EFFECTS OF THYMOSIN ALPHA-1 ON ERYTHROCYTE LIPID LEVELS AND ERYTHROCYTE MEMBRANE (Na⁺-K⁺)-ATPase ACTIVITY IN EXPERIMENTAL HYPERCHOLESTEROLEMIA

Cahide GÖKKUŞU, Evin ADEMOĞLU, Hikmet Öz
and Ümit Mutlu TÜRKOĞLU

Department of Biochemistry, Faculty of Medicine, University of Istanbul, Capa, Istanbul, Turkey

(Received August 21, 1996. Accepted February 28, 1997)

SUMMARY: Thymosin alpha-1 is an active polypeptide isolated from thymus. This polypeptide is widely used in the diagnosis and treatment of many diseases, especially immune diseases. In this present study, we examined the effects of thymosin alpha-1 on plasma and erythrocyte lipid levels and the changes in erythrocyte membrane (Na⁺, K⁺)ATPase activity in hypercholesterolemic rabbits. The erythrocyte lipid levels decreased, whereas the erythrocyte membrane (Na⁺, K⁺)ATPase activity increased significantly in these rabbits after thymosin alpha-1 injection. These findings suggest that thymosin alpha-1 is effective on both the lipid level and erythrocyte membrane (Na⁺, K⁺)ATPase activity.

INTRODUCTION

Thymus, is accepted as an endocrine gland, that synthesizes hormone-analogous polypeptides with different chemical structures (1). Among these active peptides, particularly the thymosin family, seem to play an important role in the growth and development of the lymphoid tissues and in the maintenance of the immune balance (2,3). Presently, thymosin F5 and thymosin alpha-1, which is a subgroup of thymosin F5, are extensively employed in both in vivo and in vitro studies carried out on animals and humans in the diagnosis and treatment of a

number of diseases, such as immune diseases (4-7). One of the other subjects in which the effects of thymosins are studied, is the lipid metabolism. In the recent studies carried out on different experimental models for this purpose demonstrated that thymosins decreased high cholesterol and triglyceride levels (8,9). It was revealed that thymus fraction III (F3) reduces high cholesterol levels by activating the low-density lipoprotein (LDL)-receptor pathway (10). It was also reported in another study (11) that thymic hypercholesterolemic factor (Tphf) inhibited 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which plays a role in the regulation of cholesterol synthesis.

On the other hand, it has been reported that there exists a close relationship between $(\text{Na}^+, \text{K}^+)\text{ATPase}$ activity and the degree of unsaturation of fatty acids in the erythrocyte membrane (12,13). It has been discovered that when membrane cholesterol levels are increased, $(\text{Na}^+, \text{K}^+)\text{ATPase}$ is decreased, whereas when membrane cholesterol levels are decreased, this enzyme becomes active. It has been suggested that the increased cholesterol levels inhibited the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ activity by effecting the allosteric behavior of the enzyme (14,15).

In our previous study, we showed that thymosin F5 led to a change in the plasma and erythrocyte lipid contents and erythrocyte membrane $(\text{Na}^+, \text{K}^+)\text{ATPase}$ activity in the hypercholesterolemic rabbits. In this study, we attempted to examine the effect of thymosin alpha-1 on plasma and erythrocytes lipid levels as well as erythrocyte membrane $(\text{Na}^+, \text{K}^+)\text{ATPase}$ activity in hypercholesterolemic rabbits. To compare, our results with those we obtained with thymosin F5, we chose the same experimental conditions.

MATERIALS AND METHODS

New Zealand rabbits weighing 2.5 kg were used. They were divided into two groups. The control group was fed standard chow pellets for 2.5 months. The experimental group (Group A) was fed chow pellets containing 2% (w/w) cholesterol for the same period. At the end of this period, after the blood samples were withdrawn the rabbits in the experimental group were divided into two groups. One of the groups (Group B) was fed standard chow pellets for 14 days, the other group (Group C) received 25 $\mu\text{g/kg}$ thymosin alpha-1 (supplied by A. L. Goldstein, George Washington University, Washington, DC), intraperitoneally every other day in addition to standard chow pellets for the same period. After this period, the rabbits were fasted overnight and blood samples withdrawn to heparinized test tubes from the marginal vein of the ear. Heparinized blood was centrifuged

to separate plasma and erythrocytes. Lipids of the erythrocytes were extracted by the method of Rose and Oklander (16). Total cholesterol levels of plasma and erythrocyte lipid extracts and plasma triglyceride levels were measured with enzymatic kits (Sigma, St. Louis, MO). Phospholipid levels of plasma and erythrocyte lipid extracts were assayed according to Gomori (17).

