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Biochemical Pharmacology, Vol. 31, No. 8, pp. 1658–1661, 1982.
Printed in Great Britain.

0006-2952/82/081658-04 $03.00/0

Inhibitory action of macrocyclic polyamines on lipid peroxidation in rat liver microsomes

(*Received* 9 *September* 1981; *accepted* 18 *November* 1981)

It is well known that biomembranes are susceptible to lipid peroxidation which results in their disintegration and hence loss of function [1, 2]. In addition, microsomal lipid peroxidation has been found to be catalysed by NADPH–cytochrome *c* (P-450) reductase from the studies of the reconstituted systems containing purified NADPH–cytochrome *c* (P-450) reductase [3–5]. Since some of macrocyclic polyamines had a potent inhibitory effect on NADPH-supported lipid peroxidation in rat liver microsomes [6], we have investigated the reason why some macrocyclic polyamines are more effective than other polyamines in the inhibition of lipid peroxidation.

Rat liver microsomes were prepared as described previously [7]. Protein was determined by the method of Lowry *et al.* [8] using bovine serum albumin as a standard. Lipids used as substrates for lipid peroxidation were extracted from intact rat liver microsomes by the method of Folch *et al.* [9]. Lipid phosphorus was determined by the method of Bartlett [10]. The content of iron in microsomes was measured by atomic absorption spectrometry after the wet combustion of microsomes. NADPH–cytochrome *c* (P-450) reductase was purified from phenobarbital-treated rat liver microsomes by a minor modification of the method of Yasukochi and Masters [11]. NADPH–cytochrome *c* (P-450) reductase activity was measured by the method of Phillips and Langdon [12] using cytochrome *c* as an electron acceptor.

Microsomal lipid peroxidation was determined by thiobarbituric acid (TBA) method as described previously [7]. A reaction mixture for purified NADPH–cytochrome *c* (P-450) reductase mediated lipid peroxidation consisted of 0.25 M Tris–HCl (pH 6.8), 0.25 M NaCl, 2 mM ADP, 0.1 mM EDTA, 0.2 mM $Fe(NO_3)_3$, 1.0 mg of extracted microsomal lipids, 0.016 unit of NADPH–cytochrome *c* (P-450) reductase and 0.1 mM NADPH in a final volume of 1.0 ml. Malondialdehyde formed was calculated using a molar extinction coefficient for malondialdehyde–thiobarbituric acid complex of 156 $mM^{-1}cm^{-1}$ [13]. Although chelating agents such as EDTA have been demonstrated to inhibit microsomal lipid peroxidation [14], EDTA-chelated iron is required for the reconstituted system [3, 4]. Macrocyclic polyamines were prepared with a minor modification [15] of the method of Koyama and Yoshino [16] and Martin *et al.* [17]. The structure and abbreviations of macrocyclic polyamines are shown in Fig. 1. Since these synthetic polyamines do not have primary amino groups, they are probably more stable than naturally occurring polyamines *in vivo*.

Effects of macrocyclic polyamines and spermine on microsomal lipid peroxidation are shown in Fig. 2. An inhibitory effect was obtained with 2,3,3,3-, 2,3,4,3- and 3,3,3,4-cyclic polyamines and spermine. The maximum degree of inhibition was produced by the 2,3,3,3-cyclic polyamine.

The time course of NADPH-dependent microsomal lipid peroxidation in the presence or absence of 2,3,3,3-cyclic polyamine are shown in Fig. 3. Malondialdehyde was formed at the rate of 5.4 nmole/mg/min in the control mixture after a lag period. On the other hand, the rate of malondialdehyde formation in the presence of 0.1 mM and 0.2 mM of 2,3,3,3-cyclic polyamine were 1.57 and 0.83 nmole/mg/min, respectively. Furthermore, the inhibitory action of 2,3,3,3-cyclic polyamine was virtually independent of the incubation time. These results suggest that

(1) $n_1 = n_2 = 3$, $n_3 = 4$
3,3,4 – cyclic polyamine

(1) $n_1 = n_2 = n_3 = 3$, $n_4 = 4$
3,3,3,4 – cyclic polyamine

(2) $n_1 = 2$, $n_2 = n_3 = n_4 = 3$
2,3,3,3 – cyclic polyamine

(3) $n_1 = 2$, $n_2 = n_4 = 3$, $n_3 = 4$
2,3,4,3 – cyclic polyamine

Fig. 1. Illustration of cyclic polyamines.

2,3,3,3-cyclic polyamine may not have a general property of antioxidants.

