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Effects of Dopaminergic Drugs on the Mast Cell Degranulation and Nitric Oxide Generation in RAW 264.7 Cells

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Effects of dopaminergic drugs on the degranulation of mast cells (RBL-2H3 cells) and the nitric oxide production from macrophage cells (RAW 264.7) were studied. Among the dopaminergic agonists and antagonists tested, bromocriptine, 7-OH-DPAT, haloperidol, and clozapine showed potent inhibitions of mast cell degranualtion (IC_{50} value, 5 μM). However, these dopaminergic agents did not affect the tyrosine phosphorylations of the signaling components of the high affinity IgE receptor (FcεRI), such as Syk, PLCγ1, and PLCγ2.; This suggested that these signaling components were not involved in the inhibition of the mast cell degranulation by these compounds. On the other hand, dopamine, bromocriptine, 7-OH-DAPT, and haloperidol markedly inhibited the nitric oxide production from RAW 264.7 cells (IC_{50} values, 10-20 μM). Bromocriptine, a dopamine agonist that is routinely used for the treatment of Parkinsons disease, inhibited the expression of the inducible nitric oxide synthase at an early stage of the LPS-induced protein expression in a dose-dependent manner. The results suggested that these dopaminergic agents, when used for the treatment of dopamine receptors-related diseases, such as Schizophrenia or Parkinsons disease, might have additional beneficial effects.

Key words: Dopaminergic drugs, Mast cell, Degranulation, Macrophage, Nitric oxide

INTRODUCTION

The main uses of dopaminergic drugs are for the long-term treatment of Parkinson's disease or Schizophrenia. Various adverse effects have been reported for some motor functions, such as the extrapyramidal syndrome and tardive dyskinesia, some mental functions, and the cardiovascular system. It has also been reported that dopamine-related drugs also affect the immune system. For example, the destruction of the brain dopaminergic system with 6-hydroxydopamine compromises peripheral immunity (Filipov *et al.*, 2002), and various dopaminergic drugs such as haloperidol, bromocriptine, L-DOPA, and clozapine have opposite effects (Nozaki *et al.*, 1996; McMurray, 2001; Panajotova, 1997; Carr *et al.*, 2003; Song *et al.*, 2000). However, with the exception of clozapine, which is an atypical antipsychotic with less adverse effect on motor functions (Sano *et al.*, 1990), the effects of dopaminergic drugs on the mast cell degranulation have not been reported.

Nitric oxide mediates both the host defense and inflammations. In particular, the inflammatory processes in Parkinson's disease are associated with the up-regulation of inducible nitric oxide synthase (iNOS) (Hunot *et al.*, 1996), and nitric oxide-mediated cytotoxicity in substantia nigra is one of the best-characterized mechanisms for the development of Parkinsons disease.

In this study, in order to see whether the inhibitions of mast cell degranulation are common properties of dopaminergic drugs, we determined the effects of several dopaminergic drugs on the mast cell degranulation in connection with their effects on the signaling components of the high affinity IgE receptor (FcεRI). In addition, we also determined the effects of dopaminergic drugs on the production of nitric oxide from macrophage cells in order to test whether these drugs have extra beneficiary effects on the prognosis of Parkinson's disease in which nitric oxide has detrimental effects.

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MATERIALS AND METHODS

Materials

All the dopaminergic drugs were obtained from Sigma Chemical Co. (St. Louis, MI, USA). Protein and nitrite assay kits were from Promega (Madison, WI, USA). Antibodies for iNOS and PLCγ1 were from Sigma Chemical Co, and antibodies for PLCγ2 and phospho-ERK were from Santa Cruz (Santa Cruz, CA, USA).

