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BIOCHEMICAL ALTERATIONS OF DOPAMINE RECEPTOR RESPONSES FOLLOWING CHRONIC L-DOPA THERAPY

KEITH D. WILNER, IAN J. BUTLER, WILLIAM E. SEIFERT, JR. and YVONNE C. CLEMENT-CORMIER

Departments of Pharmacology, Neurobiology, Neurology and Biochemistry, University of Texas Medical School at Houston, Houston, TX 77030, U.S.A.

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Abstract—The effects of chronic L-dopa treatment on rat striatal adenylate cyclase and dopamine-receptor binding activities were studied using an oral dose of Sinemet (250 mg L-dopa/25 mg carbidopa). The calculated average daily oral intake of L-dopa was 150 mg/rat. A 4-fold increase in the EC_{50} for dopamine on adenylate cyclase activity in homogenates of the caudate nucleus was observed in the oral drug-treated group with no change in the maximal level of enzyme activity. Binding studies using [^{3}H]spiroperidol, a dopamine-receptor antagonist, revealed a decrease in the dissociation constant from 0.26 nM in the control group to 0.069 nM in the oral drug-treated group. In addition, the B_{max} for [^{3}H]spiroperidol specific binding in the animals receiving oral L-dopa increased by 300 fmoles/mg over that observed in the control group. Dopamine-sensitive adenylate cyclase and binding activities in animals receiving a lower dose of L-dopa alone, given intraperitoneally (50 mg/kg) twice daily, were determined to be similar to control values. Analysis of the cerebrospinal fluid biogenic amine metabolites, homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), by gas chromatography/mass spectrometry revealed a 13-fold increase in HVA following oral L-dopa and a 44 per cent increase in MHPG levels. These data, which demonstrate an increased number of dopamine binding sites following long-term L-dopa therapy, are consistent with demonstrations of behavioral hypersensitivity in animals undergoing this particular drug treatment. The results also suggest that the subsensitivity of the adenylate cyclase system may reflect a side effect of this drug, due to prolonged administration.

For the past 10 years, 3,4-dihydroxy-L-phenylalanine (L-dopa) has been used successfully as a therapeutic agent for Parkinson's disease. Although substantial data exist to document the therapeutic efficacy of this drug treatment [1], a surprisingly small number of chronic L-dopa studies measuring biochemical changes in receptors appear in the literature [2–4]. Long-term drug studies in animals can provide valuable clinical information on the mechanism of drug action and can contribute to a better understanding of the side effects of such therapy. Clinical observations made by Cotzias *et al.* [5] have shown that the initial therapeutic efficacy of L-dopa is enhanced by chronic treatment, and that the maintenance dose for many patients may be reduced. However, most patients on prolonged L-dopa therapy either become refractory to the drug [6], or develop 'on–off' phenomena [7] or dyskinesias [8]. Although the precise mechanism by which this refractoriness occurs remains to be elucidated, one suggestion which has been offered to explain refractoriness during chronic L-dopa treatment is that, with the progression of the disease, there may be a decrease in the number of dopaminergic cells in the Parkinsonian patient [9, 10]. This reduction in dopamine neurons might lead to receptor hypersensitivity, but the increase in receptor activity would not be sufficient to compensate for refractoriness. Another possibility is that L-dopa-induced refractoriness may be due to changes in the molecular topography of the dopamine receptor. Thus, the administration of L-dopa in combination with other agents which interact with a specific class of dopamine receptors, such as the ergot alkaloids [11], have been used to overcome the refractoriness associated with long-term L-dopa treatment.

