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INHIBITION BY POLYAMINES OF LIPID PEROXIDE FORMATION IN RAT LIVER MICROSOMES

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SUMMARY: Both NADPH- and ascorbic acid-dependent lipid peroxidations were inhibited by spermine, the degree of inhibition being greater with the former peroxidation. The effective concentration of spermine required for inhibition was higher when larger amounts of microsomes were used. However, the activities of NADPH-cytochrome c reductase and NADPH-peroxidase were not influenced by spermine. These results suggest that spermine inhibits lipid peroxidation by binding to phospholipids in the microsomes.

Polyamines have been implicated in numerous growth processes (1,2). In this respect, the effects of polyamines on DNA replication (3), RNA synthesis (4-6), and protein synthesis (7-9) have been studied extensively.

Lipid peroxidation *in vivo* has been identified as a basic deteriorative reaction in cellular mechanisms of the aging process (10,11). Since polyamine concentration in animal tissues decreases gradually with aging and polyamines react not only with nucleic acid but also with phospholipids (1), we have investigated whether polyamines affect lipid peroxidation in rat liver microsomes.

MATERIALS AND METHODS

Materials - Polyamines (HCl salt) were purchased from Nakarai Chemicals Co. $NADP^+$, glucose 6-phosphate-Na and glucose-6-phosphate dehydrogenase (grade I) were obtained from Boehringer Mannheim GmbH. Thiobarbituric acid was purchased from Daiichi Pure Chemicals Co.; just before use, this was dissolved in 0.5% sodium acetate to made a final concentration of 0.67%.

Male Wistar rats weighing 120 - 150 g were starved for 16 to 20 hr and exsanguinated without anesthesia. The liver was removed quickly after perfusion with isotonic KCl solution, rinsed with the same solution, and homogenized in 3 volumes of the same solu-

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tion by means of 8 strokes in a motor-driven Potter homogenizer with a Teflon pestle. The homogenate was centrifuged for 20 min at 9,000 x g and supernatant fluid was recentrifuged at 105,000 x g for 1 hr. The microsomal pellet was resuspended in ice-cold isotonic KCl solution and recentrifuged for 30 min at 105,000 x g. The washed microsomal pellet thus obtained was suspended in water to give a final protein concentration of 30 to 40 mg per ml. The microsomal suspension was stored at -70° under nitrogen atmosphere and was used within 2 to 3 days. Microsomal protein was determined according to the method of Lowry et al. (12).

Assay of lipid peroxidation - Lipid peroxidation was determined by the thiobarbituric acid method, as described by Wills (13) and Kamataki et al. (14). The reaction mixture (0.5 ml) contained 100 mM Na, K-phosphate (pH 7.4), 6 mM magnesium acetate, 0.33 mM $NADP^+$, 8 mM glucose 6-phosphate, 0.045 unit of glucose-6-phosphate dehydrogenase, microsomes (600 µg of protein), and cations at the specified concentrations. When ascorbic acid-dependent lipid peroxidation was measured, 300 µM ascorbic acid replaced the $NADP^+$, glucose 6-phosphate and glucose-6-phosphate dehydrogenase. After incubation at 37° for 10 min, 0.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged. The resulting supernatant (0.5 ml) was mixed with 3.5 ml of 0.67% thiobarbituric acid, heated at 100° for 10 min, and the TBA value (absorbance at 532 nm/mg of microsomal protein) was then determined.

Assay of NADPH-cytochrome c (P-450) reductase activity and NADPH-peroxidase activity - NADPH-cytochrome c (P-450) reductase activity was measured using cytochrome c as an electron acceptor according to the method of Phillips and Langdon (15). A unit of NADPH-cytochrome c reductase is defined as one µmole cytochrome c reduced per minute.

Microsomal NADPH-peroxidase activity was measured according to the method of Hrycay and O'Brien (16). The rate of oxidation of NADPH by cumene hydroperoxide in the first minute of reaction was measured at 340 nm using an Aminco recording spectrophotometer, Model DW-2. The reaction mixture (3 ml) contained 0.1 M Na, K-phosphate (pH 7.4), 2 mg microsomal protein, 0.1 mM EDTA, 0.15 mM NADPH, and 0.5 mM cumene hydroperoxide. The reaction rates were calculated using an extinction coefficient for NADPH of 6.22 cm^{-1} mM^{-1}.

