL) and growth hormone. The purpose of this study was to investigate the PRL-lowering effect and antitumor effect of CG on estradiol-induced rat pituitary tumors *in vitro* and to elucidate these mechanisms. We compared the effects of CG with those of bromocriptine (BC) in terms of the inhibition of hormone secretion as well as antitumor effects on rat pituitary tumors. Primary cultures of dissociated pituitary tumor cells were used in these studies. A significant inhibition of prolactin (PRL) secretion was observed for both drugs within 12 h after treatment, and the inhibitory effects of CG and BC were antagonized by sulpiride or haloperidol. Inhibitory effect on PRL secretion after 12-h BC or CG pretreatment was more pronounced with CG than BC treatment at all time points. PRL secretion in group pretreated with CG was significantly suppressed at 72 h when compared to that of vehicle. Inhibition of *de novo* PRL synthesis was better demonstrated in the CG group. These findings suggest that CG has a higher affinity for the D₂ receptor of pituitary cells as compared to BC and may preferentially inhibit PRL secretion rather than PRL production. An antitumor effect of CG has been confirmed at a lower dosage than that of BC.

Key words: Cabergoline, Bromocriptine, Rat pituitary tumor, Prolactinoma, Prolactin

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THE TREATMENT of hyperprolactinemic disorders has been greatly improved since the advent of dopamine agonists. Bromocriptine (BC), an ergot derivative that is now in common clinical use, has a potent prolactin (PRL)-lowering effect [1–3] and an antitumor effect [3, 4–11]. However, BC requires administration several times daily and is not always well tolerated. Recent work in this field has been directed towards the search for drugs with more sustained action that could be given less frequently to patients and therefore improve compliance. On that basis cabergoline (CG) 1-[(6-allyl)ergoline-8 β -yl] carbonyl]-1-[3-(dimethylamino)

propyl]-3-ethylurea, an ergot derived compound with long lasting dopamine agonist effects, was developed [2, 3, 12–17]. However, little has been known about the action mechanism of CG on pituitary tumor, such as tumor shrinkage, cytotoxic effect and PRL-lowering effect. We have already examined in detail the *in vivo* effects of CG on estrogen (E₂)-induced rat pituitary tumor [18]. The continued oral administration of CG significantly reduced both the serum PRL level and the weight of the pituitary during 15 to 60 days of treatment as compared with BC. Morphologic studies revealed that CG reduced the size of the cells and of the granules, and increased the number of granules per unit cytoplasmic area [18]. The purpose of this study was to clarify the time-related changes and the mechanism of the PRL-lowering and antitumor effect of CG and BC on cultured E₂-in-

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duced rat pituitary tumors *in vitro*.

Materials and Methods

Induction of rat pituitary tumor

Female F344 rats (Charles River Japan Co., Ltd., Yokohama, Japan) at 4 weeks of age were used in all experiments. The rats were housed under controlled conditions (24 °C and artificial light from 0600 to 2000 h). A pituitary tumor was induced in each rat by subcutaneous implantation of a cholesterol pellet containing 20 mg estradiol-17 β (E₂) for 9 to 10 weeks. Such treatment increased pituitary weight to 96.7 ± 21.0 mg (mean \pm SEM) and the percentage of PRL cells to $78.5 \pm 2.2\%$.

Monolayer culture of rat pituitary tumor

While under ether anesthesia the rats were killed by decapitation and the enlarged pituitary tumor was isolated aseptically. The tumors were minced and incubated with 0.4% collagenase in phosphate buffered saline (PBS, pH 7.4) for 40 min at 37 °C with gentle shaking as previously described [19]. The monodispersed cell suspension in Ham's F 12 containing 10% fetal bovine serum was placed into 35 mm culture dishes at a density of $4-6 \times 10^5$ cells/dish. The culture dishes were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂. To determine optimal preincubation period for the best cultured tumor cell condition, pituitary tumor cells were incubated with vehicle, media with 10^{-7} -M dopamine or with 10^{-7} -M thyrotropin releasing hormone for 6 h on day 1, 3, 6, 9 and 12 of primary culture. PRL secretion of cultured tumor cells showed good response to thyrotropin releasing hormone or dopamine treatment after 3 days of the primary culture. Consequently, experimental studies were started on day 3 of culture.