Measurement of β-hexosaminidase release from RBL-2H3 cells

The procedure, which was previously described by Oak *et al.*, was followed (Oak *et al.*, 1999). RBL-2H3 cells were treated with IgE (0.5 μg/mL) overnight. The cells were washed and pre-incubated in PIPES buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA) for 10 min at 37°C. Then, the cells were treated with antigen (DNP-BSA, 1 μg/mL) for 10 min at 37°C. After centrifugation, the supernatants were added into 96-well plates, and were incubated with the substrate (1 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide) for 1 h. The reaction was stopped by adding 0.1 M Na_2CO_3, and the absorbance at 405 nm was measured with an ELISA reader.

Nitric oxide analysis

The concentration of nitric oxide was determined by measuring the amount of nitrite from the cell culture supernatant using the Griess reagent according to the manufacture's protocol (Ryu *et al.*, 1999).

Immunoblotting

RAW 264.7 cells were stimulated with either 1 μg/mL LPS alone or with the dopaminergic agents for 24 h. Cells were freeze-thawed 3 times, and were centrifuged at 45,000×g for 30 min. The supernatants which contain 20 μg of protein were analyzed by the 10% SDS-PAGE gel, and the blots were probed with antibodies for iNOS.

Phosphorylation studies using phosphorylated amino acids-specific antibodies

RBL-2H3 cells were treated with IgE overnight, and washed with the PIPES buffer containing the phosphatase inhibitors (1 mM EDTA, 1 mM sodium orthovanadate, 5 mM sodium fluoride), and then treated with DNP-BSA (100 ng/mL) for 1 min. The cells were collected in lysis buffer (200 mM boric acid, 160 mM NaCl, 0.3% Triton X-100, 1% BSA, pH 8.0, 10 μg/mL leupeptin and aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 5 mM sodium fluoride), freeze-thawed 3 times, and centrifuged at 48,000 g for 30 min. The postnuclear supernatants were immunoprecipitated with anti-phosphotyrosin, anti-phosphoserine, or anti-phosphothreonine antibodies (Santa Cruz Biotechnology or Sigma Chemical Co). The beads were boiled for 5 min in Laemmli buffer and were analyzed by Western blotting with antibodies for Syk, PLCγ1, PLCγ2, and ERK.

RESULTS AND DISCUSSION

Mast cell degranulation, a critical step in allergic diseases, was determined by measuring the β-hexosaminidase release. β-hexosaminidase is stored in the secretory granules of mast cells, and is released, along with histamine, when the mast cells are immunologically activated. It has frequently been employed as a marker molecule for examining the degranulation process of mast cells (Schwartz *et al.*, 1979).

Among the drugs tested, bromocriptine (D_2 receptor agonist), 7-OH-DPAT (selective D_3 receptor agonist), clozapine (antagonist, atypical antipsychotic), and haloperidol (D_2 receptor antagonist, typical antipsychotic) showed strong inhibitory activities upon the release of β-hexosaminidase in a dose-dependent manner (Fig. 1) with the IC_{50} value around 5 μM. In terms of their inhibitory activities on mast cell degranulation, these drugs are as potent as quercetin, a well-known flavonoid compound that is strong inhibitor of mast cell degranulation (Lee *et al.*, 1999), and they are more potent than azelastine (IC_{50} value, 26±3.2 μM), an anti-allergic drug (Fischer and Schmutzler, 1981). However, other dopamine agents showed relatively weak activities upon the release of β-hexosaminidase at 30 μM, dopamine, 10%; sulpiride (D_2/D_3 receptor antagonist, atypical antipsychotic), 21%; and U99194A (selective D_3 receptor antagonist), 26%.

In order to determine their cellular targets for the inhibition of mast cell degranulation, the effects of dopaminergic agents on the FceRI-mediated (high affinity IgE receptor) tyrosine phosphorylation of signaling components, such as Syk, PLCγ1, and PLCγ2, were tested. As shown in Fig. 2, the aggregation of FcεRI on the RBL-2H3 cells increased the tyrosine phosphorylation of PLCγ1 (A), PLCγ2 (B), and Syk (C) (compare lane 2 vs 3). However, none of these dopaminergic drugs affected the tyrosine phosphorylation of the signaling components that were tested, which suggests that their inhibitory effects on mast cell degranulation do not involve IgE receptor signaling.

