Original

Membrane Effects of Monoamine Oxidase Inhibitors on Erythrocytes and Freshly Isolated Hepatocytes

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Abstract: Effects of monoamine oxidase (MAO) inhibitors on two kinds of membranes, biological and model membranes, were studied. Erythrocytes and freshly isolated hepatocytes from rats were used to examine the effects of MAO inhibitors on biological membranes. Hydrazide and hydrazine derivatives, except isonicotinic acid, enhanced hemolysis and the stability of isolated hepatocytes membrane. Deprenyl and moclobemide stabilized the biological membranes of erythrocytes and isolated hepatocytes, and moclobemide induced slight enzyme leakage from isolated hepatocytes. Clorgyline and brofaromine had opposite effects. At lower concentrations, they remarkably inhibited hemolysis and enzyme leakage. At higher concentrations, they enhanced hemolysis and apparent enzyme leakage. To investigate effects of MAO inhibitors on a model membrane, changes of phase transition temperature of dipalmitoyl phosphatidylcholine liposomes was determined. Brofaromine induced the most extreme decline of phase transition temperature. Deprenyl and clorgyline also induced moderate decline of transition temperature, and phenylhydrazine and *p*-tolylhydrazine induced mild decline. These results suggest that the effects of clorgyline and brofaromine on the membranes of erythrocytes and freshly isolated hepatocytes might be partly due to increased fluidity of membrane phospholipid. These severe membrane effects of MAO inhibitors might be the reason for their cytotoxicity.

Key words: monoamine oxidase inhibitor, membrane, hepatocyte, erythrocyte, liposome, fluidity

Introduction

There have been many reports of the adverse effects of antidepressant drugs such as monoamine oxidase (MAO) inhibitors¹⁻⁵⁾ and tricyclic antidepressants¹⁻⁶⁾. The discovery of MAO-A and MAO-B types of the MAO enzyme, each showing distinctly different substrate preferences and inhibitor sensitivity⁷⁾, has led to an increase in the development of new and selectively acting MAO-A or MAO-B inhibitors without the hydrazine structure. The cytotoxicity of various therapeutic compounds that induce membrane action has already been reported. We previously demonstrated that the cytotoxicity of some compounds, such as tricyclic antidepressants, is positively correlated between hepatocytes and erythrocytes⁸⁾. We also reported that the membrane stabilizing action on rat erythrocytes by some compounds was related to increased fluidity of the phospholipid bilayer⁹⁾. Therefore, the effects of

therapeutic drugs on cell membrane action are clinically interesting.

The present study aims to compare the effects of MAO inhibitors on the hypotonic hemolysis of erythrocytes and leakage of enzymes from freshly isolated hepatocytes. In addition, the effects of MAO inhibitors on a model membrane were investigated to measure changes of phase transition temperature using dipalmitoyl phosphatidylcholine (DPPC) liposomes.

Materials and Methods

Materials and animals

Iproniazid was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); phenylhydrazine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan); deprenyl was provided by Dr. J. Konell of Semmelweis University, School of Medicine, (Budapest, Hungary); isonicotinic acid and pargyline were obtained from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.); clorgyline was provided by May & Baker Ltd. (Dagenham, U.K.); brofaromine (CGP11305A) was provided by Ciba-Geigy Ltd. (Basel, Switzerland); moclobemide was provided by Hoffmann-La Roche & Co., Ltd. (Basel, Switzerland).

Collagenase (type IV) and DPPC were obtained from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.). William's E medium was purchased from Flow Laboratories (Irvine, Scotland). Trypan blue (0.4%) was obtained from Gibco Laboratories Life Technologies Inc. (New York, U.S.A.). All other chemicals used in this experiment were the purest grade commercially available. In the present study, male Sprague-Dawley rats (6 weeks; 150 g) were used.

Studies of hypotonic hemolysis

Effects of MAO inhibitors on hypotonic hemolysis of rat erythrocytes were studied as previously described¹⁰⁾. MAO inhibitors were dissolved in a hypotonic buffer (about 54 mM NaCl in 10 mM phosphate buffer, pH 7.4) which usually induced lysis of 50% of the erythrocytes. The erythrocyte suspension (2×10^9 cells/ml) was incubated for 1 hr at 37°C with various concentrations of MAO inhibitors ranging from 1×10^{-6} to 1×10^{-3} M.