The enzyme reaction was performed as described by Wood and Beutler (18). Briefly, 1.5 ml of packed erythrocytes were lysed in a hemolyzing solution (7.0 mM Tris/0.1 mM Na₂EDTA, pH 7.4), and the lysate was centrifuged at 15,000 × g for 20 min. A hemoglobin-free membrane suspension (0.5 ml) was added to 1.9 ml of an incubation mixture containing 30 μmoles of Tris-HCl (pH 7.4), 2 μmoles of NaCl, with or without 10 μmoles of KCl. The reaction was started with 2 μmoles of disodium ATP and incubated at 37 C for 1 hr. ATPase activities were estimated by measuring inorganic phosphorus released during 1-hr incubation of the membrane suspensions with or without KCl. The (Na⁺-K⁺)ATPase activity was calculated from the difference between the total ATPase and Mg⁺² ATPase activities. This activity was expressed as inorganic phosphorus liberated per mg membrane protein per hour. The protein content of membrane was measured by the method of Lowry et al (19). All data were presented as mean ± S.D. Student's t test was performed for statistical analysis.

RESULTS

Lipid levels in plasma and erythrocytes as well as erythrocyte membrane ATPase activities after cholesterol-rich diet feeding are shown in Tables I, II and III. In the atherosclerotic group (Group A), cholesterol, triglyceride and phospholipid levels in plasma were significantly higher than controls ($p < 0.001$) (Table I). The erythrocytes cholesterol levels were higher by 2.75 fold, while the phospholipid levels remained unchanged in the atherosclerotic group (Table II). When the erythrocytes cholesterol/phospholipid ratio of the atherosclerotic group was compared with the data obtained with the control group, a significant increase was observed ($p < 0.001$) (Table II). In the atherosclerotic group (Group A), cholesterol-rich-diet feeding caused a significant decrease in the ATPase activities of erythrocyte membranes ($p < 0.001$) (Table III).

The data obtained after thymosin alpha-1 injections on alternate days to the hypercholesterolemic rabbits are seen in Tables I, II and III. Thymosin alpha-1 treatment caused a significant decrease in the lipid levels of plasma and erythrocytes (Group A vs. Group C), while the erythrocyte phospholipid level remained unchanged (Tables I and II). The erythrocyte cholesterol/phospholipid ra-

Table I. Effect of thymosin alpha-1 treatment on erythrocyte lipid levels in hypercholesterolemic rabbits

	Control group (n = 5)	Group A (n = 20)	Group B (n = 10)	Group C (n = 10)
Cholesterol (mg/dl)	86.6 ± 6.6	631 ± 19.0*	302.5 ± 10.3 ¹	189.9 ± 7.3 ^{1,a}
Triglyceride (mg/dl)	56.7 ± 8.3	265.1 ± 17.0*	177.8 ± 18.3 ¹	123.2 ± 7.8 ^{1,a}
Phospholipid (mg/dl)	77.5 ± 5.4	268.9 ± 20.4*	122.1 ± 8.0 ¹	90.5 ± 9.8 ^{1,a}

(A) Group: cholesterol diet for 2.5 months, (B) Group: standard diet for 14 days after cholesterol diet, (C) Group: standard diet + thymosin alpha-1 (ip) for 14 days after cholesterol diet. *p < 0.001 as compared to controls, ¹p < 0.001 in comparison of (A) with (B) and (C) groups, ^ap < 0.001 in comparison of (B) with (C) groups.

