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INDUCTION OF HYALURONIC ACID SYNTHETASE BY ESTROGEN IN THE MOUSE SKIN

MAKOTA UZUKA [a], KEISUKE NAKAJIMA [a], SABURO OHTA [a] and YO MORI [b]

[a] *Shiseido Laboratories, 1050 Nippa-cho, Kohoku-ku, Yokohama, 223 and* [b] *Department of Biochemistry, Tokyo College of Pharmacy, Hachioji-shi, Tokyo, 192-03 (Japan)*

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Summary

Hyaluronic acid synthetase activity was measured in male mouse skin following the topical application of estradiol in vivo. The enzyme activity increased in parallel with the hyaluronic acid content of the skin, and showed a similar response in the skin of ovariectomized female mice. The increase in enzyme activity was reduced by the anti-estrogen agents, tamoxifene citrate and clomiphene citrate, which block competitively the binding of estrogen to the estrogen receptor. The increase in hyaluronic acid synthetase activity was also reduced by topical application of cycloheximide or by subcutaneous injection of actinomycin D. The results suggest that the stimulation of hyaluronic acid synthesis in mouse skin in response to estrogen treatment is mediated through estrogen receptors and involves the induction of the enzyme hyaluronic acid synthetase.

Introduction

In previous papers we have shown that glycosaminoglycans present in mouse skin consisted largely of hyaluronic acid and dermatan sulfate and that estrogen dramatically increased only the synthesis of hyaluronic acid [1]. Concerning the mechanism of the action of estrogen in mouse skin, we have shown that mouse skin contained an estrogen receptor in the cytosol fraction and that the estrogen receptor complex translocated into the nucleus where it might interact with chromatin [2]. Recently, the results obtained in our laboratory

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)benzene.

have shown that the increase in the rate of hyaluronic acid production in mouse skin after estradiol treatment was parallel to an increase in the estrogen receptor and that an increase in hyaluronic acid induced by estrogen in the mouse skin was inhibited by simultaneous administration of anti-estrogen [1]. Therefore, it seems reasonable to believe that the action of estrogen on the increase in hyaluronic acid may be mediated by the estrogen receptor in the mouse skin.

In order to clarify further the mode of action of estrogen on hyaluronic acid synthesis in mouse skin, we have measured the activity of hyaluronic acid synthetase in mouse skin after estrogen treatment.

Materials and Methods

Chemicals. UDP-*N*-acetyl-[U-^{14}C]glucosamine, specific activity 336 mCi/mmol, and UDP-[U-^{14}C]glucuronic acid, specific activity 307 mCi/mmol, were purchased from The Radiochemical Centre, Amersham. Anti-estrogens, tamoxifene citrate and clomiphene citrate, were donated by Dr. Hirakawa, Department of Obstetrics and Gynecology, Toho University. Other chemicals were obtained from the following sources: UDP-*N*-acetylglucosamine, UDPglucuronic acid, 17β-estradiol, actinomycin D and cycloheximide from Sigma Chemical Co. (St. Louis, MO), sodium hyaluronate (hog skin) and hyaluronidase (*Streptomyces hyalurolyticus*) from Seikagaku Kogyo Co. (Tokyo, Japan).

Animals. Male dd/y mice and ovariectomized dd/y mice were fed with a standard laboratory diet and water ad libitum throughout the experiment. The mice were treated with estradiol alone or with estradiol and anti-estradiol or antibiotic at the times indicated in the text. Mice were killed and the back skin was removed and used to determine hyaluronic acid and for the assay of hyaluronic acid synthetase.

Determination of hyaluronic acid

Glycosaminoglycan was isolated from mouse skin as described in the previous report [1]. Hyaluronic acid contained in glycosaminoglycan was measured by using a cellulose acetate strip by the method of Kondo et al. [3] with a slight modification as described in our previous paper [1].

Assay of hyaluronic acid synthetase

Skin specimens from the mouse back region were immediately dropped into liquid nitrogen and then cut 20 μm in thickness at —20°C in a cryostat. Frozen sections were vacuum-dried for 24 h, weighed by an electromicro balance, and transferred into the incubation tubes for subsequent enzyme assay. The enzyme preparation obtained by this method showed no change in activity due to freezer storage for long periods.

