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# Screening Test(I) of Several Antipsychotic Agents on NO Formation

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## ABSTRACT

A number of neurological syndromes(e.g. tardive dyskinesia) are developed as a consequence of chronic treatment with neuroleptics or antipsychotic agents. Despite the long and succesful use of phenothiazine derivatives and related agents in the treatment of certain states of mental disease, the mechanisms of these drugs are still poorly understood. One current hypothesis from extensive reviews is that these compounds might significantly interfere with the cyclic nucleotide system in brain (Levin and Weiss, 1977; Nowicki *et al.*, 1991; Haley *et al.*, 1992). Nitric oxide (NO), one of an interesting messenger molecule and aberrant transmitter, is believed to play a important role in biological functions of cyclic nucleotides in nervous system. It has been reported that calcium-dependent NO synthesis in endothelial cytosol is mediated by calmodulin which is supposed to be tightly related to pharmacological actions of antipsychotic agents.

In the present study, the effect of several antipsychotic agents on the activity of NO synthesis and formation of cyclic GMP were investigated. These agents inhibited both the formation of [$^3$H]L-citrulline and that of [$^3$H]cyclic GMP by concentration-dependent manner, and their inhibiting patterns are so similar to that of calmodulin antagonist.

**Key Words:** Nitric Oxide(NO), Tardive dyskinesia, Phenothiazine antipsychotics, Cyclic nucleotides, Calmodulin

## INTRODUCTION

Nitrogen monoxide(NO) has been implicated in a number of diverse physiological and biochemical processes including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation and penile erection. Neuron responds to NO generation by an increase in their cyclic GMP levels (Ignarro, 1992), and NO acts as an intracellular second messenger in the brain to transmit information from one neuron to another (Snyder, 1992).

Currently, the evidences that the generation of nitric oxide (NO) through a $Ca^{2+}$ and calmodulin-dependent pathway plays various important roles in nervous system have been rapidly accumulated. An enzyme responsible for synthesizing NO, NO synthase, catalyses formation of NO from L-arginine via a $Ca^{2+}$/ calmodulin-dependent mechanism. Therefore, the increase in intracellular $Ca^{2+}$ resulted from activations of voltage-gated $Ca^{2+}$ channels, ligand-gated $Ca^{2+}$ channels or from the mobilization of intracellular $Ca^{2+}$ stores could affect this enzyme.

One of the most consistent finding has been reported that phenothiazine derivatives antipsycho-

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tic agents selectively inhibit calmodulin-induced enzyme activation in rat brain (Prozialeck & Weiss, 1982; Weiss *et al.*, 1982). The wide distribution of calmodulin in the brain and its mediation of a variety of biological functions have suggested that antagonism of this $Ca^{2+}$ binding protein by antipsychotics might be implicated in the pharmacoogical actions.

Because the NO signal transduction system has been most intensely studied in mammarian tissues especially in cerebellum (Bredt and Snyder, 1989) and culture cell-lines (Dawson *et al.*, 1991), we studied whether these drugs might affect the activity of NO synthase for their pharmacological mechanisms both in rat cerebellum and in N1E-115 culture cells.

Our results showed that several antipsychotics attenuated NO sythase activity correlated to inhibition of calmodulin in both preparations.

## MATERIALS AND METHODS

### Drugs

Radioactive agents and antipsychotic agents used in this study were as followings: L-[2, 3, 4, 5-$^3$H]arginine monohydrochloride was purchased from Amersham, Arlington,, IL). [8-$^3$H]guanine and [8-$^{14}$C]guanosine 3′, 5′-cyclic monophosphate were from Moravek Biochem, Inc. (Brea, CA), NADPH was from Calbiochem (La Jolla, CA). Clozapine was kindly provided by Sandoz Pharmaceutical Corp. (East Hanover, NJ). Other compounds were obtained from Sigma (St. Louis, MO).