The effects of the dopaminergic drugs on nitric oxide production were determined by measuring their effects on the production of nitrites (NO_2^-) from the murine macrophage cell line, RAW 264.7 cells. When RAW 264.7 cells were incubated with 1 μg/mL of lipopolysaccharide (LPS) for 24 h, the nitrite content in the media increased up to 4 to 5 times. However, when the cells were treated with 1-50 μM of dopaminergic drugs for 24 h, the nitrite content

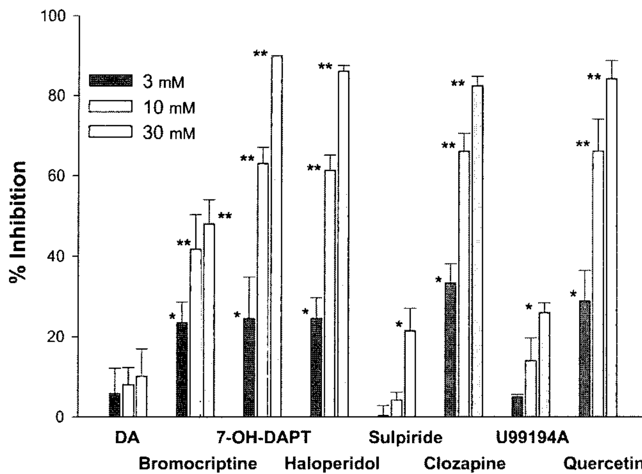

Fig. 1. Inhibitory effects of dopaminergic agents upon the release of β-hexosaminidase. RBL-2H3 cells were treated with 0.5 μg/mL IgE overnight. The cells were treated with dopaminergic drugs for 20 min, and then were stimulated with 1 μg/mL DNP-BSA for 10 min.

% Inhibition
=(Treated-Blank-Spontaneous)/(Control-Blank-Spontaneous)

Control: normal allergen-IgE response was evoked with test material not added.
Treated: normal allergen-IgE response was evoked with test material added.
Blank: only the test material and substrate were added into the ELISA plate.
Spontaneous: allergen-IgE response was not evoked when the test material was not added.

Each data point represents a mean±S.E.M. of triplicate determinations, and distinct triplicates were measured for each determination (n=3). The Student's *t*-test was used for statistical analysis. *: $p<0.1$ compared with the control group. **: $p<0.01$ compared with the control group.