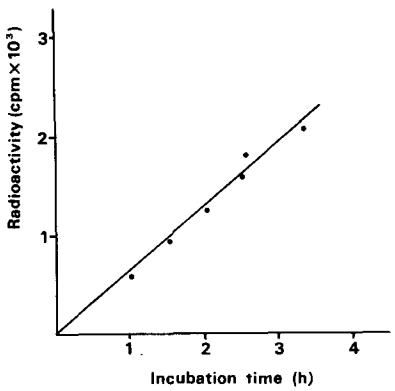
The enzyme was assayed according to the method of Ishimoto et al [4]. The reaction mixture contained in a total volume of 125 μl: 25 μl 0.5 M Tris-HCl buffer, pH 7.2, 15 μl 0.2 M $MgCl_2$, 10 μl 0.01 M UDPglucuronic acid or 0.01 M UDP-[U-^{14}C]glucuronic acid, 5 μl 0.4 mM UDP-*N*-acetyl-[U-^{14}C]glucosamine or 0.4 mM UDP-*N*-acetylglucosamine and the enzyme preparation (from about 1.5 mg dry skin tissue). The mixture was incubated at 37°C for 2 h, then heated at

100°C for 2 min to stop the reaction. After the incubation mixture was homogenized by a microglass homogenizer, the homogenate was lyophilized. Water (2—3 drops) was added and the resultant solution subjected to descending paper chromatography on Whatman No. 3MM filter paper in isobutyric acid/1 M NH_4OH (5 : 3, v/v). After development for 48 h, the spotted origins of the chromatogram were cut out, and the radioactivity was measured in 10 ml scintillation fluid (4.0 g PPO and 0.3 g POPOP per l toluene) in a Beckman liquid Scintillation Counter. Specific activity of the enzyme was expressed as pmol *N*-acetyl-[^{14}C]glucosamine or [^{14}C]glucuronic acid incorporated into hyaluronic acid/h per mg protein or mg dry tissue. The protein content was determined by the method of Lowry et al. [5], using bovine serum albumin as standard.

Results

There was marked incorporation of label into the spotted origin of the chromatogram when UDP-*N*-acetylglucosamine and UDPglucuronic acid were incubated with the enzyme preparation from mouse skin tissue. The radioactivity at origin was reduced to the background level when the reaction mixture was chromatographed after digestion with *Streptomyces* hyaluronidase. When the heated enzyme preparation was used, there was no radioactivity at the spotted origin of the chromatogram which corresponded to hyaluronic acid. Under the assay conditions indicated in Materials and Methods, the enzyme activity was proportional to the time of incubation and to the amount of enzyme added (Figs. 1 and 2). These results indicated that the enzyme from mouse skin tissue was active in the synthesis of hyaluronic acid.

The hyaluronic acid synthetase activity and hyaluronic acid content after estradiol treatment were measured daily for 72 h (Fig. 3). Hyaluronic acid



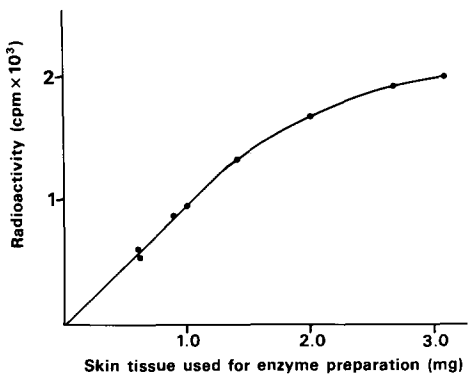


Fig. 1. Effect of incubation time on hyaluronic acid synthetase activity. Enzyme preparation was obtained from lyophilized skin tissue as described in Materials and Methods. Incubation mixture in a total volume of 125 µl contained 25 µl 0.5 M Tris-HCl buffer, pH 7.2, 5 µl 0.4 mM UDP-*N*-acetyl-[U-^{14}C]-glucosamine, 10 µl 0.01 M UDPglucuronic acid, 15 µl 0.2 M $MgCl_2$ and 1.0 mg enzyme preparation (lyophilized dry skin tissue). The mixture was incubated at 37°C for the times indicated.

Fig. 2. Effect of amount of enzyme preparation on hyaluronic acid synthetase activity. Enzyme preparation was prepared and the amount of enzyme preparation indicated was used for the assay of hyaluronic acid synthetase activity as described in Fig. 1.