Reagents

CG was kindly provided by Farmitalia Carlo Erba (Milan, Italy). Estradiol-17 β , BC, and sulpiride were purchased from Sigma Chemical Co. (St. Louis, MO). Haloperidol was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). CG and BC were dissolved in ethanol and further diluted with culture medium. The final concentration

of ethanol was 0.04% in experimental media and controls. Cultured cells were treated with CG and BC at the concentrations of 10^{-5} , 10^{-7} and 10^{-9} M.

Inhibitory effect on PRL secretion

Quadruplicate cultures were used for each experimental group. One hour prior to the beginning of an experiment, one ml of fresh medium was replaced and the cultures incubated. The medium was then removed and the cells were further incubated for 3, 6, 12, 24, 48, or 72 h in one ml of fresh medium with or without reagents at the concentrations noted above. Media were changed every 24 h. Sampled media were centrifuged $125 \times g$ at 4°C for 10 min to remove cell debris. Collected supernatants were frozen for radioimmunoassay. To elucidate the mechanism involved in the inhibition of PRL secretion by dopamine agonist, cultured cells were co-incubated with CG or BC and sulpiride, a selective D₂ antagonist, or haloperidol, a nonselective dopaminergic antagonist. The cells were treated for 3 h in one ml of medium with or without the following substances: 10^{-7} M CG, 10^{-7} M BC, 10^{-4} M sulpiride and 10^{-6} M haloperidol. After treatment media were collected and stored as described above.

Recovery of PRL secretion after withdrawal of D2 agonist

After 12 h pre-incubation with cabergoline or bromocriptine, cultured cells were rinsed twice with fresh medium and subsequently cultured in medium without a dopamine agonist for an additional 12 h. The medium was collected and stored as above.

PRL assay

PRL was measured by radioimmunoassay (RIA) using a double antibody method. The PRL standards (NIDDK rat PRL RP-3, AFP-4459B) were provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK Bethesda, MD) and the anti-rat PRL serum (HAC-RT26-01RBP85) by Dr. Wakabayashi (Institute for Molecular & Cellular Regulation, Gunma University, Maebashi, Japan). Radioiodinated rat PRL and goat anti-rabbit IgG were purchased from Du Pont/

NEN Research Products (Boston, MA) and Bio Makor (Rehovot, Israel), respectively.

Analysis of de novo synthesis of PRL

Monodispersed cells at a density of 6×10^5 cells/dish were precultured for 3 days. Culture media were replaced with leucine-free Eagle's minimum essential medium (MEM) (Nissui Pharma Co., Tokyo, Japan) supplemented with $10 \mu\text{Ci/ml}$ of [^3H] L-leucine (Du Pont/NEN Research Products, Boston, MA). After a three to 24 h incubation the dishes were rinsed with PBS and frozen at -20°C . The thawed cells were scraped from the dish and homogenized by sonication in ice-cold PBS. The samples were diluted 1:4 with PBS containing 1% bovine serum albumin (BSA-PBS), mixed with $100 \mu\text{l}$ of a 1:400 dilution of anti-rat PRL serum (HAC-RT26-01RBP85) in PBS containing 50 mM EDTA and 0.5% normal rabbit serum, and further incubated at room temperature for 24 h. The antibody-bound rat PRL was incubated for 12 h after addition of goat anti-rabbit IgG dissolved in $400 \mu\text{l}$ of PBS containing 50 mM EDTA, 3.5% polyethylene glycol (EDTA-PBS) and $100 \mu\text{l}$ of BSA-PBS. The mixture was centrifuged at $1500 \times g$ for 20 min at 4°C . The precipitate was washed twice with EDTA-PBS containing 0.25% Triton X-100, dissolved in $250 \mu\text{l}$ of 0.5 N NaOH and neutralized with $250 \mu\text{l}$ of 0.5 N HCl. A $200 \mu\text{l}$ aliquot was mixed with 10 ml of scintillation fluid (Clear-sol I; Nacalai Tesque Inc., Kyoto, Japan). The radioactivity in samples was measured in duplicate in a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). Specific counts were calculated from the difference between total count and nonspecific count (14250 dpm).