Isolated hepatocytes of rat

Hepatocytes were isolated from rats by a collagenase perfusion technique¹¹⁾, and viable hepatocytes were suspended in a mixture of William's E medium, supplemented as previously described¹⁰⁾. The viability of isolated hepatocytes determined by trypan blue exclusion was in the range of 90–95%. MAO inhibitors in a range of 1×10^{-6} M to 1×10^{-3} M were dissolved in the William's E medium. The isolated hepatocyte suspension (1×10^5 cells/ml) was incubated for 1 hr at 37°C with MAO inhibitors. Activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) which leaked from the isolated hepatocytes were determined by the method of Ohkawa *et al.*¹²⁾. The morphological changes of isolated hepatocytes treated with MAO inhibitors were observed under a phase-contrast microscope after dying with trypan blue.

Phase-transition temperature study

DPPC at 1 mM concentration was dispersed in 10 mM phosphate buffer (pH 7.4) using a Sonifier cell disruptor 200 (Branson), as previously described⁹⁾. This method has been generally accepted to produce liposomes of DPPC. MAO inhibitors ranging from 1×10^{-6} to 1×10^{-3} M were dissolved in the DPPC liposomal suspension. The phase transition temperature of each liposomal suspension with MAO inhibitors was measured to an accuracy of 0.01°C with a thermistor probe (Takara Thermistor Instruments, Co., Ltd.). The midpoint

between the beginning of the decrease in the scattering and reaching a plateau was taken as the phase transition temperature for each concentration of MAO inhibitors. A decrease in phase transition temperature without MAO inhibitors was plotted against the concentration of each MAO inhibitor. The concentration of each MAO inhibitor necessary to induce a 1.0°C decline of phase transition temperature was calculated from the midpoint of each slope.

Statistical significance of the difference between the control and test values was calculated by Student's *t*-test.

Results

Effects of MAO inhibitors on the hypotonic hemolysis of rat erythrocytes are shown in Fig. 1. In the present study, the value of about 50% hypotonic hemolysis without treatment of MAO inhibitors was used as the control. Treatment with iproniazid at a concentration range of 2×10^{-4} to 1×10^{-3} M enhanced hemolysis significantly more than the control. In the treatment with *p*-tolylhydrazine, slight protection of hemolysis was observed at a con-

Fig. 1. Effect of MAO inhibitors on hypotonic hemolysis of rat erythrocytes. Erythrocyte suspension was incubated for 1 hr at 37°C with various concentrations of MAO inhibitors in a range of 1×10^{-6} M to 1×10^{-3} M. Data are expressed as percentage of the mean value of control. Statistically significant changes are indicated by: * $p < 0.01$ and ** $pp < 0.001$ compared to the values obtained from the control (Student's *t*-test). ■—■, iproniazid; ▲—▲, *p*-tolylhydrazine; ●—●, deprenyl; △—△, clorgyline; ○—○, brofaromine; □—□, moclobemide.

Table 1. Effect of monoamine oxidase inhibitors on leakage of glutamic oxaloacetic transaminase from freshly isolated hepatocytes.

Compounds	Concentrations			
	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
	% of control			
Hydrazines				
Isonicotinic acid	106.3±14.9	104.8±17.3	117.7±23.6*	126.5±34.6*
Iproniazid	100.3±22.8	109.8±13.0	95.0±24.1	85.3±7.8
Phenylhydrazine	95.7±10.7	112.8±27.7	100.1±18.6	49.8±14.6***
p-Tolylhydrazine	93.5±8.8	94.7±19.0	83.1±27.8	30.7±4.8***
Propinylamines				
Deprenyl	95.0±13.3	94.4±11.8	74.2±10.4***	83.1±8.0***
Pargyline	96.0±14.9	104.8±22.8	112.0±1.8	101.2±39.8
Clorgyline	72.6±6.2**	97.2±24.7	500.8±61.8***	1337.8±70.4***
Others				
Brofaromine	94.4±27.7	96.9±31.3	179.6±67.6	582.0±22.7***
Moclobemide	98.3±19.6	115.2±12.6	103.8±9.5	117.9±24.0*

Hepatocytes were suspended in William's E medium and incubated for 1 hr at 37°C after the addition of various concentrations in a range of 1×10^{-6} to 1×10^{-3} M. Data are expressed as percentage of the mean value of the control. Each value represents the mean±SD of 5 to 10 samples. Significant differences from the control value are: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

Table 2. Effect of monoamine oxidase inhibitors on leakage of glutamic pyruvic transaminase from freshly isolated hepatocytes.