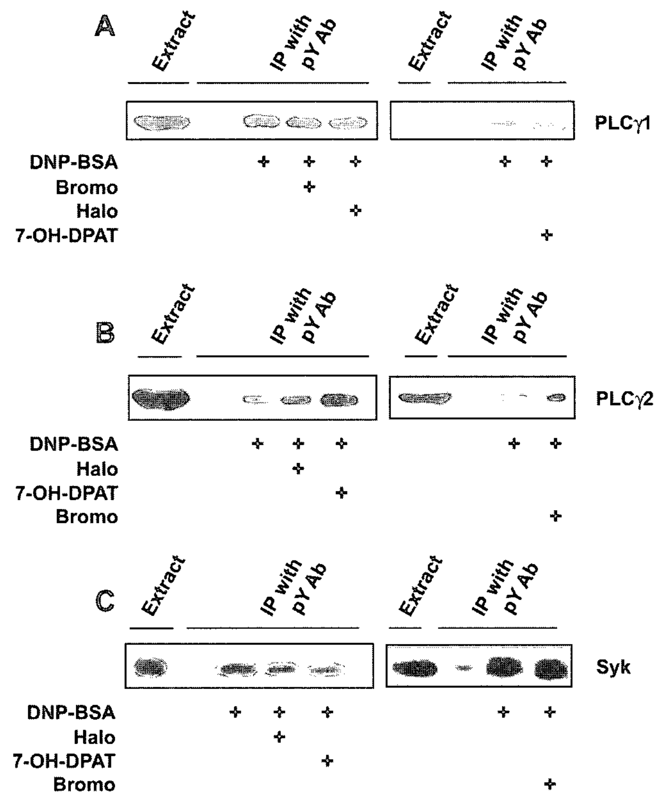

Fig. 2. Effects of dopaminergic drugs on the FcεRI-mediated tyrosine phosphorylation of PLCγ1, PLC-γ2, and Syk. RBL-2H3 cells were stimulated with 1 μg/mL DNP-BSA and/or with 25 μM 15,16-dihydrotanshinone-I for 20 min. The cell supernatants were immunoprecipitated with agarose beads that were conjugated to phosphotyrosine antibodies. The samples were analyzed by a 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and blotted with antibodies for PLCγ1, PLCγ2, and Syk at 1:1,000 dilutions. The same results were obtained with three independent immunoprecipition studies.

was significantly reduced in a dose dependent manner, and the IC_{50} value was approximately 10-20 μM (Fig. 3A for agonists, Fig. 3B for antagonists). All the dopaminergic agonists tested, with the exception of quinpirole, strongly inhibited LPS-induced nitric oxide production. In contrast, for antagonists, haloperidol and clozapine less efficiently inhibited the nitrite production.

Since bromocriptine is widely used for the treatment of Parkinsons disease, the effects of bromocritpine on the iNOS expression were further studied through immunoblotting of inducible nitric oxide synthase after bromocriptine treatment. In order to test cell viability, the cells were treated with bromocriptine for 24 h, which is long enough to induce cell death. As shown in Fig. 4A, the inhibition of nitric oxide production did not involve the cytotoxicity.

To determine the stage at which bromocriptine affects the production of nitric oxide, the cells were treated with bromocriptine at various time points after LPS stimulation. Bromocriptine inhibited the expression of iNOS in a dose-dependent manner when they were treated at the same time (Fig. 4B). However, once the cells were pre-stimulated with LPS, the effects of bromocriptine on the iNOS expression were markedly reduced (Fig. 4C), suggesting that bromocriptine affects the initial stage of LPS-induced expression of certain genes that are involved with the production of nitric oxide. Considering that iNOS plays an important role in the the development of Parkinsons disease in terms of cytotoxicity, the activity of bromocriptine inhibiting nitric oxide production will be additionally beneficial for the treatment of Parkinsons disease.

The effects of dopaminergic drugs on mast cell degranulation or nitric oxide production seem to be unrelated with dopamine receptors or with their effects on motor functions because i) the antagonists did not block the activities of agonists from inhibiting mast cell degranulation (data not shown), ii) both the agonists and antagonists showed inhibitory effects, iii) and there were no differences between typical and atypical antipsychotics.