MTT assay

MTT assay was performed using previously described techniques [20]. The monodispersed cell suspension (0.5 ml) was placed into a 24-well dish at a density of 1.0×10^5 cells/well. Cultured tumor cells were treated with BC or CG at 10^{-5} , 10^{-7} , 10^{-9} M from day 3 of culture. Culture medium with or without reagents was changed every 24 h.

For the MTT assay, media were replaced with $500 \mu\text{l}$ of PBS containing 0.1% sodium succinate and 0.4% 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and incubated for 3 h at 37°C in a CO_2 incubator. After incubation

the MTT solution was aspirated and MTT formazan was dissolved with $500 \mu\text{l}$ of dimethyl sulfoxide. A $100 \mu\text{l}$ aliquot was transferred in duplicate into a 96-well microplate and optical density (OD) was measured with a microplate reader (EAR340AT, SLT Labinstrument, Co., Ltd., Austria) with a test wavelength of 570 nm and a reference wavelength of 620 nm. Linearity in OD of the MTT assay under our experimental conditions occurred at 2.3×10^4 to 7.0×10^5 cells/well and the values in every experimental group were within the linear range.

Statistical method

Data were reported as the mean \pm SEM. Differences between two groups were statistically analyzed by Mann-Whitney U test. A level of P less than 0.05 was accepted as statistically significant.

Results

PRL secretion from cultured pituitary cells

Pituitary tumor cells showed higher basal PRL values on day 3 and 6 of primary culture and showed the best response of PRL secretion to thyrotropin releasing hormone or dopamine treatment on day 3. Experimental studies were therefore started on day 3 of culture.

BC and CG did not significantly decrease PRL secretion at any concentration within the first 3 h of treatment. The inhibitory effect of these reagents on PRL secretion began to appear at 6 h after treatment, and after 12 h a significant decrease in PRL secretion was detected at all concentrations of both agents (Fig. 1). These inhibitory effects became to be almost constant at 24 h after treatment. Although dose-dependent inhibition was observed in groups treated with BC, no difference in the inhibitory effect at the highest concentration (10^{-5} M) was evident between BC and CG. In contrast, the effect at the lower concentrations (10^{-7} M and 10^{-6} M) was more pronounced with CG than BC (Fig. 1).

The inhibitory effect of CG and BC at the same concentration was antagonized by concomitant treatment with 10^{-4} M sulpiride or 10^{-6} M haloperidol (Fig. 2). Also, sulpiride stimulated PRL