Biphasic alterations between dyskinetic episodes and the re-emergence of Parkinsonism are complex incidents which have been termed 'on–off' phenomena. This event is best defined as a rapid, unpredictable oscillation between dyskinetic and akinetic states which are unrelated to the timing of L-dopa administration [7, 12–14]. The biochemical manifestations of these phenomena are still unclear, but it has been suggested that a sudden change in either the effective dopamine concentration at the receptor sites or a desensitization of brain dopamine receptors due to elevated dopamine levels in L-dopa-treated Parkinson's patients may be responsible for the on–off phenomena [15, 16]. One approach to elucidating the mechanism by which L-dopa precipitates these side effects is to assess the biochemical integrity of the dopamine receptor following long-term L-dopa therapy. Several parameters have been used successfully in the past to characterize the biochemical and molecular changes in the dopamine receptor. The ones selected for this study were dopamine-sensitive adenylate cyclase and dopamine receptor binding using [^{3}H]spiroperidol. For comparative purposes, two routes of drug administration, an oral dose of Sinemet (250 mg L-dopa, 25 mg carbidopa)

and an intraperitoneal (i.p.) injection of a low dose of L-dopa twice daily (50 mg/kg), were also included in this study.

It has been discovered that long-term drug therapy which alters the level of one neurotransmitter in the central nervous system (CNS) may effect changes in other transmitter systems [17]. This response may be a compensatory change which has therapeutic benefit or an unwanted secondary side effect of the drug regimen. Therefore, in addition to studying the biochemical responsiveness of the dopamine receptor following chronic L-dopa treatment, data are presented on the CNS norepinephrine-sensitive adenylate cyclase in control and drug-treated animals along with cerebrospinal fluid (CSF) measurements of γ-aminobutyric acid (GABA) and several neurotransmitter metabolites.

MATERIALS AND METHODS

For the chronic i.p. injection study (21 days), male Sprague–Dawley rats (Timco Laboratories, Houston, TX, 150–250 g) were housed twelve to a cage. Food and water were available *ad lib.* throughout the experiment. L-dopa stock solution was prepared weekly by dissolving 15 mg L-dopa/ml in 0.2 N HCl. The i.p. injection dose, 50 mg/kg, was made fresh daily by diluting 1 vol. of stock L-dopa solution with 2 vol. of normal saline to a final concentration of 5 mg/ml and a pH of 3.2. Randomly, 25 per cent of the rats were weighed daily and an average dose was computed based on the average weight. Intraperitoneal injections were given twice daily (morning and late afternoon). The average dose computed for the morning injections were used also for the afternoon injections. Control animals were injected with physiological saline (pH 3.2).

For the chronic oral study (11 days), rats were housed four to a cage. Sinemet tablets, containing 250 mg L-dopa and 25 mg carbidopa, were pulverized and homogenously mixed with the standard diet, using a Hobart mixer, so that the final concentration of the drug was 250 mg L-dopa/20 g of laboratory chow. Controls were given only the standard diet. The control rats consumed approximately 21 g of food/day, while the experimental rats on the average consumed 12 g of food/day. Thus, the average consumption of L-dopa was about 150 mg/rat/day. These values were calculated by monitoring the daily food intake.

CSF was collected from individual rats anesthetized with an i.p. injection of chloral hydrate (400 mg/kg). The cisterna magna was punctured with a blunted Butterfly needle attached to a cannula, and the CSF was collected by gravity into a glass microfuge tube on ice [18]. Using this procedure, approximately 300 μl of CSF can be collected. CSF collected for GABA analysis was blood free; some samples in which the metabolites were analysed contained trace amounts of blood. The samples were centrifuged to remove blood elements prior to storage. Preliminary studies have demonstrated that these small quantities of blood do not alter CSF metabolite levels significantly (I. J. Butler, unpublished observations).

To assess the metabolism of the biogenic amines, the metabolites homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) were determined in CSF by combined gas chromatography/mass spectrometry (g.c./m.s.), using stable isotope-labeled internal standards. The procedure used was a modification of the method used by Swahn *et al.* [19]. The deuterated analogs of the metabolites, HVA-d_2, 5-HIAA-d_2 (Merck & Co., Rahway, NJ) and MHPG-d_3 (LKB Instruments, Inc., Pleasant Hill, CA) (100 ng each), were added to 150–250 μl of rat CSF (which had been stored with ascorbic acid at −80°). After adjusting the pH to 2.5 with 0.1 N HCl, the solution was saturated with NaCl and extracted three times with 2 ml ethyl acetate. The organic layers were combined, evaporated under N_2, and the residue was redissolved in a small amount of methanol and transferred to a 1-ml conical reaction vial fitted with a Teflon-lined screw cap. After the methanol was evaporated *in vacuo*, the residue was derivatized with a mixture of pentafluoropropionic anhydride (PFPA, 50 μl) and 2,2,3,3,3-pentafluoropropanol (PFPOH, 50 μl) for 1.5 hr at 60°. Following evaporation of the reagents *in vacuo*, the residue was dissolved in 10 μl ethyl acetate containing 1% PFPA. Quantitative analyses were performed on a Finnigan model 3300 GC/MS, using electron impact ionization. Chromatographic separation was achieved using a glass column (1.5 m × 2 mm i.d.) packed with 3% OV-17 on 80/100 mesh Gas-Chrome Q (Applied Science Laboratories, State College, PA). Quantitation was accomplished by selectively monitoring the ions at *m/e* 458/461 (MHPG/MHPG-d_3), 460/462 (HVA/HVA-d_2) and 438/440 (5-HIAA/5-HIAA-d_2).