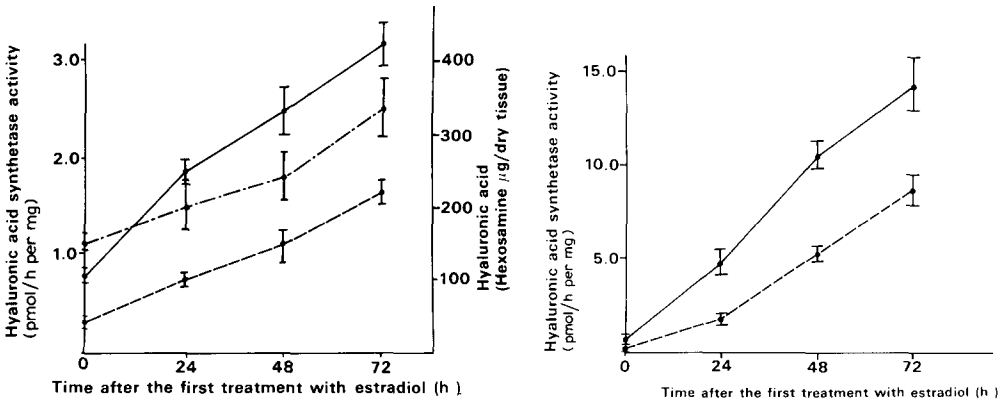


Fig. 3. Effects of estradiol on hyaluronic acid and hyaluronic acid synthetase activity in mouse skin. Male mice were treated topically on the back with 0.1 ml 50% ethanol, containing estradiol (5 μg), repeatedly every 24 h. At 24 h after each treatment, the back skin was removed and the content of hyaluronic acid and the activity of hyaluronic acid synthetase were determined. The plot at 0 time represents the value treated with vehicle alone at 24 h prior to killing. Each bar represents mean ±S.D. (n = 5). ———, hyaluronic acid synthetase activity per mg protein; - - - - - -, hyaluronic acid synthetase activity per mg defatted dry skin tissue; — · — ·, hyaluronic acid content per mg defatted dry skin tissue.

Fig. 4. Effect of estradiol on the hyaluronic acid synthetase activity in ovariectomized mouse skin. 3-week-old mice were ovariectomized. After 2 weeks, the mice were treated topically with 0.1 ml 50% ethanol containing estradiol (5 μg) repeatedly every 24 h. At 24 h after each treatment, the back skin was removed and hyaluronic acid synthetase activity was determined. The solid line represents the hyaluronic acid synthetase activity calculated on the basis of units per mg protein (———), while the broken line represents those which were calculated on the basis of units per mg dry skin tissue (- - - - -). The plot at 0 time represents the values obtained from mice treated with vehicle alone at 24 h prior to killing. Each bar represents mean ± S.D. (n = 5).

TABLE I

EFFECT OF ANTI-ESTROGEN ON THE INCREASE IN HYALURONIC ACID SYNTHETASE ACTIVITY INDUCED BY ESTRADIOL

Male mice were treated topically on the back with 0.1 ml 50% ethanol solution containing estradiol (5 μg) or anti-estrogen (1000 μg) plus estradiol (5 μg) repeatedly every 24 h. At 24 h after each treatment, the back skin was removed and the hyaluronic acid synthetase activity was determined. The values shown are estradiol being eliminated at 0 h. Upper values are for dry tissue, while lower values are for protein. Each value represents mean ± S.D. (n = 5).

Time after first treatment (h)	Hyaluronic acid synthetase activity after treatment with:		
	Estradiol (pmol · h^{-1} · mg^{-1})	Tamoxifene citrate estradiol (pmol · h^{-1} · mg^{-1})	Clomiphene citrate estradiol (pmol · h^{-1} · mg^{-1})
0	0.46 ± 0.11 1.21 ± 0.13		
24	0.93 ± 0.30 2.64 ± 0.25	0.54 ± 0.06 1.56 ± 0.17	0.58 ± 0.04 1.61 ± 0.26
48	1.46 ± 0.01 3.59 ± 0.57	0.80 ± 0.13 2.34 ± 0.23	0.59 ± 0.25 1.41 ± 0.33
72	1.50 ± 0.07 3.67 ± 0.60	0.78 ± 0.08 2.31 ± 0.25	0.56 ± 0.06 1.38 ± 0.14

TABLE II

EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON THE INCREASE IN HYALURONIC ACID SYNTHETASE ACTIVITY INDUCED BY ESTRADIOL

Male mice were treated topically on the back with 0.1 ml 50% ethanol solution containing estradiol (5 μg) every 24 h. The mice were simultaneously injected with 0.1 ml saline containing actinomycin D (25 μg) 24 h prior to killing or were treated with 0.2 ml 0.1% acetone containing cycloheximide (200 μg) with a 12 h interval prior to killing. The skin was removed and hyaluronic acid synthetase activity was determined. All the values are shown as being eliminated estradiol at 0 h. Upper values are for dry tissue, while lower values are for protein. Each value represents mean ± S.D. (n = 5).