### Cell culture

Neuroblastoma N1E-115 cells were grown in Dulbecco's modified Eagle Medium containing 10% bovine calf serum, penicillin (100 U/ml) and streptomycin (20 $\mu$g/ml). Cells were maintained at 37℃ in 10% $CO_2$ and 90% humidified air up to stationary phase (El-Fakahany and Richelson, 1980). Subculture was achieved by Puck's $D_1$ solution on day 14 (El-Fakahany and Richelson, 1980). Culture medium was changed on day 3, 5 and every day thereafter by adding 10 ml of fresh medium and removing 10 ml of conditioned medium. Cells were used for experiments on day 16~20.

### Measurement of [$^3$H]L-citrulline formation in cytosolic preparations of rat cerebellum

Assay was modified based on the method described by Bredt and Snyder (1989). Sprague-Dawley rats (250~300 g, male) were decapitated. Brain was removed and cerebellum was dissected quickly. The cerebellum was homogenized in 20 mM Hepes containing 0.5 mM EGTA, 1 mM dithiothreitol and 0.32 M sucrose at 23,000 rpm (Polytron/PT3000, Brinkmann) for 30 sec. The homogenates were then centrifuged at 20,000×g for 15 min. Resulting supernatant was passed through 1 ml columns of Dowex AG50W-X8 ($Na^+$ form) to remove endogenous L-arginine. Aliquots of the obatined cytosol (100 $\mu$g protein) were incubated with a buffer containing 20 mM Hepes, 0.5 mM EGTA, 1 mM dithiothreitol, 0.32 M sucrose, 0.5 mM $Ca^{2+}$ (1 $\mu$M free $Ca^{2+}$), 200 $\mu$M NADPH, 1 $\mu$M L-arginine, 1 $\mu$Ci/ml [$^3$H]L-arginine in the absence or presence of the test substances at 37℃ for 45 min. Reaction was stopped by adding 2 ml of 20 mM Hepes buffer plus 2 mM EDTA (pH 5.5). Samples were passed and collected through the Dowex AG50W-X8 ($Na^+$ form) columns followed by washing the columns with 2 ml of water. [$^3$H]L-citrulline was counted using liquid scintillation spectrometry. The level of [$^3$H] L-citrulline conversion was defined after subtracting the blank value which is the nonspecific radioactivity in the absence of enzyme.

### Measurements of [$^3$H]cyclic GMP formation in N1E-115 cells

N1E-115 cells were detached and collected as mentioned above, and then resuspended in hepes salt buffer. Assay was performed according to the method described by El-Fakahany and Richelson (1980). After 45 min incubation of the cells with [$^3$H]guanine (10 $\mu$Ci/ml) at 37℃, the cells were washed twice with 10 ml of hepes salt buffer to remove excess radioactivity and then distributed into 24-well plates in aliquots of $2\times10^5$ cells/well. Stimulation of cyclic GMP formation was initiated by addition of 100 $\mu$M sodium nitroprusside in the absence or presence of increasing concentrations of the test substances in a final volume of 300 $\mu$l. Reactions were stopped by adding 30 $\mu$l of

50% trichloroacetic acid to precipitate proteins. [$^{14}$C]cyclic GMP (=1500 dpm) was added to each well as internal standard. Separation of cyclic GMP was achieved using Dowex AG 50W-X2 columns (hydrogen form). [$^{3}$H] and [$^{14}$C]cyclic GMP were measured by liquid scintillation spectrometry and the recovery of [$^{14}$C]cyclic GMP usually ranged between 60~80%.

**Cell counts and protein determination**

The number of N1E-115 cells was estimated using a coulter counter (Coulter Electronics, FL). Protein concentration was determined by the method of Lowry (Lowry *et al.*, 1951) using bovine albumin as standard.

**Data analysis**

The concentration required to inhibit 50% of the maximal response ($IC_{50}$) was calculated by Hill transformation of the data. All data are presented as mean±standard errors. Student t test was employed for statistical analysis.