Cerebrospinal fluid concentrations of GABA were determined by chemical ionization g.c./m.s., by a modification of the procedure of Ferkany *et al.* [20]. The rat CSF (200–300 μl) was lyophilized following the addition of GABA-d_2 (Merck & Co., 20 ng). The residue was then extracted three times with 300 μl of 80% ethanol. Following evaporation of the combined ethanol extracts *in vacuo*, the residue was esterified with a mixture of $SOCl_2$/methanol (1:5) at room temperature for 30 min. The reagents were removed *in vacuo*, and the residue was derivatized with heptafluorobutyric anhydride (HFBA) for 1 hr at 45°. Following removal of the reagents *in vacuo*, the heptafluorobutyryl-GABA methyl ester was dissolved in 10 μl ethyl acetate. Quantitative analyses were performed using chemical ionization g.s./m.s. Chromatographic separation was achieved using a glass column (1.5 m × 2 mm i.d.) packed with 10% Silar 5C (Applied Science Laboratories), which was programmed from 140 to 210° at 6°/min. Methane was used as the carrier/reagent gas. The ions at *m/e* 282/284 were selectively monitored to quantitate GABA and its deuterated analog.

For the measurement of dopamine-sensitive adenylate cyclase, the animals were decapitated, and the caudate nucleus was rapidly dissected and assayed for enzyme activity as described previously [21]. The method of Cote and Kebabian [22] was used for the measurement of cerebellar norepinephrine-sensitive adenylate cyclase, with the following modification: cerebellar tissue was homogenized in

10 vol. of a solution of 2 mM Tris maleate–2 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA). Also, the standard assay mixture was pre-incubated at 0° for 20 min. before the addition of the mixture of ATP and GTP, 1.5 mM and 30 nM respectively.

[^{3}H]Spiroperidol (23.6 Ci/nmole, New England Nuclear, Boston, MA) was used in all binding studies. The binding assay was performed on the P_2 fraction of the striatum as previously described [23]. Protein was determined by the method of Lowry *et al.* [24]. Student's *t*-test was used for statistical analysis of the data.

RESULTS

The effect of long-term L-dopa therapy on striatal adenylate cyclase activity is shown in Fig. 1. The apparent EC_{50} for dopamine stimulation of adenylate cyclase activity increased from 5 μM in controls to 20 μM in those animals receiving a high dose of the drug (oral group), with no change in the maximal level of enzyme activity compared to the control group. No detectable change in dopamine-sensitive adenylate cyclase activity was observed following intraperitoneal injections of a low dose of L-dopa for 21 days as compared to control animals (data not shown). In the cerebellum, an increased affinity for norepinephrine-stimulated adenylate cyclase activity was observed with both high and low dose L-dopa (Fig. 2). The maximum level of enzyme activity was observed to decrease following oral and i.p. administration of L-dopa by 20 and 80 per cent, respectively, compared to control animals.