Compounds	Concentrations			
	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
	% of control			
Hydrazines				
Isonicotinic acid	114.5±17.5	104.0±19.2	107.6±32.9	115.0±7.3**
Iproniazid	107.0±18.7	119.4±23.2	101.0±20.8	64.6±7.3*
Phenylhydrazine	120.4±3.6	89.6±27.5	75.7±32.7	39.3±2.8***
p-Tolylhydrazine	94.5±13.9	96.0±29.5	53.6±7.6***	6.7±7.3***
Propinylamines				
Deprenyl	109.6±19.2	106.6±14.1	88.8±14.9	90.6±9.7
Pargyline	99.3±10.8	94.2±20.4	80.4±4.2***	70.2±10.0***
Clorgyline	87.7±17.8	88.3±12.9*	202.2±30.8**	438.4±29.7***
Others				
Brofaromine	106.5±16.5	86.7±10.7*	94.3±11.4	370.3±18.4***
Moclobemide	100.8±12.8	106.0±15.8	94.7±13.3	97.1±42.4

Conditions are shown in Table 1. Each value represents the mean±SD of 5 to 10 samples. Significant differences from the control value are: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

centration of 1×10^{-6} to 6×10^{-3} M, while slight enhancement of hemolysis was observed at more than 2×10^{-4} M. However, treatment with deprenyl at 4×10^{-4} to 1×10^{-3} M, and with moclobemide at 1×10^{-3} M induced protection of hypotonic hemolysis. Clorgyline and brofaromine induced opposite effects on hypotonic hemolysis: 1) Treatment with 2×10^{-6} to

Fig. 2. Effect of MAO inhibitors on phase transition temperature of 1 mM DPPC liposomal suspension. Data are expressed as the decline in temperature from the phase transition temperature of the control.

■—■, iproniazid; ▲—▲, *p*-tolylhydrazine; ●—●, deprenyl; △—△, clorgyline; ○—○, brofaromine; □—□, moclobemide.

1×10^{-4} M clorgyline or with 4×10^{-6} to 4×10^{-4} M brofaromine inhibited hypotonic hemolysis. 2) They substantially enhanced hypotonic hemolysis about 296% and 270%, respectively at the higher concentrations.

Effects of MAO inhibitors on the leakage of GOT and GPT from rat isolated hepatocytes are shown in Tables 1 and 2. In this study, isolated hepatocytes untreated MAO inhibitors were used as control. The amounts of GOT and GPT leaked from control isolated hepatocytes were 22.8 ± 7.2 (mean $\pm$ SD) KU/ml and 5.8 ± 2.2 KU/ml, respectively. In the isolated hepatocytes treated with iproniazid and pargyline, there was no apparent effect on the leakage of GOT. The treatment with isonicotinic acid at concentrations of 10^{-4} and 10^{-3} M, or with moclobemide at 10^{-3} M, induced a significantly greater increase in GOT leakage than that from the control. Treatment with 10^{-3} M phenylhydrazine or *p*-tolylhydrazine decreased GOT leakage to less than that of control. In the isolated hepatocytes treated with 10^{-6} M clorgyline, a significant decrease in GOT leakage was observed. At 10^{-4} M and 10^{-3} M, clorgyline induced increases of about 500% and 1337%, respectively. Treatment with brofaromine at 10^{-3} M significantly increased GOT leakage from isolated hepatocytes. Treatment with deprenyl or

Table 3. Effects of monoamine oxidase inhibitors on biological membranes and model membrane.

Compounds	Erythrocytes		Hepatocytes		Fluidity
	MSA	lysis	MSA	leakage	
Hydrazines					
Isonicotini acid	+	—	—	+	—
Iproniazid	—	‡	+	—	—
Phenylhydrazine	+	+	‡	—	+
<i>p</i> -Tolylhydrazine	+	+	‡	—	+
Propinylamine					
Deprenyl	‡	—	‡	—	‡
Pargyline	+	—	+	—	—
Clorgyline	‡	‡	+	‡	‡
Others					
Brofaromine	‡	‡	+	‡	‡
Moclobemide	‡	—	+	+	—

Effects of MAO inhibitors to biological membrane (erythrocytes and isolated hepatocytes) and DPPC liposomes. The grades of effects are: —, no effect; +, mild increase in membrane stabilizing action (MSA) and in hemolysis and leakage, and slight increase in membrane fluidity; ++, moderate increase in MSA and in hemolysis and leakage, and mild increase in membrane fluidity; ###, prominent increase in MSA and severe increase in hemolysis and leakage and prominent increase in membrane fluidity.