Since NADPH-dependent lipid peroxidation is catalysed by NADPH–cytochrome *c* (P-450) reductase, the effect of 2,3,3,3-cyclic polyamine on the activity of the reductase was investigated. As can be seen in Table 1, 2,3,3,3-cyclic polyamine did not affect the activity of the reductase. In addition, the peroxidation induced by cumene hydroperoxide which has been considered to be dependent on cytochrome P-450 [18, 19], was not affected by the addition of 2,3,3,3-cyclic polyamine suggesting that the cyclic polyamine does not affect the propagation step of lipid peroxidation proposed by Svingen *et al.* [19]. The effect of 2,3,3,3-cyclic polyamine on lipid peroxidation catalysed by purified NADPH–cytochrome *c* (P-450) reductase was also studied. As shown in Table 1, 2,3,3,3-cyclic polyamine was an inhibitor of reconstituted system in which membrane structure is known to be absent. It was found, however, that the effect of cyclic polyamine on lipid peroxidation catalysed by purified NADPH–cytochrome *c* (P-450) reductase was less inhibitory than that on microsomal lipid peroxidation. Since spermine or spermidine could not inhibit lipid peroxidation in the reconstituted system (data not shown), these results suggest that 2,3,3,3-cyclic polyamine has another inhibitory effect which is different from the inhibitory effect caused by polyamine interaction with microsomal lipids [7, 20].

As shown in Fig. 4, 2,3,3,3-cyclic polyamine inhibited lipid peroxidation semi-competitively with respect to the amounts of endogenous and exogenously added lipids, although spermine could competitively inhibit lipid peroxidation [20].

Another inhibitory site by 2,3,3,3-cyclic polyamine in lipid peroxidation was then studied. Since ferrous ion has been shown to be essentially required for reconstitution of NADPH–cytochrome *c* (P-450) reductase supported lipid peroxidation [3, 4], we have examined whether the inhibition of lipid peroxidation by 2,3,3,3-cyclic polyamine was partially caused by the chelating action of this compound. The content of iron in our microsomes was 22 nmole/mg. The inhibitory effect of 2,3,3,3-cyclic polyamine on lipid peroxidation in rat liver microsomes decreased as the concentration of ferric ion added was increased (Fig. 5a). However, the inhibition of lipid peroxidation by 2,3,3,3-cyclic polyamine was still observed when the ferric ion was added to the reaction mixture at a concentration of 400 μM. In addition, in the presence of 100 μM ferric ion, the inhibition of lipid peroxidation by 2,3,3,3-cyclic polyamine

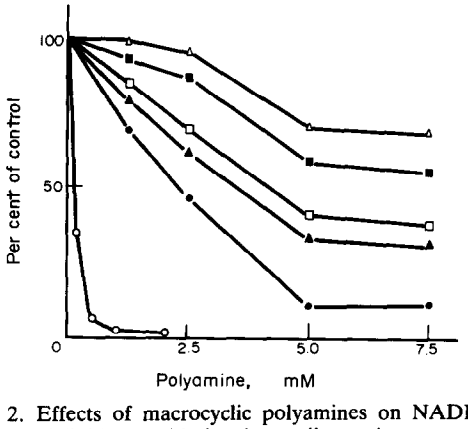

Fig. 2. Effects of macrocyclic polyamines on NADPH-supported lipid peroxidation in rat liver microsomes. A standard mixture for microsomal lipid peroxidation contained 80 mM Na,K–phosphate (pH 7.4), 6 mM $MgCl_2$, 0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 unit of glucose-6-phosphate dehydrogenase and microsomes (0.55 mg of proteins). Incubation was carried out at 37° for 10 min aerobically in the presence of spermidine (△), 3,3,4-cyclic polyamine (■), 3,3,3,4-cyclic polyamine (□), spermine (▲), 2,3,4,3-cyclic polyamine (●), and 2,3,3,3-cyclic polyamine (○).

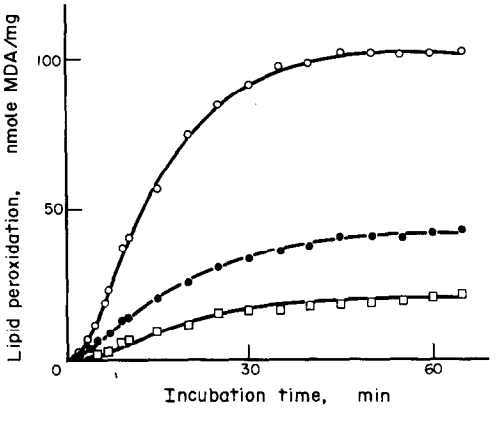

Fig. 3. Effect of 2,3,3,3-cyclic polyamine on the time course of NADPH-dependent microsomal lipid peroxidation. Malondialdehyde formed was measured in the presence (0.1 mM ●, 0.2 mM □) or absence (○) of 2,3,3,3-cyclic polyamine. Other experimental details were as described in Fig. 2.