## RESULTS

**Effect of antipsychotic drugs on [$^{3}$H]L-citrulline formation**

The effects of several antipsychotic agents on formation of [$^{3}$H]citrulline in cytosolic preparation of rat cerebellum were examined. Thirty percent of [$^{3}$H]L-arginine could be converted into [$^{3}$H]L-citrulline in the presence of 1 $\mu$M free $Ca^{2+}$, 200 $\mu$M NADPH and 1 $\mu$M L-arginine after 45 min incubation at 37°C.

We used 7 current antipsychotic durgs to observe the effects of these durgs on NO synthase in rat cerebellar cytosol. Data shows that all of tested drugs inhibited the formation of L-citrulline from arginine by usual dosage in this experiment. To determine the relationship between attenuation of the activity of NO synthase and antagonism of calmdulin, we used calmidazolium as one of classic calcium antagonists. In this experiment, all of the tested agents were less potent than calmidazolium ($IC_{50}$=2.09±0.87 $\mu$M). Among the tested agents in this experiment, trifluoperazine was more potent than any other tested drugs in inhibiting L-citrulline formation in this cytosolic

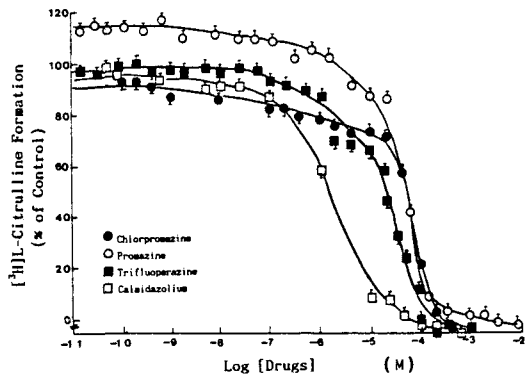


**Fig. 1.** Typical patterns of several antipsychotic drugs on [$^{3}$H]L-citrulline formation in rat cerebellar cytosol.
Data shown are the means+SEM 4 to 6 independent experiments performed in triplicate and are represented as percentage of control which is the conversion of [$^{3}$H]L-arginine into [$^{3}$H]L-citrulline in the absence of test substancs (19±1×10 dpm).

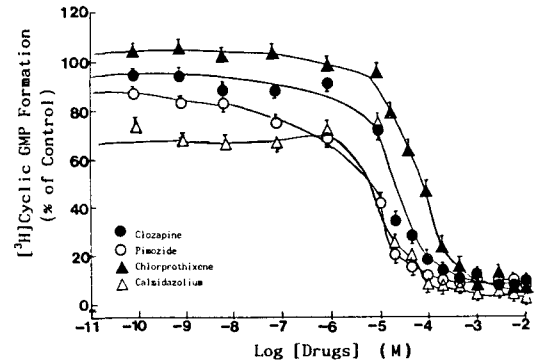


**Fig. 2.** Typical patterns of several antipsychotic drugs on [$^{3}$H]cyclic GMP formation in intact N1E-115 cells.
Data shown are the means+SEM from 4 to 6 independent experiments performed in triplicate and are represented as percentage of control which is the level of stimulated [$^{3}$H]cyclic GMP formation in the absence of the antipsychotics[(78±19×10 dpm) above basal for sodium nitroprusside].