moclobemide induced no apparent effect in the leakage of GPT from isolated hepatocytes, whereas treatment with 10^{-3} M iproniazid or phenylhydrazine, or with 10^{-4} M and 10^{-3} M *p*-tolylhydrazine or pargyline significantly decreased GPT leakage below that of the control. Treatment with 10^{-5} M clorgyline or brofaromine significantly decreased by GPT leakage, and treatment with 10^{-4} or 10^{-3} M clorgyline or 10^{-3} M brofaromine induced a remarkable increase in GPT leakage from isolated hepatocytes. These effects of MAO inhibitors on GPT leakage were similar to the effects on GOT leakage from the isolated hepatocytes.

After treatment with MAO inhibitors, isolated hepatocytes were dyed with trypan blue and the morphological changes were observed by phase contrast microscopy. Non-treated isolated hepatocytes were clearly round shaped, and few could be dyed with trypan blue, that is, most hepatocytes were viable (data not shown).

The decline of phase transition temperature of each MAO inhibitor was plotted against the control without MAO inhibitors (Fig. 2). The phase transition temperature of control liposomes was $41.74 \pm 0.05^\circ\text{C}$ (mean $\pm$ SD). DPPC liposomal suspension in 10^{-3} M iproniazid or moclobemide induced a slight decline in phase transition temperature. DPPC liposomal suspension in 10^{-3} M *p*-tolylhydrazine depressed the phase transition temperature to 40.80°C . Moreover, DPPC liposomes in 10^{-4} to 10^{-3} M clorgyline, deprenyl or brofaromine remarkably depressed the phase transition temperature. The calculated concentrations of isonicotinic acid and iproniazid necessary to induce a 1°C decline of the phase transition temperature were 1.29×10^{-2} M and 4.80×10^{-2} M, respectively. The necessary concentration of brofaromine was only 1.73×10^{-4} M, which was less than the 3.02×10^{-4} M of clorgyline.

All effects of MAO inhibitors on the erythrocytes, isolated hepatocytes, and DPPC liposomes are summarized in Table 3. Membrane stabilizing action (MSA) was evaluated from

the decrease in hemolysis and enzyme leakage. Membrane fragility was evaluated from the increase in hemolysis and enzyme leakage from hepatocytes. Fluidity was evaluated from the decline of the phase transition temperature of the liposomal bilayer. We propose the degree of MSA, lysis, leakage and fluidity to be four grades. Hydrazine derivatives such as iproniazid and phenylhydrazine induced increase of hemolysis. However, these derivatives induced a decrease of the leakage of enzymes from isolated hepatocytes, which indicates membrane stabilizing action. Moclobemide and propinylamine derivatives such as deprenyl and pargyline stabilized the action of erythrocyte and isolated hepatocyte membranes. However, the effects of low concentrations of clorgyline and brofaromine were opposit to the effects of high concentrations. At low concentrations, they both induced a decrease in low hemolysis and enzyme leakage; at high concentrations they greatly enhanced these actions. Phase transition of DPPC liposomes that contained brofaromine occurred at the lowest temperature. Deprenyl and clorgyline also induced a decline of phase transition temperature to below the temperature of *p*-tolylhydrazine and phenylhydrazine. Other MAO inhibitors had no apparent effect on the phase transition temperature of DPPC liposomes.

Discussion

The basic structure of cell membranes, including erythrocytes and hepatocytes, is a phospholipid bilayer with a hydrophilic surface and a lipophilic portion inside¹³⁾. Severe membrane actions induced by some lipophilic medicines such as local anaesthetic agents and tricyclic antidepressants are reported to cause cytotoxicity^{8,14)}. In the present study, membrane actions of MAO inhibitors against hypotonic hemolysis of erythrocytes and isolated hepatocytes indicated three patterns: stabilizing effects, leakage effects and combined effects.