Table 1. Effects of 2,3,3,3-cyclic polyamine on NADPH-cytochrome *c* (P-450) reductase activity, cumene hydroperoxide induced lipid peroxidation and NADPH-cytochrome *c* (P-450) reductase supported lipid peroxidation in reconstituted system*

Addition	NADPH-cytochrome *c* (P-450) reductase activity (unit/mg)	Cumene hydroperoxide induced lipid peroxidation (nmole MDA/mg/min)	NADPH-cytochrome *c* (P-450) reductase supported lipid peroxidation in reconstituted system (μmole MDA/unit/min)
None	0.119 ± 0.004	1.218 ± 0.021	0.499 ± 0.016
2,3,3,3-Cyclic polyamine (0.5 mM)	0.118 ± 0.003	1.116 ± 0.020	0.422 ± 0.015
2,3,3,3-Cyclic polyamine (1.0 mM)	—	1.080 ± 0.017	0.355 ± 0.011
2,3,3,3-Cyclic polyamine (2.0 mM)	0.108 ± 0.001	—	0.199 ± 0.001

* The assay mixture (1 ml) for cumene hydroperoxide induced lipid peroxidation contained 80 mM Na,K–phosphate (pH 7.4), 0.55 mg of microsomal proteins and 0.1 mM cumene hydroperoxide. Other experimental details were as described in the text.

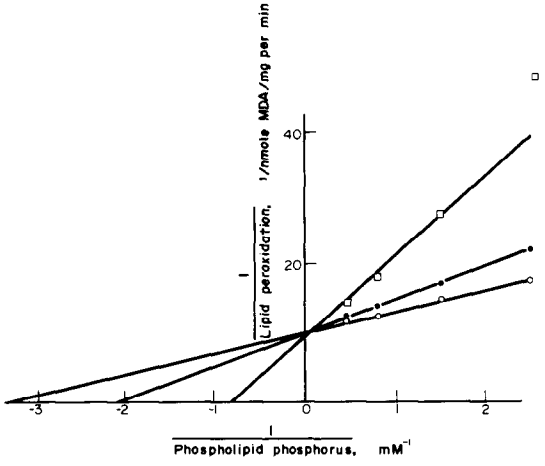

Fig. 4. Double reciprocal plots of microsomal lipid peroxidation against the concentration of phospholipids. The formation of malondialdehyde was measured in the presence (0.1 mM ●, 0.5 mM □), or absence (○) of 2,3,3,3-cyclic polyamine. Other experimental details were as described in Fig. 2.

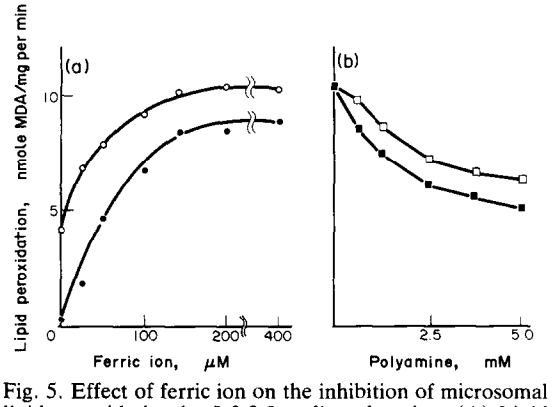

Fig. 5. Effect of ferric ion on the inhibition of microsomal lipid peroxidation by 2,3,3,3-cyclic polyamine. (A) Lipid peroxidation was measured as described in Fig. 2 in the presence (●) or absence (○) of 0.5 mM 2,3,3,3-cyclic polyamine. The concentration of ferric ion employed was specified in the figure. (B) Lipid peroxidation was measured in the presence of 100 μM ferric ion. 2,3,3,3-Cyclic polyamine (■); spermine (□).

was nearly equal to that by spermine (Fig. 5b). These results suggest that the chelating action of 2,3,3,3-cyclic polyamine with ferrous ion was at least partially responsible for the inhibition of lipid peroxidation in these experimental conditions.

From these results it was concluded that the inhibitory effect of 2,3,3,3-cyclic polyamine might have been due to both its chelating action with ferrous ion and its interaction with microsomal lipids.

Faculty of Pharmaceutical Sciences
Chiba University
Yayoi-cho, Chiba 260
Japan

MITSUKAZU KITADA
KAZUEI IGARASHI*
SHIGERU OHMORI
HARUO KITAGAWA
SEIYU HIROSE
YOSHIO KANAKUBO

Institute of Pharmaceutical Sciences
Hiroshima University School of Medicine
Hiroshima, 734 Japan

TAKASHI YATSUNAMI
EIICHI KIMURA

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* To whom correspondence should be addressed.