RESULTS

Effect of polyamines on NADPH dependent lipid peroxidation - As shown in Fig. 1, the addition to the reaction mixture of either spermine or spermidine inhibited lipid peroxidation markedly; however, the spermine was considerably more inhibitory. The addition of putrescine showed a minimal inhibitory effect. The effect of Mg^{2+} and of K^+ on lipid peroxidation was studied also. As shown in Fig. 2, the addition of less than 5 mM Mg^{2+} did not cause inhibition, but the addition of more than 5 mM Mg^{2+} gradually

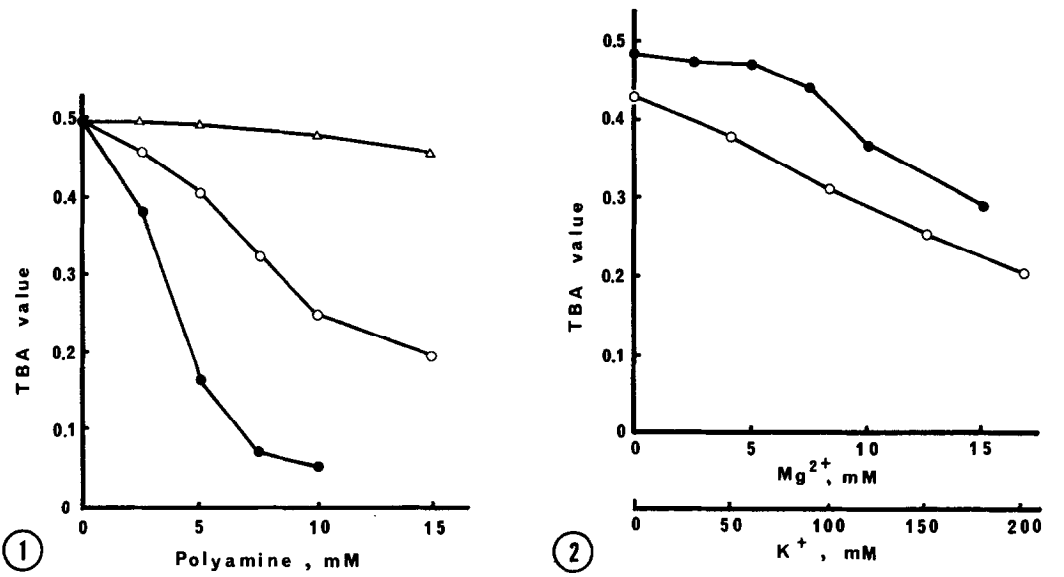

Fig. 1. Effect of polyamines on NADPH dependent lipid peroxidation. The assays were carried out under standard conditions, except that polyamines were added to the reaction mixture as specified. ●, spermine; o, spermidine; △, putrescine.

Fig. 2. Effect of K^+ and Mg^{2+} on NADPH dependent lipid peroxidation.
The assays were carried out under standard conditions, except that various concentrations of K^+ or Mg^+ were added to the reaction mixture. ●, Mg^{2+}; o, K^+.

inhibited lipid peroxidation. Although the concentration of K^+ required for inhibition of lipid peroxidation was high, a linear relationship between $[K^+]$ and inhibition was observed. Lipid peroxidation was not inhibited significantly by Na^+(data not shown). The results of Table I show that both spermidine and spermine can further inhibit lipid peroxidation even in the presence of inhibitory concentrations of Mg^{2+}(10 mM) and K^+(150 mM).

In Fig. 3, it can be seen that as the microsomal content was increased the concentration of spermine required for inhibition of NADPH dependent lipid peroxidation was also increased. However, the activities of NADPH-cytochrome c reductase and NADPH-peroxidase were not influenced by polyamines (Table II). This

Table I. Effect of polyamines on NADPH dependent lipid peroxidation in the presence of 10 mM Mg^{2+} and 150 mM K^{+}.

Addition	TBA value (A_{532}/mg/10 min)	% of control
None	0.204	
Spermine (5 mM)	0.123	60.3
Spermine (10 mM)	0.053	26.0
Spermidine (7.5 mM)	0.170	83.3
Spermidine (15 mM)	0.108	52.9
Putrescine (10 mM)	0.201	98.5
Putrescine (20 mM)	0.178	87.3

The assays were carried out under standard conditions, except that 10 mM Mg^{2+}, 150 mM K^{+} and various concentrations of polyamines, as specified in the table, were added to the reaction mixture.