The data showing analysis of the CSF for the following neurotransmitter metabolites, HVA, 5-HIAA and MHPG, appear in Table 1. A marked increase in the catecholamine metabolite, HVA, was detected following oral L-dopa, but not with a low dose of the drug. This change in CSF HVA levels represented a 1300 per cent increase following oral L-dopa. In addition, a 44 per cent increase in MHPG-levels was observed following oral L-dopa (Table 1), with no change in GABA levels compared to control. Although there was a decrease in CSF 5-HIAA after oral L-dopa, the levels did not reach statistical significance. L-dopa given intraperitoneally led to a significant decrease (27 per cent) in the norepineph-

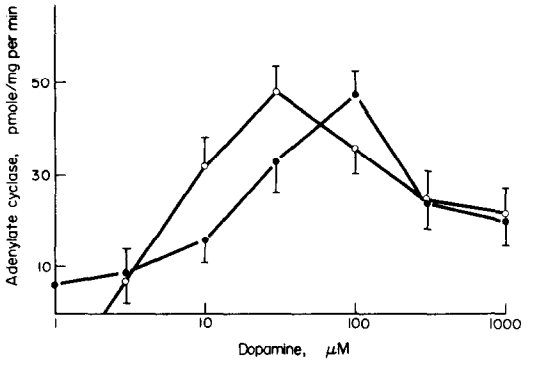

Fig. 1. Effect of orally administered L-dopa on dopamine-sensitive adenylate cyclase activity in homogenates of rat striatum. Enzyme activity was measured as described in Materials and Methods. Values shown represent the means ± S.E.M. for triplicate samples from four separate experiments. The data are plotted as an increase above basal activity. Key: (○——○) control; and (●——●) L-dopa.

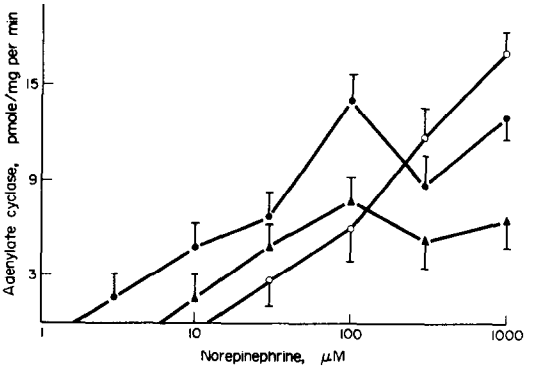

Fig. 2. Effect of L-dopa on norepinephrine-sensitive activity in homogenates of rat cerebellum. Enzyme activity was measured as described in Materials and Methods. Values shown represent the means ± S.E.M. for triplicate determinations from four separate experiments. The data are plotted as an increase above basal activity. Key:(○——○) control; (●——●) oral L-dopa; and (▲——▲) i.p. L-dopa.

Table 1. CSF metabolites and GABA levels in chronic L-dopa-treated animals*

Drug treatment	MHPG	HVA	5-HIAA	GABA
Oral administration				
None (9)	25 ± 2	72 ± 6	372 ± 48	25 ± 2 (8)
L-Dopa (6)	36 ± 7	956 ± 433	296 ± 54	27 ± 3 (10)
	P<0.2	P<0.025	P<0.4	P<0.5
Intraperitoneal administration				
None (8)	30 ± 2	71 ± 4	275 ± 30	
L-Dopa (10)	22 ± 2	62 ± 3	249 ± 10	
	P<0.01	P<0.2	P<0.5	

* Mean values are given in ng/ml of CSF and the numbers in parentheses represent the number of animals used for the control and drug-treated groups. The CSF from each animal was analysed separately for metabolites, but the data show the average value determined for all animals in the group, except for GABA in which the CSF from two animals was pooled for each data point.