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Biochemical Pharmacology, Vol. 31, No. 8, pp. 1661–1663, 1982.
Printed in Great Britain.

0006-2952/82/081661-03 $03.00/0

Specific 5-hydroxytryptamine binding to rat platelets as a system to evaluate tricyclic antidepressants in plasma

(*Received* 3 *August* 1981; *accepted* 20 *November* 1981)

Tricyclic antidepressants (TAD) are the major drugs now used in the treatment of mental depression. However only 65% of the patients given TAD show a clinical improvement [1]. Interindividual variation in plasma steady-state TAD levels as a consequence of the differences in the metabolism of these compounds has been claimed to be the reason for the lack of effects in some patients [2]. The routine determination of blood levels of TAD is therefore of great importance especially since both low and high plasma values has been reported to be correlated with a poor clinical response [3]. A number of different procedures are currently used to monitor blood levels of TAD; these are often time-consuming and complex [1, 4, 5] and an obvious place exists in clinical medicine for a simple and rapid biological assay to detect TAD and their active metabolites in plasma. The binding of 5-hydroxytryptamine (5-HT) to rat platelets at 4° demonstrated the presence of three saturable sites. Interestingly, the medium affinity site was found to be extremely sensitive to the inhibition by clomipramine and imipramine [6]. We report here the use of such a system as a bioassay to evaluate the concentration of TAD in plasma.

Materials and methods

Blood was obtained from rats (200–300 g body wt) anesthetized with ether by puncture of the carotid artery. Samples were anticoagulated with trisodium citrate (final concentration in blood 2 mg/ml) and centrifuged at room temperature for 10 min at 180 *g*. The supernatant platelet-rich plasma (PRP) was removed and kept for a maximal time of 2 h at room temperature. Cell-free plasma was prepared by centrifugation of the PRP for 20 min at 10,000 *g*.

For the radioreceptor assay polypropylene incubation tubes received, in order, 0.04 ml of PRP containing an average of 40×10^6 platelets, either 0.05–0.20 ml of plasma (from drug treated or drug-free animals) or 0.1 ml of drug for the standard curve, 0.05 ml of 1 μM 5-hydroxy-[G-^{3}H]tryptamine creatinine sulphate (15.5 Ci/mmole; Radiochemical Centre, Amersham, U.K.) and a modified Tyrode solution (containing 136 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose and 3.5 g/l bovine albumin) to 1 ml total volume. After 2 min at 4° the bound [^{3}H]-5-HT was separated from the free fraction by centrifugation at 14,700 *g* for 2 min in the cold. The pellet was then rapidly washed (< 5 sec) with 1 ml of Tyrode solution and further digested with 0.25 ml 19 M formic acid in an oven heated at 80° for 10 min. The pellet was dissolved in PCS (Radiochemical Centre) and counted in a Packard Tri-Carb 2425 liquid scintillation counter. Blanks without PRP were run in parallel. Moreover, for each assay the amount of unspecific binding was evaluated by adding a 100-fold excess of unlabelled 5-HT. Under these conditions, approximately 5% of the radioactivity remained in the pellet and this value was subtracted from the total [^{3}H]-5-HT bound in order to correct for extracellular space and for that which was unspecifically bound to the platelets. Remarkably, as compared with experiments described in previous reports [6, 7] a higher sensitivity of this system to the TAD came about when the PRP was diluted with Tyrode solution.

For the *in vivo* experiments, rats were killed after being treated intraperitoneally with a dose of the drug. Plasma was obtained as indicated above.

Source of drugs. Nortriptyline hydrochloride, amitriptyline hydrochloride, and chlordiazepoxide hydrochloride from Hoffman-La Roche (Basel, Switzerland); clomipramine hydrochloride, imiprazine hydrochloride and desipramine hydrochloride from Ciba-Geigy (Basel, Switzerland); viloxazine hydrochloride from ICI-Farma (Madrid, Spain); and chlorpromazine hydrochloride from Barcia (Madrid, Spain). All drugs used were dissolved in 0.9% (w/v) NaCl.

Results

A number of preliminary experiments confirmed that [^{3}H]-5-HT binding to platelets was reached after 2 min of incubation at 4°. Under the conditions selected for the assay no significant active transport occurs [6] and Scatchard analysis of the binding within the concentration range 0.0001–10 μM of [^{3}H]-5-HT gave a curvilinear relationship from which three sites could be resolved. The apparent dissociation constants for these sites were 10^{-9} M, 4×10^{-7} M and 3.5×10^{-6} M. On the basis that, as in previous reports [6, 7], the number of high affinity sites was found to be much lower than the medium affinity sites, a