Time after first treatment (h)	Hyaluronic acid synthetase activity after treatment with:		
	Estradiol (pmol · h^{-1} · mg^{-1})	Actinomycin D estradiol (pmol · h^{-1} · mg^{-1})	Cycloheximide estradiol (pmol · h^{-1} · mg^{-1})
0	0.32 ± 0.02	0.66 ± 0.09	0.39 ± 0.11
	0.81 ± 0.05	1.56 ± 0.09	0.93 ± 0.27
24	0.75 ± 0.11	0.63 ± 0.10	0.61 ± 0.13
	1.86 ± 0.27	1.23 ± 0.20	1.54 ± 0.21
48	1.11 ± 0.16	0.63 ± 0.20	0.62 ± 0.08
	2.29 ± 0.28	1.19 ± 0.37	1.35 ± 0.01
72	1.66 ± 0.22	0.59 ± 0.02	0.78 ± 0.11
	3.23 ± 0.44	1.33 ± 0.06	1.50 ± 0.02

induced by estradiol treatment in mouse skin increased with the number of treatments with estradiol, as previously demonstrated [1]. An increase in hyaluronic acid synthetase activity on the basis of dry skin tissue or protein was proportional to the hyaluronic acid content of dry skin tissue. Fig. 4 represents the hyaluronic acid synthetase activity in the ovariectomized mouse skin after estradiol treatment. The hyaluronic acid synthetase activity was increased in proportion to the number of estradiol treatments, as observed in male mouse skin tissue. As shown in Table I, anti-estrogens, tamoxifene citrate and clomiphene citrate, counteracted the increment of the hyaluronic acid synthetase activity induced by estradiol treatment, whereas the anti-estrogen alone had no effect on the activity of hyaluronic acid synthetase. Cycloheximide or actinomycin D was employed to examine the mechanism of increase in the hyaluronic acid synthetase activity by estradiol treatment. As shown in Table II, the treatment with either actinomycin D or cycloheximide in combination with estradiol significantly reduced the increase in the hyaluronic acid synthetase activity.

Discussion

Although many studies regarding the effect of estrogen on hyaluronic acid synthesis have been made [6—9], none of them demonstrate a primary effect of estrogen on hyaluronic acid synthesis. It has been considered that the change in hyaluronic acid content represents only one of many changes occurring in connective tissues. We have already suggested that the estrogen receptor contained in the mouse skin may play an important role in the hyaluronic acid biosynthesis [1,2]. Although many enzymes are known to be involved in the

hyaluronic acid synthesis pathway, we gave special attention to the hyaluronic acid synthetase activity since the treatment with estradiol increases only the amount of hyaluronic acid but did not increase that of dermatan sulfate. The hyaluronic acid synthetase activity in cultured cells [10,11], group A *Streptococci* [12,13] and some kinds of tissue [14,15] could be easily measured, but the enzyme activity in the skin is completely lost by homogenization. However, recent study shows that the activity of hyaluronic acid synthetase was maintained in the lyophilized rat skin [16]. After some trials we also found that the activity of hyaluronic acid synthetase in mouse skin could be measured by using lyophilized tissue.

In our previous paper [1], we have shown that hyaluronic acid in mouse skin gradually increased in proportion to the number of topical applications with estradiol. In the present paper, we examined the relationship between the amount of hyaluronic acid and the activity of hyaluronic acid synthetase in mouse skin. We found that the hyaluronic acid synthetase activity in mouse skin treated with estradiol was closely correlated to the amount of hyaluronic acid. It was therefore concluded that hyaluronic acid production is mainly regulated by the level of hyaluronic acid synthetase. Since the synthesis of hyaluronic acid is influenced by estrogen, the effect of estradiol on the activity of hyaluronic acid synthetase in skin tissue was also examined in ovariectomized mouse, in which the hyaluronic acid synthetase activity in skin tissue was increased by estradiol treatment. These results were similar to the results obtained with male mice treated with estradiol.

We have demonstrated previously that the cytoplasm of the mouse skin cell contains the specific estrogen receptor of which the properties are similar to those of the other estrogen target tissues [2] and that anti-estrogens inhibit the increase in hyaluronic acid induced by estradiol in the mouse skin [1]. It is accepted currently that the action of estrogen on the target tissue is initially mediated through the specific receptor in the cytoplasm, and anti-estrogen blocks competitively the action of estrogen at the estrogen receptor level[17, 18]. We observed that an anti-estrogen such as tamoxifene citrate or clomiphene citrate inhibited the activity of hyaluronic acid synthetase in mouse skin after estradiol treatment. Therefore, this result clearly showed that the action of estrogen on the activity of hyaluronic acid synthetase is mediated through the estrogen receptor contained in mouse skin tissue. Actinomycin D or cycloheximide was employed to examine whether an increase in hyaluronic acid synthetase activity induced by estrogen depends on de novo synthesis of enzyme or on the activity of existing enzyme. These drugs inhibited an increase in the hyaluronic acid synthetase activity after estradiol treatment in the mouse skin, indicating that new synthesis of mRNA and protein may be required for an increase in hyaluronic acid synthetase activity. The findings strongly suggest that the increase in hyaluronic acid induced by estradiol may be interpreted by the steroid hormone receptor theory.

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