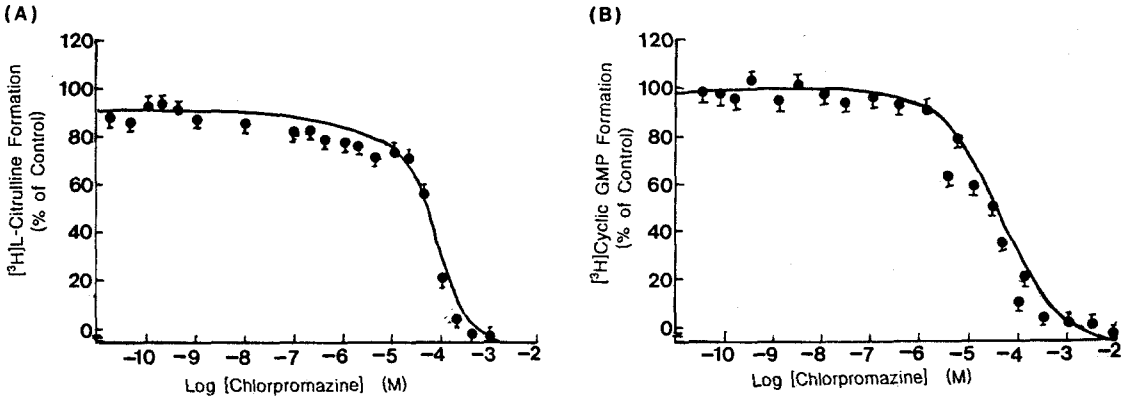


**Fig. 3.** Comparison of the dose-response effects of chlorpromazine on inhibition of NO synthase.
(A) Effect of [$^3$H]L-citrulline formation in rat cerebellar cytosol
(B) Effect of [$^3$H]cyclic GMP formation in intact N1E-115 cells

preparation of rat cerebellum ($IC_{50} = 6.54 \pm 0.45$ $\mu$M), and this drug showed higher than unity (Hill slope $= 1.91 \pm 0.10$). The potency of tested agents in inhibition of L-citrulline formation was compared to that of antagonism of calmodulin-activated phosphodiesterase derived from published literature (Prozialeck and Weiss, 1982).

As illustrated in Fig. 1, there was an excellent correlation between inhibitory responses and the concentrations, suggesting that the observed effects on NO synthase activity might be mediated by antagonism of calmodulin. The inhibition of NO synthase by antipsychotic agents in cytosolic preparations of rat cerebellum seemed not to be related to the concentration of L-arginine (Fig. 4), and differed from those of the competitive NO synthase inhibitors such aas N-methyl-L-arginine (data not shown). The concentration-dependent effects of chlorpromazine both in inhibiting the conversion of [$^3$H]L-arginine into [$^3$H]L-citrulline and in inhibiting [$^3$H]cyclic GMP formation were shown in Fig. 3. Cholorpromazine showed the more potent inhibition (about 7~8 times) of [$^3$H] citrulline formation ($IC_{50} = 14.96 \pm 1.99$ $\mu$M) than that of [$^3$H]cyclic GMP formation ($IC_{50} = 108.78 \pm 16.86$ $\mu$M).

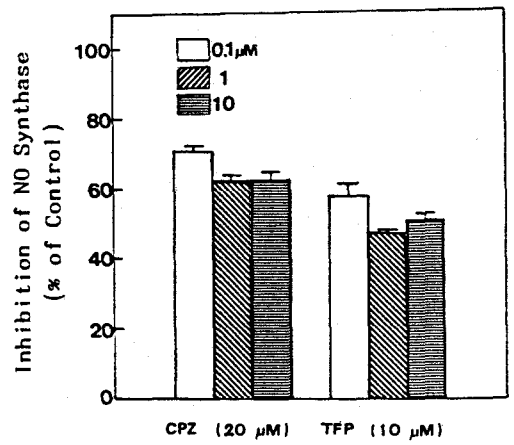


**Fig. 4.** Effects of concentration of L-arginine on NO synthase.
Data shown are the means+SEM from 5 or 6 independent experiments performed in triplicate and are represented as percentage of control which is the value in the absence of inhibitors ($35 \pm 1 \times 10$, $21 \pm 2 \times 10$ and $4 \pm 0.3 \times 10$ dpm in the presence of 0.1, 1 and 10 $\mu$M L-arginine) respectively.
CPZ; chlorpromazine
TFP; trifluoperazine