Table II. Effect of polyamines on the activities of NADPH-cytochrome c reductase and NADPH-peroxidase.

Addition	NADPH-cytochrome c reductase (unit/mg of protein)	NADPH-peroxidase (n moles NADPH oxidized/min/mg of microsomal protein)
None	0.111	14.47
Spermine (1.25 mM)	0.109	-
Spermine (2.5 mM)	0.112	15.60
Spermine (5 mM)	0.108	15.60
Spermidine (5 mM)	0.112	14.31
Spermidine (10 mM)	0.118	17.21
Putrescine (30 mM)	0.113	16.54

The assays were carried out under standard conditions, except that polyamines were added to the reaction mixture as specified in the table.

suggests that the effect of spermine on lipid peroxidation may be induced through the binding of spermine to phospholipid.

When Tris-HCl buffer was used instead of Na, K-phosphate buffer, similar results were obtained; however, polyamines were found to be slightly less effective (data not shown).

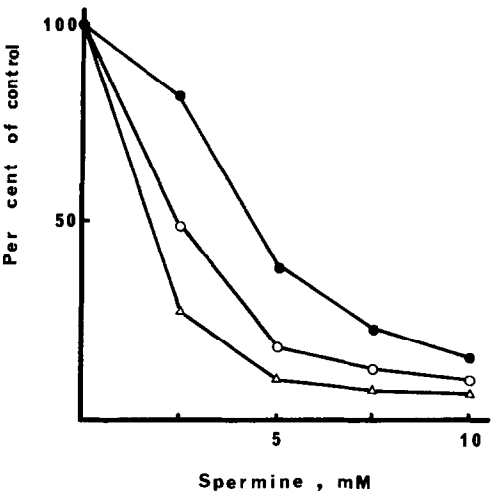

Fig. 3. Effect of spermine and microsomal concentrations on NADPH dependent lipid peroxidation.
The assays were carried out under standard conditions, except that microsomes and spermine were added to the reaction mixture as specified. ●, microsomes containing 685 µg protein (.499); o, microsomes containing 370 µg protein (.483); Δ, microsomes containing 200 µg protein (.375). The value in parentheses is the TBA value without spermine.

Effect of polyamines on ascorbic acid dependent lipid peroxidation - The effect of polyamines on lipid peroxidation, independent of NADPH regeneration, was studied also (Fig. 4). It was found that either in ascorbic acid- or in ascorbic acid and Fe^{2+}-dependent lipid peroxidation spermine or spermidine was inhibitory. However, the inhibition by polyamines of ascorbic acid dependent lipid peroxidation was less than that of NADPH dependent lipid peroxidation. The addition of putrescine caused only slight inhibition (data not shown). Since ascorbic acid dependent lipid peroxidation is non-enzymatic (17), these results support further the hypothesis that polyamines cause the inhibition of lipid peroxidation through the binding of polyamines to phospholipids.