Table II. Effect of thymosin alpha-1 treatment on erythrocyte lipid levels in hypercholesterolemic rabbits

	Control group (n = 5)	Group A (n = 20)	Group B (n = 10)	Group C (n = 10)
Cholesterol (mg/dl)	1.2 ± 0.01	3.3 ± 0.06*	2.4 ± 0.02 ¹	2.02 ± 0.03 ^{1,a}
Phospholipid (mg/dl)	4.4 ± 0.30	4.0 ± 0.7	4.1 ± 0.08	4.3 ± 0.01
Cholesterol/ phospholipid (mg/dl)	0.3 ± 0.03	0.8 ± 0.08	0.6 ± 0.3	0.5 ± 0.3 ^{1,a}

(A) Group: cholesterol diet for 2.5 months, (B) Group: standard diet for 14 days after cholesterol diet, (C) Group: standard diet + thymosin alpha-1 (ip) for 14 days after cholesterol diet. *p < 0.001 as compared to controls, ¹p < 0.001 in comparison of (A) with (B) and (C) groups, ^ap < 0.001 in comparison of (B) with (C) groups.

tio was decreased by 1.33-fold in group C rabbits (Table II). The erythrocyte (Na⁺-K⁺)ATPase activity showed a significant increase in thymosin alpha-1-injected rabbits when compared to hypercholesterolemic animals (Group A vs.

Table III. Effect of thymosin alpha-1 treatment on erythrocyte lipid levels in hypercholesterolemic rabbits

	Control group (n = 5)	Group A (n = 20)	Group B (n = 10)	Group C (n = 10)
Total ATPase ($\mu\text{molPi}/\text{mg}/\text{hr}$)	1.74 ± 0.03	$0.97 \pm 0.07^*$	1.10 ± 0.06^1	$1.63 \pm 0.09^{1,a}$
Mg ²⁺ ATPase ($\mu\text{molPi}/\text{mg}/\text{hr}$)	0.33 ± 0.01	$0.79 \pm 0.05^*$	0.86 ± 0.11^2	$1.23 \pm 0.01^{1,a}$
Cholesterol/ phospholipid ($\mu\text{molPi}/\text{mg}/\text{hr}$)	0.41 ± 0.02	$0.18 \pm 0.12^*$	0.24 ± 0.17	$0.40 \pm 0.09^{1,b}$

(A) Group: cholesterol diet for 2.5 months, (B) Group: standard diet for 14 days after cholesterol diet, (C) Group: standard diet + thymosin alpha-1 (ip) for 14 days after cholesterol diet. * $p < 0.001$ as compared to controls, ¹ $p < 0.001$ in comparison of (A) with (B) and (C) groups, ^a $p < 0.001$ in comparison of (B) with (C) groups.

Group C) (Table III). On the other hand, when the results obtained with group B were compared to those with group C, group C exhibited more significant results (Tables I, II and III).

DISCUSSION

Thymosin alpha-1 is a polypeptide employed in the diagnosis and treatment of many diseases such as chronic active B hepatitis (7), AIDS (20), lung carcinoma (21) due to its immunologic effects. Although there have been intensive studies on the immunologic effects of thymosins, its nonimmunologic effects were not investigated except for a few studies. Emphases were given to this point recently, in which it was indicated that thymosins have stimulating effects on pituitary hormones pituitary-gonadal axis and it has a hyperglycemic effect due to the stimulation of adrenal gland (22-24). Moreover, various studies carried out on thymosins revealed that thymosin F3 and thymosin F5 decrease high cholesterol and triglyceride levels in serum and liver (8,9). Besides, it was shown that the

ATPase activity is decreased in thymectomized rabbits and this decrease is improved by thymic extracts (25,26).

The changes in plasma lipid levels may affect the erythrocyte membrane lipid composition in a short time (27,28). It is known that there is a relation between the lipid composition and the fluidity and permeability of the membranes. The increase in the membrane cholesterol content reduces the membrane fluidity, whereas a decrease causes an elevation in the membrane fluidity and permeability in the experimental animals. It was shown that the osmotic fragility changed and hemolytic anemia occurred in these animals. It has also been reported that activities of some enzymes are dependent on the membrane lipid composition. (Na⁺-K⁺)ATPase is an integral membrane protein, which is responsible for maintenance of the sodium and potassium balance of the cells. It has been demonstrated that the erythrocyte membrane lipid composition affects the activity of (Na⁺-K⁺)ATPase when the membrane cholesterol levels are increased and the (Na⁺-K⁺)ATPase activity is decreased, whereas when membrane cholesterol levels are decreased, this enzyme becomes active (25). It is suggested that cholesterol causes changes in the allosteric behavior of the enzyme by reducing the molecular area of phospholipids or affecting the composition of phospholipids and/or interacting with the membrane proteins (29).