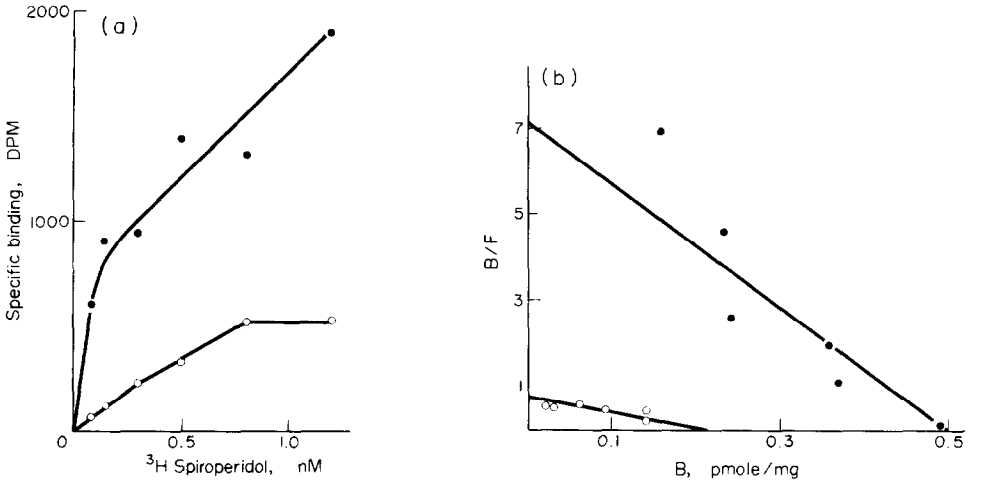

Fig. 3. Panel a: Saturation curve of specific [^{3}H]spiroperidol binding to homogenates of rat striatum following orally administered L-dopa. The binding assay was performed as described in Materials and Methods. Key: (○——○) control; and (●——●) L-dopa. The data represent the values from pooled striata from ten animals and are from a representative experiment. The average S.E.M. was less than 10 per cent. Panel b: Scatchard plot of the data which appear in panel a.

rine metabolite, MHPG, with no significant change in HVA or 5-HIAA levels.

Binding studies using [^{3}H]spiroperidol revealed significant receptor changes following the high dose of L-dopa. A decrease in the dissociation constant from 0.26 nM in control animals to 0.069 ± 0.01 nM for [^{3}H]spiroperidol was observed following oral drug administration (Fig. 3a). Moreover, an increase from 200 ± 15 fmoles/mg protein of specifically bound [^{3}H]spiroperidol in the striatum of control rats to 500 ± 65 fmoles/mg protein was detected after oral L-dopa (Fig. 3b). Binding studies to striatal membranes following a low dose of L-dopa showed no difference between the control and the drug-treated groups (data not shown).

DISCUSSION

Chronic L-dopa therapy is often associated with the on–off phenomena. It has been proposed that these changes in the clinical efficacy of the drug treatment may be due to alterations of L-dopa absorption from the gut, reduction in L-dopa transport across the blood–brain barrier, changes in dopamine distribution and metabolism in the CNS, or modifications of striatal dopamine receptor activity [25]. Previous studies with chronic administration of specific dopamine receptor antagonists reveal an induced supersensitivity of dopaminergic mechanisms in the brain and an increase in dopamine receptor numbers [26]. Thus, the expected result from long-term agonist therapy is a decrease or subsensitivity of dopamine receptors. Support for subsensitivity of the dopamine receptor following chronic agonist treatment can be derived from data demonstrating a decrease in striatal dopamine receptor sites, and a decrease in cAMP formation in striatal slices using the dopamine agonist, bromocriptine [26]. The results presented here also demonstrate a subsensitivity of the dopamine-stimulated adenylate cyclase system, following long-term L-dopa treatment, as evidenced by a 4-fold increase in the EC_{50} of dopamine for the activation of striatal adenylate cyclase.

It has been suggested that the effects of large amounts of L-dopa consumed by animals over long periods simulate the behavioral hypersensitivity seen after chronic treatment with dopamine agonists such as apomorphine. The behavioral excitatory effects of L-dopa correlate with an increase in central dopamine levels, a decrease in serotonin levels, and no change or a slight change in brain norepinephrine content [27–32]. These biochemical results are consistent with the CSF biogenic amine metabolite measurements as reported in Table 1. Also, the significant increases in dopamine binding sites shown here correlate with the behavioral observations of L-dopa-induced dopamine receptor hypersensitivity [33]. Yet, the possibility exists that L-dopa-induced hypersensitivity in animals may be similar to the hypersensitivity induced by haloperidol, a dopamine antagonist [34]. It has been suggested that long-term L-dopa therapy may lead to the production of compounds which may themselves exhibit antagonist action on the dopamine receptor [35]. Sourkes [35] has proposed that chronic L-dopa therapy can lead to the formation of isoquinoline alkaloids, condensation products of dopamine. Although Sandler *et al.* [36] have demonstrated the presence of these alkaloids in the urine, others have suggested that these compounds may be formed in the CNS [37, 38]. Davis and Walsh [39] have shown that tetrahydroprotoberberines (THPB) may be formed from dopamine and acetylaldehyde *in vitro*, and it has been demonstrated recently that THPB are competitive antagonists of dopamine-sensitive adenylate cyclase, with a potency comparable to chlorpromazine [40]. The results presented in this report, showing that dopamine binding sites are increased following chronic L-dopa therapy in a manner similar to that following chronic haloperidol treatment, support the hypothesis that high concentrations of