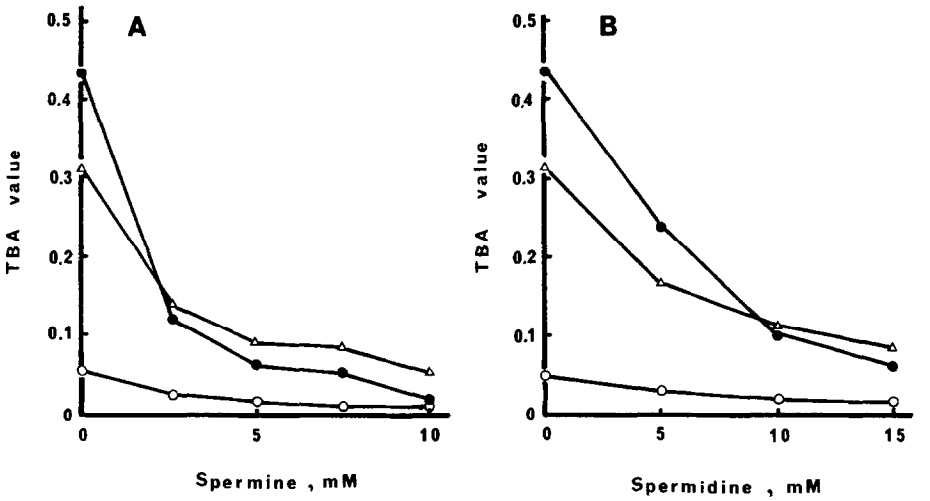

Fig. 4. Effect of polyamines and Fe^{2+} on ascorbic acid dependent lipid peroxidation.
The assays were carried out under standard conditions, except that spermine (A) or spermidine (B) was added to the reaction mixture. ●, NADPH dependent lipid peroxidation; o, ascorbic acid dependent peroxidation; Δ, ascorbic acid dependent lipid peroxidation in the presence of 20 μM Fe^{2+}.

DISCUSSION

It is of interest that polyamines not only stimulate macromolecule synthesis but also inhibit lipid peroxidation, since polyamines gradually decrease with aging. Therefore, we are studying the effect of polyamines on other basic deteriorative reactions in cellular mechanisms of the aging process.

Among the polyamines studied spermine was the most effective in inhibiting lipid peroxidation. This is in contrast to the effect of polyamines on protein synthesis, a reaction in which spermidine was the most effective stimulant (18). These results suggest that phospholipids may have a relatively higher affinity for spermine than do nucleic acids. It should be also mentioned that the inhibitory effect of spermine was much stronger than that of Mg^{2+} or of K^{+}.

Although polyamines did not influence the activities of NADPH-cytochrome c reductase and NADPH-peroxidase, NADPH dependent lipid peroxidation was inhibited more by spermine than ascorbic acid dependent lipid peroxidation. The reason for this phenomenon is under investigation.

It has been reported that the inhibition of lipid peroxidation by EDTA causes the stimulation of the activities of drug-metabolizing enzymes in rat liver microsomes (19). We are now studying whether or not polyamines can exert a similar relationship.

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REFERENCES

1. Cohen, S. S. (1971) Introduction to the Polyamines, Prentice-Hall, Englewood Cliffs, N. J.
2. Tabor, C. W. and Tabor, H. (1976) Ann. Rev. Biochem. 45, 285-306.
3. Schwimmer, S. (1968) Biochim. Biophys. Acta, 166, 251-254.
4. Raina, A. and Janne, J. (1970) Fed. Proc. 29, 1568-1574.
5. Russel, D. H. (1970) Ann. N. Y. Acad. Sci. 171, 772-782.
6. Chiu, J. F. and Sung, S. C. (1972) Biochim. Biophys. Acta, 281, 535-542.
7. Igarashi, K., Sugawara, K., Izumi, I., Nagayama, C. and Hirose, S. (1974) Eur. J. Biochem. 48, 495-502.
8. Konicki, D., Kramer, G., Pinphanichakarn, P. and Hardesty, B. (1975) Arch. Biochem. Biophys. 169, 192-198.
9. Igarashi, K., Kashiwagi, K., Kishida, K., Watanabe, Y., Kogo, A. and Hirose, S. (1979) Eur. J. Biochem. in the press.
10. Packer, L., Deamer, D. W. and Heath, R. L. (1967) Advances in Gerontological Research (Strehler, B. L., ed.) Vol. 2, pp. 77-120, Academic Press. New York.
11. Barber, A. A. and Bernheim, F. (1967) Advances in Gerontological Research (Strehler, B. L., ed.) Vol, 2, pp 355-403, Academic Press, New York.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
13. Wills, E. D. (1966) Biochem. J. 99, 667-676.
14. Kamataki, T., Ozawa, N., Kitada, M., Kitagawa, H. and Sato, R. (1974) Biochem. Pharmacol. 23, 2485-2490.
15. Phillips, A. H. and Langdon, R. G. (1962) J. Biol. Chem. 237,2652-2660.
16. Hrycay, E. G. and O'Brien, P. J. (1973) Arch. Biochem. Biophys. 157, 7-22.
17. Hochstein, P. and Ernster, L. (1963) Biochem. Biophys. Res. Commun. 12, 388-394.
18. Atkins, J. F., Lewis, J. B., Anderson, C. W. and Gesteland, R. F. (1975) J. Biol. Chem. 250, 5688-5695.
19. Kamataki, T. and Kitagawa, H. (1973) Biochem. Pharmacol. 22, 3199-3207.