In the present study, we investigated the effects of thymosin alpha-1 in the plasma and erythrocyte membrane lipid content and the erythrocyte membrane (Na⁺-K⁺)ATPase activity of the hypercholesterolemic rabbits. Thymosin alpha-1 caused a significant decrease in the plasma and erythrocytes lipid levels and an increase in the erythrocyte membrane (Na⁺, K⁺)ATPase activity of hypercholesterolemic rabbits (Table II). The results of this study are in agreement with our previous reports with thymosin F5, but thymosin alpha-1 played a much more effective role in the reduction of high plasma and erythrocyte lipid levels than did thymosin F5 even at lower doses and for shorter time.

These beneficial effects of thymosin alpha-1 may be due to (i) the activating effects on the enzymes of bile acid synthesis, (ii) reduction of endogenous cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase, (iii) the increase in the esterification of cholesterol by inhibiting the oxidation of fatty acids, and (iv) the reduction of high plasma cholesterol levels by activating the LDL receptor pathway.

In conclusion, thymosin alpha-1 has beneficial effects on the improvement of impaired (Na⁺, K⁺)ATPase activity by reducing high plasma and erythrocyte

lipid levels in hypercholesterolemic rabbits. Further studies are needed to evaluate the mechanism of the action.

ACKNOWLEDGMENTS

We are indebted to Professor A. L. Goldstein (George Washington University, Washington, D.C.) for his generous gift of thymosin alpha-1.

REFERENCES

1. Oastes, K. K. and Goldstein, A. L. (1984): Thymosins: hormones of the thymus gland. *TIPS*, 5, 347-352.
2. Goldstein, A. L. and White, A. (1971): Role of thymosin and other thymic factors in the development, maturation and functions of lymphoid tissue. *Curr. Top. Exp. Endocrinol.*, 1, 121-149.
3. Trainin, N. (1974): Thymic hormones and immune response. *Physiol. Rev.*, 54, 272-315.
4. Barret, D. J., Wara, D. W., Amman, A. J. and Cowan, M. J. (1980): Thymosin therapy in the DiGeorge Syndrome. *J. Pediatr.*, 97, 66-72.
5. Lavastida, M. A., Goldstein, A. L. and Daniels, J. C. (1981): Preliminary report: thymosin administration in autoimmune disorders. *Thymus*, 2, 287-291.
6. Low, T. L. K. and Goldstein, A. L. (1984): Thymosins: structure, function and therapeutic applications. *Thymus*, 6, 27-42.
7. Sherman, K. E., Jones, C. C., Goldstein, A. L. and Naylor, P. (1991): Low thymosin alpha-1 concentrations in patient chronically infected with the hepatitis B virus. *Viral Immunol.*, 4, 195-199.
8. Gokkusu, C., Omidfar, H. and Oz, H. (1989): Effect of thymosin fraction 5 (F₅) on plasma and erythrocyte lipid levels in atherogenic rabbits. *Med. Sci. Res.*, 17, 959-960.
9. Mondola, P., Santangelo, F., Falconi, C. and Reichi, D. (1983): Effect of a thymus fraction on the hematic Apo B of rats fed on a diet rich cholesterol. *Horm. Metabol. Res.*, 15, 518-519.
10. Mondola, P., Santillo, M., Santangelo, F., Caporale, C., Belfiore, A. and Bifulco, M. (1989): Thymus fraction (FIII) effect on cholesterol metabolism: modulation of the low-density lipoprotein receptor pathway. *Int. Biochem.*, 21, 627-630.
11. Mondola, P., Santillo, M., Santangelo, F., Belfiore, A., Gambardella, P. and Bifulco, M. (1992): Effect of a new calf thymus protein on 3-hydroxy-3-