In this study, hydrazine derivatives such as phenylhydrazine and *p*-tolylhydrazine at high concentrations gradually enhanced hemolysis. Phenylhydrazine is well known to cause hemolysis both *in vitro* and *in vivo*^{15,16)}. The formation of hydrogen peroxide in erythrocytes has been demonstrated to be associated with the hemolytic action of phenylhydrazine¹⁷⁾. These results of the present study support the idea that hydrogen peroxide is formed at concentrations that exceed the detoxication capacity of erythrocytes, and this partly contributes to the cytotoxicity of phenylhydrazine¹⁶⁾. In the assay of biochemical activity in freshly isolated hepatocytes, phenylhydrazine and *p*-tolylhydrazine showed only a membrane stabilizing action. These phenomena were morphologically evident; that is, a few isolated hepatocytes of phenylhydrazine and *p*-tolylhydrazine treatments took up trypan blue but most hepatocytes were viable (data not shown). More recently, we reported that these inhibitors decrease the protein synthesis in primary cultured hepatocytes of rat¹⁸⁾. Therefore, the decrease in GOT and GPT leakage in phenylhydrazine and *p*-tolylhydrazine treatments might be partly due to the suppression of synthesized proteins. Iproniazid also induced slight membrane damage in erythrocytes, whereas it slightly stabilized hepatocyte membranes. It seems that these various effects of hydrazide derivatives on biological membranes were caused by differences in the phospholipid constituents of the membranes of erythrocytes and isolated hepatocytes. From the determination of the phase transition temperature, these inhibitors had no effects on the fluidity of DPPC liposomes. Therefore, their membrane actions may have induced some influence on the proteins of the cell membranes but not on the phospholipids.

Brofaromine and clorgyline exerted the maximum membrane stabilization at the lowest concentrations. Depression of the phase transition temperature of DPPC liposomes by bro-

faromine and clorgyline indicates that these agents partly penetrate the bilayers and increase the fluidity of the bilayer membrane. In model membranes of DPPC liposomes, they are crystalline below the phase transition temperature (41.0°C) and in a liquid crystalline phase above that temperature. In general, the acyl chains of phospholipids are arranged in parallel with slight mobility in the crystalline phase. In the liquid crystalline phase, the disorder in the conformation of the lipid tails is characterized by increased gauche rotations, that is, their acyl chains increase mobility and thus the lipid bilayer becomes thin¹⁹⁾. In addition, membrane fluidity is influenced not only by temperature but also by various environmental factors, such as chemical compounds or pH^{20,21)}. An increase in fluidity of the bimolecular membrane of phospholipid induces expansion of the bilayer membrane^{22,23)}. Therefore, we can imagine that the volume can increase because the cell membrane of erythrocytes and hepatocytes is expanded by treatment with brofaromine and clorgyline, and so more water remains in these cells until lysis and leakage occur. In this study, the two MAO inhibitors, at concentrations below those causing lysis and leakage, substantially decreased hemolysis and enzyme leakage. From these results, the increase in membrane fluidity by brofaromine and clorgyline is one reason for their stabilizing effects on the erythrocyte and hepatocyte membranes. However, it is important to realize that if the fluidity of cell membranes is increased too much, cell membranes overexpand and become unstable. Thus, brofaromine and clorgyline exerted lytic and leakage effects on the erythrocytes and hepatocytes at higher concentrations. The effects of clorgyline were more severe than those of brofaromine. However, the phase transition temperature of DPPC liposome in clorgyline was higher than that in brofaromine at 10^{-3} M. That is, the increase in the fluidity of DPPC liposomes by clorgyline was less than that by brofaromine. Thus, these membrane actions may affect not only phospholipids, but also other components of the cell membranes, such as protein. Morphological features of isolated hepatocytes treated with these MAO inhibitors induced bumpy surfaces within trypan blue; and at 10^{-3} M, decreased viable cell numbers and the amorphous remains of hepatocytes were observed (data not shown). The fact that the low concentration of brofaromine and clorgyline was enough to depress the phase transition temperature indicates their marked stabilizing effect on the cell membranes compared to other MAO inhibitors. This is similar to the action of potent local anesthetics such as tetracaine or bupivacaine, which induce a marked decline of the phase transition temperature and increase in the stabilization of the cell membrane, as opposed to weak local anesthetics such as procaine²⁴⁾. After single oral administrations of brofaromine (100 mg) to healthy volunteers, the maximum plasma concentration of brofaromine reaches about 2×10^{-6} M²⁵⁾. In this study, treatment of erythrocytes and isolated hepatocytes with 10^{-6} to 10^{-4} M brofaromine stabilized the cell membranes. This suggests that exposure to brofaromine at a plasma concentration of 10^{-6} M does not induce a clinical effect on the cell membrane.

In conclusion, the present study showed that MAO inhibitors possess an ability to produce various membrane effects. The potency of MAO inhibitors on hypotonic hemolysis and freshly isolated hepatocytes was clorgyline > brofaromine > other MAO inhibitors. These results suggest that brofaromine induces membrane damage at high concentrations, thus it should be used carefully in clinical applications.

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