**Effects of antipsychotic agents on [$^3$H]cyclic GMP formation**

We used the sodium nitroprusside which spontaneously generates NO (Loiacono and Beart, 1992) to activiates guanylate cyclase for generation of cyclic GMP. We tried to observe more

than 10 antipsychotic drugs and their structure-related agents, but we had considerable results from 7 antipsychotic drug. Promazine, chlorpromazine and trifluoperazine inhibited the cyclic GMP response to sodium nitroprusside in N1E-115 cells (Fig. 3). Hill slope of the inhibition curves of these durgs were $0.68 \pm 0.25$, $1.9 \pm 0.64$ (greater than one) and $1.74 \pm 0.33$ (greater than one), respectively. More lipid-soluble anti-psychotic agents —clozapine, pimozide, chlorprothixene and haloperidol than promazine, chlorpromazine and trifluoperazine— were chosen for comparison. When these drugs were used to observe the effect of antipychotics on cyclic GMP formation, we found significant inhibition of sodium nitroprusside-stimulated cyclic GMP formation in intact N1E-115 cells (Fig. 2). Hill slopes of clozapine and pimozide were almost close to one but those of chlorprothixene and haloperidol were similar to that of trifluoperazine.

## DISCUSSION

It is now well known that NO synthase catalyzes the conversion of L-arginine into L-citrulline, with a concomitant release of the second messenger molecule NO. The expression of the activity of the neuronal NO synthase is dependent upon the presence of NADPH and $Ca^{2+}$/calmodulin (Moncada *et al.*, 1991).

In this experiment, we tried to examine one mechanism of the pharmacological effects of several antipsychotic drugs using similar lines of essay. It was observed that antipsychotic drugs inhibited the activity of neuronal NO synthase and NO-triggered cyclic GMP responses. In contrast to the effects of competitive NO synthase inhibitors, these antipsychotic drugs were not affected by increasing concentrations of L-arginine. In our results, there were the correlativities between pharmacological potencies of antipsychotic actions and in inhibiting potencies of NO synthase. Despite these antipsychotic drugs being not unities in pharmachological potencies, they shared their typical shapes of inhibiting patterns which has similarity of that of calmidazolium on NO synthase both in rat cerebellar cytosol and in N1E-115 culture cells.

There is an evidence that purified neuronal NO synthase could be phosphorylated by a $Ca^{2+}$/calmodulin-dependent protein kinase (Bredt *et al.*, 1992). Previous studies have been reported that calmodulin antagonist and related agents inhibited the activity of the constitutive NO synthase in brain (Bredt and Snyder, 1990), endothelial cells (Förstermann *et al.*, 1991; Schini and Vanhoutte, 1992) and endothelial cytosol of aorta (Busse and Mulsch, 1990).

Since our results in both assays showed the significant inhibitions of NO synthase, we assume that these antipsychotic drugs might affect calcium-related protein or cyclic nucleotide related to NO synthase which activates NO formation. It is interesting to observe that the $IC_{50}$ values of chlorpromazine, trifluoperazine and promazine in the inhibition of cyclic GMP formation induced by sodium nitroprusside which spontaneously generates NO in intact N1E-115 cells were significantly higher than those obtained from rat cerebellar cytosol in formation of L-citrulline. This difference might be explained by the specific abilities of different antipsychotic potencies to affect the basal versus the stimulated activity of NO synthase whether direct or indirect way. In addition, we cannot rule out the possibility that the attitudes of access of these drugs to the intracellular enzyme might be hindered in intact N1E-115 cells.

It has been reported that inhibitors of NO synthase antagonize cyclic GMP formation elicited by ionomycin or sodium nitroprusside in cells with high potency (Pou *et al.*, 1990), and we also observed that antipsychotic drugs attenuated the cyclic GMP formation induced by sodium nitroprusside in intact N1E-115 cells.