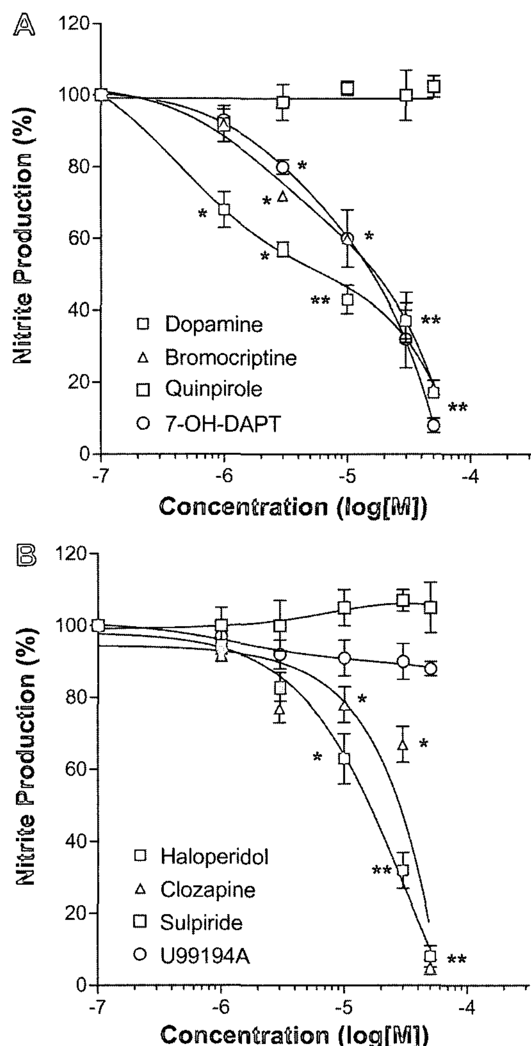

Fig. 3. Effects of dopaminergic drugs on the production of nitric oxide from RAW 264.7 cells. **A**, **B**. Effects of dopaminergic agonists and antagonists on the production of nitric oxide.

% NO production
= (Treated-Blank-Spontaneous)/(Control-Blank-Spontaneous)

Control: cells were treated with LPS.
Treated: cells were treated with LPS and the test material.
Blank: only the test material and Griess reagent were added.
Spontaneous: only the Griess agent was added.

Each data point represents a mean±S.E.M. of triplicate determinations, and distinct triplicates were measured for each determination (n=3). The Students *t*-test was used for the statistical analysis. *: p<0.1 compared with the control group. **: p<0.01 compared with the control group.

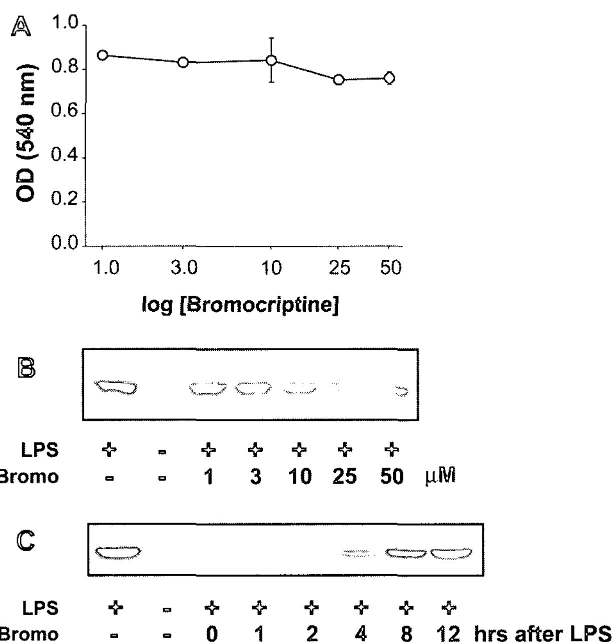

Fig. 4. Effects of bromocriptine on the cell viability and on iNOS expression from RAW 264.7 cells. **A**. Effects of bromocriptine on the viability of RBL 2H3 cells. Cells were treated with 0-50 μM of bromocriptine for 24 h. XTT labeling mixture (50 μL) was added followed by incubation for 5 h at 37°C in 5% CO_2. Each data point represents a mean±S.E.M. of triplicate determinations. **B**. Dose-response relationship of bromocriptine on iNOS expression. Samples were prepared as described in the Materials and Methods. The immunoblots were probed with antibodies for iNOS at 1:1000 dilutions, and an alkaline phosphatase-conjugated anti-rabbit IgG was used as the secondary antibody at 1:5000 dilutions. **C**. Time course for the effects of dopaminergic drugs on LPS-induced iNOS expression. LPS(+)/Bromo (-), positive control; LPS(-)/Bromo(-), negative control.

In summary, our results provide important information for the clinical uses of dopaminergic drugs. Since a majority of the dopaminergic drugs that we tested, irrespective of them being agonists or antagonists, strongly inhibited mast cell degranulation and nitric oxide production in macrophages, and because these drugs are usually used on a long-term basis, an extra consideration should be taken for the patients whose the immune functions are impaired.

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