- methylglutaryl-CoA reductase activity in rat hepatocyte cells (BRL-3A). *Comp. Biochem. Physiol., B*, 103B, 431-434.
12. Giraud, F., Claret, M., Bruckdorfer, K. and Chailley, B. (1981): The effect of membrane lipid order and cholesterol on the internal and external cationic sites of the $\text{Na}^+ + \text{K}^+$ pump in erythrocytes. *Biochim. Biophys. Acta*, 647, 249-258.
 13. Kimelberg, H. K. and Papahadjopoulos, D. (1972): Phospholipid requirements for $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity: head-group specificity and fatty acid fluidity. *Biochim. Biophys. Acta*, 282, 277-292.
 14. Bloj, B., Merero, R. D. and Farias, R. N. (1973): Membrane fluidity, cholesterol and allosteric transitions of membrane-bound $\text{Mg}^{2+}\text{-ATPase}$, $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and acetylcholinesterase from rat erythrocytes. *FEBS Lett.*, 38, 101-105.
 15. Claret, M., Garay, R. and Giraud, F. (1978): The effect of membrane cholesterol on the sodium pump in red blood cell. *J. Physiol.*, 274, 247-263.
 16. Rose, H. G. and Oklander, M. (1965): Improved procedure for extraction of lipids from human erythrocytes. *J. Lipid Res.*, 6, 428-431.
 17. Gomori, G. (1942): Modification of colorimetric phosphorus determination for use with photoelectric colorimeter. *J. Lab. Clin. Med.*, 27, 955-960.
 18. Wood, L. and Beutler, E. (1967): Temperature dependence of sodium potassium activated erythrocyte adenosine triphosphatase. *J. Lab. Clin. Med.*, 70, 288-294.
 19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 256-275.
 20. Naylor, P. H., Friedman-Kien, A., Hersh, E., Erdos, M. and Goldstein, A. L. (1986): Thymosin α_1 and thymosin β_4 in serum: comparison of normal, cord, homosexual and AIDS serum. *Int. J. Immunopharmacol.*, 8, 667-676.
 21. Mastino, A., Favali, C., Grelli, S., Rasi, G., Pica, F., Goldstein, A. L. and Garaci, E. (1992): Combination therapy with thymosin alpha-1 potentiates the anti-tumor activity of interleukin-2 with cyclophosphamide in the treatment of the Lewis lung carcinoma in mice. *Int. J. Cancer*, 50, 493-499.
 22. Chesnokova, W. M. and Ignatieva, E. V. (1990): Involvement of thymic hormones in the regulation of the pituitary-adrenal axis in mice. *Endocrinol. Exp.*, 24, 133-141.
 23. Hall, N. R., O'Grady, M. P. and Menzies, R. A. (1992): Thymic regulation of the hypothalamic-pituitary-gonadal axis. *Int. J. Immunopharmacol.*, 14, 353-359.
 24. Milenkovic, L. and McCann, S. M. (1992): Effect of thymosin alpha-1 on pituitary hormone release. *Neuroendocrinology*, 55, 14-19.
 25. Gokkusu, C. and Oz, H. (1990): Influence of thymic fraction 5 on erythrocyte $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity in hypercholesterolemic rabbits. *J. Vit. Nutr. Res.*, 60, 398-401.

26. Potop, I., Boeru, V. and Mreana, G. (1966): The effect of thymus extracts on phosphorus compounds in muscle and serum and on serum calcium. *Biochem. J.*, 101, 454-459.
27. Cooper, R. A. (1977): Abnormalities of cell membrane fluidity in the pathogenesis of disease. *N. Engl. J. Med.*, 297, 371-378.
28. Schouten, J. A., Beynen, A. C., Popp-Snijders, C. and Van der Veen, E. A. (1984): Lipid composition of erythrocytes isolated from cholesterol-fed rabbits. *Nutr. Rep. Int.*, 29, 1223-1232.
29. Torkhovskaya, T. I., Artemova, L. G., Khodzhakuliev, B. G., Rudenko, T. S., Polesskii, V. A. and Azizouq O. A. (1980): Structural and functional changes in erythrocyte membranes in experimental atherosclerosis. *Biyull. Eksp. Biol. Med.*, 89, 675-678.