From above results, we could assume that these drugs might affect in part to generate the cyclic GMP by direct inhibition of guanylate cyclase. The less potent effects on antagonizing cyclic GMP synthesis than those on inhibiting L-citrulline formation by antipsychotic drugs used in this experiment (except clozapine and pimozide) might be related to their suppression behaviers on phosphodiesterase activity or on NO synthase as a consequence of inhibition of calmodulin, it is directly or not. Even though we do not clearly explain why only pimozide and clozapine were shown more potent effects on inhi-

bition of cyclic GMP formation than on that of L-citrulline formation, we could assume that these highly lipid-soluble drugs might more significantly affect the biological functions in intact cell-lines than in cytosoic preparations.

We do know that the observed net influences of these antipsychotic drugs on NO synthase were resulted from the summation of complicated effects both on the synthesis and on degradation of NO whether it was direltly or not, therfore, still many important and serious questions to be solved are remained. The estimates of the free concentrations of antipsychotic drugs in the brain of patients might not be easier than measuring in plasma. There is a report that the free concentrations of these drugs in CSF of patients are approximately 2nM (Seeman, 1992).

So, from above report, we could assume that these drugs might alter the activity of neuronal NO synthase depending on calcium by these experimental doses. AS the chronic treatment with antipsychotics in vivo results in a significant increase in the activity of brain calmodulin (Bon *et al.*, 1992; Lau and Gnegy, 1982), it is also remained to be investigated whether these drugs alter the action or sensitivity of this enzyme to $Ca^{2+}$ after long-term treatment. Now we are doing studies for these drugs in different preparations according to specific conditions and tests.

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=국문초록=

# 수종 정신병치료제들의 NO형성에 대한 검색(I)

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정신병치료제들을 장기투여하여 치료를 시도하였을 때에 생기는 여러 부작용은 그 정도 또한 매우 심각하기때문에 그들의 치료효과와 함께 야기되는 부작용들을 따로 생각할 수가 없게 되었다. 특히 정신병치료는 그 자체에 대한 병인적 원인을 정확히 알 수 없기에 증상에 따른 대증요법이 일괄적으로 사용되므로, 이러한 현재의 치료방법으로는 부작용들이 더 치명적이 될 수 있기 때문에 일차적으로 이들의 공통적약리작용기전들을 연구하는 것은 매우 필요하다.

최근 NO(Nitric oxide)에 대한 많은 연구들에 의하면, 이들이 중추신경계에서 중요한 second messenger 또는 mediator로 신경활동에 영향을 나타내는 것으로 보고되고 있다. 그러므로 저자들은 먼저 이들 약물들과 NO와의 관계를 연구하고자, 중요한 몇종의 정신병치료제들을 택하여 NO생성에 어떤 영향을 미치는 가를 검색하여 다음과 같은 일차 결과를 얻었다.

1. 정신병치료제 수종(chlorpromazine, trifluoperazine promazine, pimozide, clozapine, chlorprothixene, haloperidol)을 택하여 쥐의 소내에서 [$^3$H]L-arginine으로부터 [$^3$H]L-citrulline의 생성양을 측정하여 calmodulin antagonist(calmidazolium)와 비교하였다.

2. 이들을 N1E-115 cell에 투여하여 [$^3$H]cyclic GMP양을 측정하고 그 결과를 calmida-zolium과 비교하였다.

3. 이들 약물들은 citrulline과 cyclic GMP 모두의 생성양을 의의있게 억제하였으며 그 기전은 calmidazolium과 매우 유사하였다.

위의 일차적 검색결과에 의하면, 정신병치료약물들의 약리작용기전중에 일부는 중추신경계내의 NO생성 및 cyclic GMP생성에 영향을 나타내는 것으로 사료되며, 이에는 calcium ion이 상당히 중요한 역활을 하는데, 특히 소뇌에서의 NO생성의 감소는 이들 약물들의 치명적 부작용인 tardive dyskinesia와 매우 깊은 관련을 추측할 수 있다. 그러나, 더 많은 약물들의 검색으로 일관적인 기본 결과가 필요되고 또 각개 약물의 특정적 기전이 연구되기 위하여 현재 실험중이다.