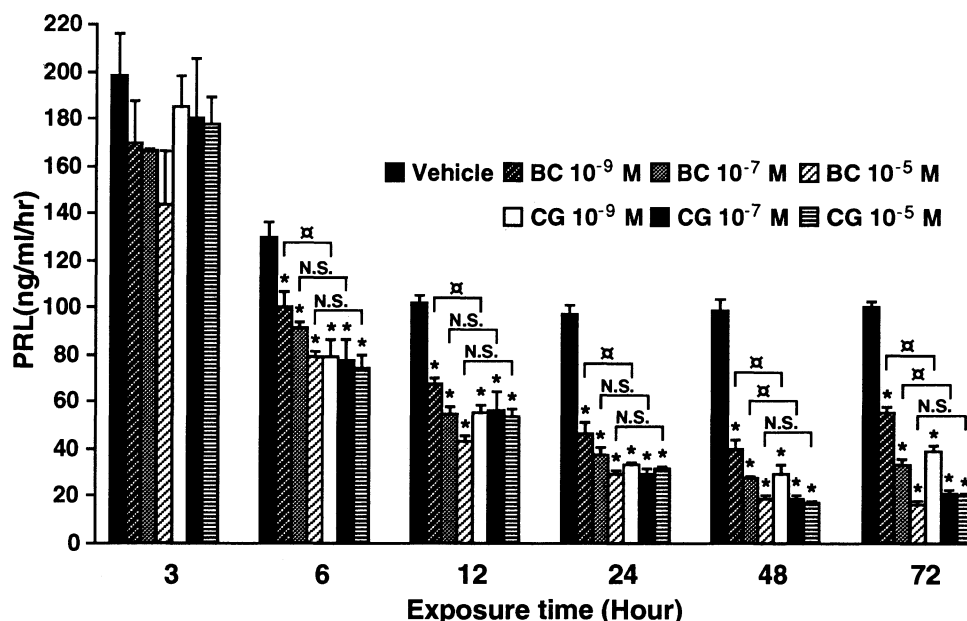


Fig. 1. Inhibitory effect of bromocriptine (BC) and cabergoline (CG) on prolactin (PRL) secretion. No statistically significant difference at the $P < 0.05$ level between any groups treated for 3 h. * $P < 0.05$ vs. comparable vehicle, $\square P < 0.05$. N.S., not significant.

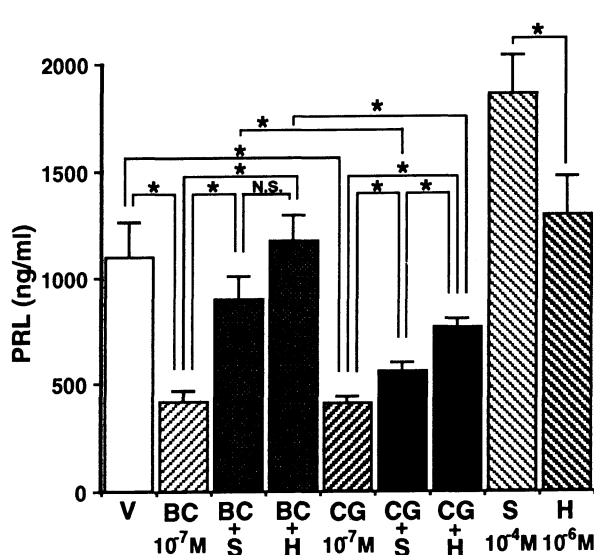


Fig. 2. Effects of the dopamine (D_2) antagonists sulpiride (S) and haloperidol (H) on inhibition of prolactin (PRL) secretion. Inhibitory effect of 10^{-7} M BC or CG was antagonized by a 3 h co-incubation with 10^{-4} M sulpiride (S) or 10^{-6} M haloperidol (H). * $P < 0.05$. N.S., not significant; V, vehicle.

secretion, while haloperidol did not. These findings demonstrate that CG also inhibits PRL secretion via D_2 receptors.

Recovery of PRL secretion after withdrawal of D_2 agonist

Inhibitory effect on PRL secretion after withdrawal of D_2 agonists was more pronounced with CG than BC treatment at all time points. Although no significant difference between these two groups was detected, PRL secretion in the group pretreated with CG was significantly suppressed at 72 h when compared to that with vehicle alone (Fig. 3). These results suggest that CG has a higher affinity binding to the D_2 receptor of the tumor cells than BC.

Analysis of *de novo* synthesis of PRL

De novo synthesis of PRL was measured in terms of immunoprecipitable radioactivity of [3 H] PRL per dish. Both CG and BC suppressed *de novo* synthesis of PRL within the first 3 h after treatment. This effect of BC persisted 24 h after treatment, while CG did not suppress *de novo* synthesis of PRL in tumor cells after 6 h of treatment (Fig. 4).