dopamine may lead to the production of antagonist-like compounds.

It has been proposed that the dopamine receptor exists in two forms: D_1, which is coupled to dopamine-sensitive adenylate cyclase, and D_2, which is uncoupled [41]. Binding studies have indicated that D_2-type dopamine receptors have a higher affinity than D_1 dopamine receptors for ergot alkaloid derivatives and butyrophenone-type dopamine antagonists. The data presented reveal that these sub-classes of dopamine receptors are affected differently by L-dopa therapy. An increase in D_2-type receptors was detected, whereas D_1 subsensitivity was observed following long-term L-dopa therapy. Since treatment with bromocriptine, an ergot alkaloid, has been shown to result in D_1-type receptor subsensitivity, the finding that long-term L-dopa therapy selectively increases D_2-type receptors may explain, in part, the efficacy of combination L-dopa and ergot alkaloid treatment.

CSF levels of biogenic amine metabolites have been used to estimate serotonin, norepinephrine and dopamine metabolism in the human CNS. In patients with Parkinson's disease, the concentrations of HVA and 5-HIAA in the CSF are lower before therapy than in control subjects [34]. Following long-term treatment of these patients with L-dopa, either alone or in combination with a peripheral decarboxylase inhibitor, CSF HVA levels are observed to increase [42]. In addition, treatment with L-dopa alone and especially in combination with a decarboxylase inhibitor will decrease the concentration of 5-HIAA in the CSF [43, 44]. Similar changes in the serotonin system have been observed in animals. For example, decreased CNS serotonin levels have been observed in rats following L-dopa treatment in combination with a peripheral decarboxylase inhibitor [45] and in mice on L-dopa alone [28]. The data presented in this study also suggest a decrease in CSF 5-HIAA levels.

The interplay of brain monoamines must also be taken into account when interpreting the results of studies which have the potential of altering more than one neurotransmitter system. Most animal studies suggest that L-dopa administration does not produce an increase in brain norepinephrine [27–32]. However, under conditions where brain amine levels had been depleted, norepinephrine levels increased following L-dopa treatment, but a greater per cent change was detected with dopamine. Recent evidence has suggested that L-dopa may actually result in a reduction of norepinephrine in noradrenergic neurons [17]. The data presented here reveal that chronic i.p. administration of a low dose of L-dopa decreased brain norepinephrine, as evidenced by a 27 per cent decrease in the CSF metabolite MHPG, and also increased the affinity for norepinephrine-stimulated adenylate cyclase in the cerebellum. Whether or not alterations in the norepinephrine system represent a beneficial effect of L-dopa therapy or a secondary side effect is not known. The influence of chronic L-dopa therapy on the brain norepinephrine system, particularly on the norepinephrine-sensitive adenylate cyclase activity, as described, suggests that interdependent occurrences rather than isolated changes in neurotransmitter systems happen, not only in affective disorders but also in chronic drug therapy.

In summary, the data showing dopamine receptor hypersensitivity following chronic oral L-dopa therapy support the behavioral findings of others and are consistent with the idea that this drug treatment may result in the formation of endogenous compounds, which may themselves be inhibitors of the dopamine receptor. These studies also provide evidence that the expected subsensitivity of the dopamine receptor following chronic treatment with agonists may be linked to a decreased affinity of the striatal adenylate cyclase for dopamine. The results also suggest a role for L-dopa-induced norepinephrine receptor changes in the manifestations of drug-induced side effects.

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