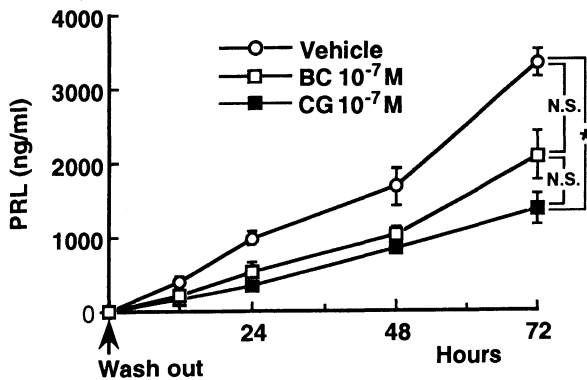


Fig. 3. Recovery of prolactin (PRL) secretion after 12 h incubation and withdrawal of 10^{-7} M bromocriptine (BC) or cabergoline (CG). * $P < 0.05$. N.S., not significant.

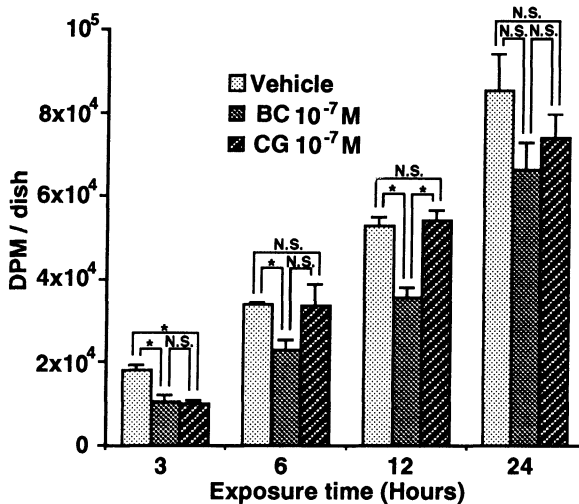


Fig. 4. Effect of bromocriptine (BC) and cabergoline (CG) on *de novo* synthesis of prolactin (PRL). Ordinate indicates the disintegrations per minutes (DPM) of intracellular [3 H] PRL/dish. * $P < 0.05$. N.S., not significant.

Antitumor effect

Antitumor effect of D_2 agonists was evaluated with the MTT assay. In the CG group, an antitumor effect was demonstrated on the 4th and the 6th day of the treatment at the concentrations of 10^{-5} to 10^{-9} M (Fig. 5). BC had an antitumor effect only at the highest concentration after 6 days of treatment. CG had a greater antitumor effect on cultured pituitary tumor cells as compared to BC.

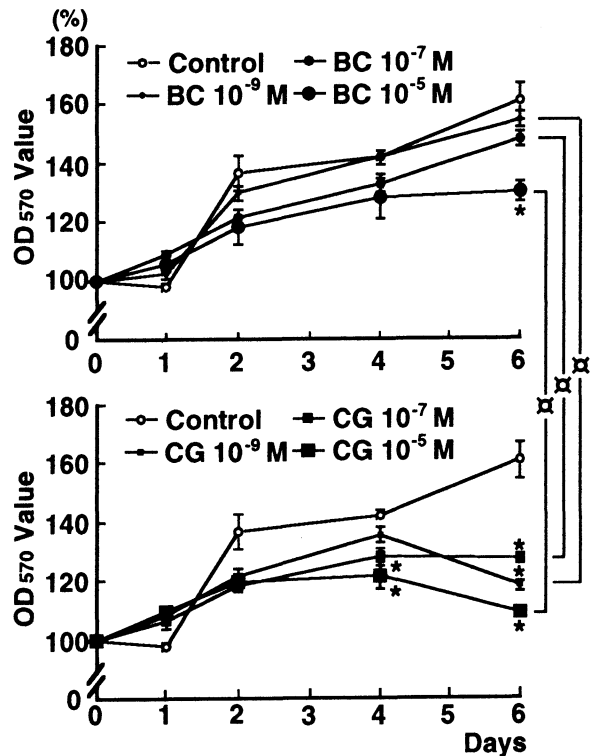


Fig. 5. Antitumor effect of bromocriptine (BC, upper) and of cabergoline (CG, lower) evaluated by chromogenic assay. * $P < 0.05$ vs. comparable control, \square $P < 0.05$.

Discussion

BC was the first compound that was effective in the treatment of hyperprolactinemic disorders. Prolonged administration of this agent results in rapid tumor shrinkage [2, 4, 9, 13, 21–23]. This agent also has a potent PRL-lowering effect and cytotoxic effect in *in vitro* studies of human pituitary adenomas [6, 24–27].

It has been well established that chronic E_2 treatment in rats induces PRL-secreting pituitary tumors. Such tumors also possess D_2 dopamine receptors similar to those present in normal tissue [28–30]. We have compared the effectiveness of CG and BC by using E_2 -induced rat pituitary tumors as a model for human prolactinomas [18].

In the present study CG had a marked PRL-lowering effect on cultured rat pituitary tumor cells. This effect was equal to that of BC and is in agree-

ment with the findings of previous studies [15, 16] that utilized rat pituitary cells *in vitro*. The fact that the PRL-lowering effect of CG, like BC, is blocked by dopamine receptor antagonists demonstrated that this effect is mediated by D₂ receptors of lactotrophs. The present study also demonstrated that the inhibitory effect on PRL secretion occurred at lower concentrations of CG than BC. In addition, the recovery of PRL secretion after withdrawal of CG was slower than that of BC. These findings suggest that CG has a higher affinity for the D₂ receptor of pituitary cells as compared to BC. A previous study has shown that the affinity of CG for dopamine receptors is about twice that of BC in rat striatal tissue [31].

BC reduces the rate of PRL synthesis, probably as a result of increased intracellular PRL content within lactotrophs. The present results agree well with those of previous studies [9, 32, 33]. In contrast, CG reduced the rate of PRL synthesis within 3 h of treatment, but thereafter it did not inhibit *de novo* synthesis of PRL, regardless of continued inhibition of PRL secretion from tumor cells. These findings support our results that the chronic administration of CG increases the number of PRL-immunoreactive secretory granules per unit cytoplasmic area in *in vivo* study [18]. Although the reason for these differences remains unknown, one possibility is that CG may preferentially inhibit PRL secretion rather than PRL production.

In vitro treatment of adenoma cells with BC induces numerous intracellular vacuoles, probably originating from dilated rough endoplasmic reticulae [6, 26, 34], and results in cell death. Such a direct cytotoxic effect by BC is more pronounced on PRL-secreting adenoma cells than on growth hormone-secreting adenoma cells [6]. From immunoelectron microscopic observation, we previously verified that CG induced degenerating PRL tumor cells with vacuolized and fragmented rough endoplasmic reticula in *in vivo* treatment [18]. In the present study the antitumor effect of CG or BC on cultured pituitary cells was examined by MTT assay, a quantitative colorimetric assay for

mammalian cell cytotoxicity and proliferation [20, 35, 36]. The present result confirmed that CG has more potent cytotoxic effects against pituitary tumor cells than BC. However, we have not yet tested by the appropriate inhibition studies whether the antitumor effect of CG is mediated by D₂ dopamine receptors alone.

The many drugs that have been reported to suppress PRL secretion and shrink prolactinomas include pergolide [5, 37, 38], CV 205-502 [39-43], injectable BC [44], lisuride [22, 45], metergoline [45], terguride [46, 47], dehydroergocryptine [48], mesulergin [49], and CG [2, 3, 12, 13]. Of these drugs pergolide, CV 205-502, an injectable form of BC and CG are reported to be long-acting and will suppress PRL levels for approximately 24 h, 24 h, 28 days and 7 days, respectively. Their side effects are similar to those of BC [22, 44, 48], but CV 205-502 and CG are generally better tolerated than BC [2, 12, 13, 17, 42, 43].

We were able to demonstrate *in vitro* that CG has several advantages in terms of PRL-lowering effect and antitumor effect on E₂-induced rat pituitary tumors. These advantages might be attributable to a higher affinity for the D₂ receptor of pituitary tumor cells and its chemical stability [18] as compared with that of BC.

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